The Effect of Acute and Chronic Beetroot Juice Supplementation on Submaximal Running and 1500 m Running Performance in Elite Distance Runners

by

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A Thesis
presented to
The University of Guelph

In partial fulfillment of requirements
for the degree of
Master of Science
in
Human Health and Nutritional Sciences

Guelph, Ontario, Canada

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ABSTRACT

THE EFFECT OF ACUTE AND CHRONIC BEETROOT JUICE SUPPLEMENTATION ON SUBMAXIMAL RUNNING AND 1500 M RUNNING PERFORMANCE IN ELITE DISTANCE RUNNERS

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University of Guelph, 2013

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This thesis examined the effects of acute and chronic beetroot (BR) supplementation on \(\text{VO}_2\) during submaximal treadmill running and 1500 m time-trial performance in well-trained runners. Ten elite male middle distance runners (\(\text{VO}_2\) peak, 80 ± 5 ml/kg/min) participated in a randomized, double-blind, crossover design, and supplemented with 210 ml of BR or a nitrate-free BR placebo (PL) for 8 days separated by at least one week. On days 1 (acute) and 8 (chronic), subjects completed a submaximal treadmill run and 1500 m time-trial on an indoor 200 m track. Acute and chronic BR supplementation had no effect on \(\text{VO}_2\) while running at 50, 65 and 80% \(\text{VO}_2\) peak, despite large and significant increases in plasma nitrate. The time to complete the 1500 m run was also unaffected by acute or chronic BR. In summary, elite distance runners did not benefit from acute or chronic BR supplementation.
Acknowledgements

I would like to thank my family for their unwavering support throughout not only this thesis but my entire life. I would not have been able to accomplish so much without you.

Thank you to my advisor, Dr. Lawrence Spriet, for teaching me so much about the scientific process. Most importantly, thank you for challenging me to seek out and pursue what I am passionate about. Your guidance has left a lasting impression on my life.

A special thank you to all my lab mates, Jamie, Chris, Matt, Mark, Tanya, Samantha, Sebastian, Tara and Ian. I’m glad to have shared one of the most exciting times in my life with you.

Thank you to everyone who has made this experience so memorable.
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List of Symbols and Abbreviations

NO – Nitric Oxide

$\text{NO}_2^-$ - Nitrite

$\text{NO}_3^-$ - Nitrate

NOS – Nitric Oxide Synthase

$\text{VO}_2$ – Oxygen uptake

$\text{VCO}_2$ – Carbon Dioxide Production

PO – Power Output

$\text{PO:VO}_2$ – Whole body power output to oxygen uptake ratio

P/O – Ratio of the rate of mitochondrial ATP resynthesis to oxygen utilization

ATP – Adenosine Triphosphate

ADP – Adenosine Diphosphate

PCr – phosphocreatine

SERCA – Sarco-endoplasmic Reticulum Calcium ATPase
Chapter 1: Review of Literature

Role of Nitrate, Nitrite and NO in Human Physiology

Nitric oxide (NO) is a potent signaling molecule that effects cellular and organ function in many tissues of the body. NO plays an important role in regulation of vascular tone, neurotransmission, immune defense, energy metabolism and other processes (Lundberg et al., 2008). NO is produced endogenously by nitric oxide synthases (NOS) from the oxidation of the amino acid L-arginine that is produced within the body in sufficient quantities in healthy adults. NO signaling is fast acting (half life = 2 – 6 s) as it is rapidly oxidized to nitrite (NO$_2^-$). Nitrite is transported in the blood and can be converted back to NO or oxidized to nitrate (NO$_3^-$) (Bailey et al., 2012). Thus, NO$_2^-$ reduction to NO represents an alterative pathway for the generation of NO that complements the classical NOS-derived production (Lundberg et al., 2008). Interestingly, nitrite represents a bioactive source of NO that can be augmented through diet via exogenous inorganic nitrate ingestion. Nitrate, endogenous or dietary, enters the entero-salivary circulation when it is taken up by the salivary glands and concentrated in saliva. In the mouth, commensal anaerobic bacteria use nitrate as an electron acceptor during respiration to reduce nitrate to nitrite (Lundberg et al., 2004). These bacteria are integral to the bioavailability of nitrite because human cells are not capable of reducing nitrate (Lundberg et al., 2008). Importantly, the rise in plasma [NO$_2^-$] following dietary nitrate ingestion is ablated when the entero-salivary circulation is interrupted by spitting (Webb et al.,
or by eliminating the nitrate reducing bacteria in the mouth through the use of antibacterial mouthwash (Govoni et al., 2008). Most swallowed nitrite is reduced to NO in the stomach but some enters the systemic circulation. Nitrite may be reduced to NO by deoxyhemoglobin, deoxymyoglobin, xanthine oxidoreductase, ascorbate, polyphenols, and proteins of the electron transport chain (ETC). NO$_2^-$ reduction to NO is facilitated by hypoxia and low pH (Lundberg et al., 2008). Thus, the conversion of nitrite to NO preserves NO production in hypoxia when NOS activity is limited by the decrease in pO$_2$. In this way, nitrite maintains NO mediated vasodilatation in hypoxic conditions (Lundberg et al., 2008). Figure 1 illustrates how the classical L-arginine – NOS pathway and the Nitrate-Nitrite-NO pathway work in synergy to facilitate NO mediated effects in various target tissues. Until recently, nitrate and nitrite were considered inert by products of NO production via NOS. However, the role of nitrite in human health is an exciting new area of investigation (Bailey et al., 2012).
Nitrate supplementation has gained considerable attention as a dietary means to increase NO bioavailability. Vegetables comprise 60 – 80% of dietary intake of nitrates (Lundberg et al., 2004). Beetroot or dark leafy green vegetables, such as spinach, contain the highest concentrations of nitrates. The typical nitrate ingestion for a western diet is 93 mg/day (Ysart et al., 1999). The Acceptable Daily Intake (ADI) of nitrate represents the amount of nitrate an individual can ingest daily over a lifetime without an appreciable health risk. The current ADI for nitrate is 3.7 mg/kg/day which equates to 222 mg or ~3.6 mmol NO$_3^-$ for a 60 kg individual (Lundberg et al, 2008). This amount corresponds to a large serving of beetroot or spinach (~120 g). However, juice sources represent a more potent form of dietary intake. Seventy ml of concentrated nitrate-rich beetroot (BR) juice is equivalent to a 200 – 250 g serving of beetroot (Lundberg and Govoni, 2004). Therefore, through
dietary supplementation via BR it is possible to ingest amounts of NO\textsubscript{3} that far exceed the ADI. However, the proposed negative impacts of nitrate consumption are questionable and nitrate ingestion from vegetable sources is not considered to have any negative health consequences (Lundberg et al., 2004). In fact, nitrate ingestion has been reported to have many beneficial effects on human health (Lundberg et al., 2004, 2008; Bailey et al., 2012).

Nitrate supplementation represents a practical method to increase NO bioavailability and can play an important role in optimizing human health. Cardiovascular and metabolic disease states have been characterized by reduced NOS production of NO. Furthermore, humans with impaired NOS activity have reduced exercise tolerance. Therefore, nitrate supplementation presents a possible treatment to enhance exercise in these populations and improve health (Bailey et al., 2012). Improvement in vasodilatation via NO mediated effects of nitrate supplementation could confer protection from ischemic-reperfusion injury in the liver, heart, brain and kidney. Furthermore, nitrate ingestion could reduce pulmonary hypertension through blood pressure reducing effects observed following supplementation (Webb et al., 2008). Interestingly, the habituation following chronic exposure to organic nitrates such as nitroglycerin is not observed for inorganic dietary sources of nitrate (Lundberg et al., 2008). Furthermore, dietary nitrate supplementation also has profound effects on contracting rodent and human skeletal muscle (Bailey et al., 2012). Nitrate supplementation has been shown to modulate sarco-endoplasmic reticulum calcium handling and improve
muscle perfusion and oxygen distribution (Hernandez et al., 2012; Ferguson et al., 2012, 2013) in rodent muscle. In addition, respiratory efficiency was improved in mitochondria isolated from healthy humans following nitrate supplementation (Larsen et al., 2011). Nitrate supplementation with nitrate-rich BR has also been shown to reduce the ATP cost of muscle force production during exercise in human subjects (Bailey et al., 2010). Remarkably, nitrate supplementation has been shown to improve whole body exercise efficiency (Larsen et al., 2007, 2009, 2011; Bailey et al., 2009, 2010; Lansley et al., 2010, 2011; Cermak et al., 2012a; Kelly et al., 2013). Specifically, an increase in the ratio of external power output to oxygen consumption (PO:VO₂) is observed during cycling and running following nitrate supplementation. So, at any given fixed rate of work the rate of oxygen consumption is decreased resulting in enhanced exercise tolerance as measured by time to exhaustion tests (Bailey et al., 2009; Lansley et al., 2010; Kelly et al., 2013). In the same way, for any given sustainable metabolic rate of oxygen utilization the power output is increased leading to enhanced performance by reducing the time to complete a specific distance or amount of work in time trial tests (Lansley et al., 2011; Cermak et al., 2012a). This review will discuss in detail the physiological responses to exercise following nitrate supplementation and the underlying mechanisms that may account for improved exercise efficiency and performance.
Physiological Effects of Nitrate Supplementation at Rest and During Exercise

Resting Metabolic Rate and Blood Pressure

At this point only one study has investigated the effect of nitrate supplementation on resting metabolic rate (RMR). In recreationally active men, RMR was unchanged following 500 ml of BR or placebo (Kelly et al., 2013). However, it is not clear from this single observation if RMR is in fact unchanged or if the measurements taken were not sufficiently sensitive to detect a change.

A reduction in resting BP has consistently been shown in normotensive and recreationally active men supplemented with 5 - 7 mmol NO\textsubscript{3} in the form of sodium nitrate or BR (Larsen et al., 2007; Bailey et al., 2009; Lansley et al., 2010; Vanhatalo et al., 2010; Webb et al., 2008; Kapil et al., 2010; Wylie et al; 2013b.). Systolic BP, being reduced 5 - 9 mmHg in nearly all studies, appears to be most sensitive to the vasodilatory effects of nitrate supplementation, while diastolic and mean arterial pressure (MAP) are reduced occasionally (Kapil et al., 2010; Wylie et al., 2013b).

Cardio-respiratory and Metabolic Responses to Exercise

At the onset of exercise the ATP demand of skeletal muscle increases greatly so that muscular contraction can meet the required external power output. The increase in ATP demand is met by varying contributions from phosphocreatine (PCr) breakdown, anaerobic glycolysis and oxidative phosphorylation (Wasserman
et al., 1967). PCr breakdown and anaerobic glycolysis provide ATP instantaneously at the onset of exercise, or during the transition to higher power outputs, while oxidative phosphorylation is activated more slowly. For low and moderate intensity steady state exercise, eventually the ATP demand of exercise is matched completely by aerobic respiration (Whipp and Wasserman, 1972). Consequently, at the onset of exercise there is an exponential increase in VO$_2$ until steady state is attained within 2-3 min. Supplementation with 5 - 7 mmol NO$_3^-$ for 1 – 6 days, in the form of pharmacological sodium nitrate or nitrate rich BR juice has consistently been shown to reduce the VO$_2$ primary amplitude and steady state VO$_2$ of low and moderate intensity exercise by 3-15% in recreationally active men (Figure 2). This reduction is observed during constant work rate cycling (Larsen et al., 2007, 2009, 2011; Bailey et al., 2009; Vanhatalo et al., 2010; Wylie et al., 2013b), walking and running (Lansley et al., 2010) and knee extensor exercise (Bailey et al., 2010).
Similar to that observed during low intensity cycling and running, a 7% reduction in end exercise $VO_2$ and $VO_2$ at task failure was observed during severe intensity constant work rate running (Lansley et al., 2010)(Figure 3). In addition, maximal $VO_2$ during incremental combined arm and leg ergometry was reduced by 3% without a reduction in maximal work rate (Larsen et al., 2009). However, the $VO_2$ kinetics during high intensity exercise are complicated by the development of the slow component that elevates $VO_2$ above the steady state (Coyle, 1999).

Furthermore, the nature of nitrate supplementation on $VO_2$ kinetics during exercise above the ventilatory threshold appears to be related to the activated muscle mass. Contrary to running, no reduction was observed for end exercise $VO_2$ or $VO_2$ at task failure for high intensity constant work rate cycling (> 80% $VO_2$ peak) (Bailey et al.,...
2009; Larsen et al., 2007; Wylie et al., 2013b; Kelly et al., 2013) or maximal VO\textsubscript{2} during incremental cycling where the same maximal power output was attained (Vanhatalo et al., 2010). Rather, a reduction in the slow component amplitude has been observed for severe intensity cycling (Bailey et al., 2009; Kelly et al., 2013) and high intensity knee extensor exercise (Bailey et al., 2010). Although the VO\textsubscript{2} kinetics may be different between running and cycling, an improvement in exercise efficiency was still observed during high intensity time trial cycling. Contrary to constant work rate exercise, where the power output is fixed, the power output during time trial cycling is variable and increases or decreases when the cyclist’s self selected cadence increases or decreases, respectively. Interestingly, VO\textsubscript{2} was not significantly different between the placebo and BR supplemented groups during 4 and 16.1 km cycling time trials. However, BR supplementation increased power output resulting in an improvement in exercise efficiency measured as a 7 - 11\% increase in the PO:VO\textsubscript{2} ratio (Lansley et al., 2011). In other words, for the same given sustainable metabolic rate a greater external work rate was achieved following BR supplementation. Interestingly, the effect of nitrate supplementation on VO\textsubscript{2} is not only dependent on exercise intensity and modality but also the nitrate dose and the subject’s training status. In this section the effects of nitrate supplementation on VO\textsubscript{2} were observed following a 5 – 7 mmol NO\textsubscript{3}\textsuperscript{-} dose in recreationally active men. The influence of increasing dose and training status on the efficacy of nitrate supplementation will be discussed in subsequent sections of this review.
Figure 3 – Time to exhaustion and oxygen uptake during severe intensity running following Placebo or Beetroot supplementation. Time to exhaustion and end exercise VO$_2$ and VO$_2$ at exhaustion was significantly reduced following Beetroot supplementation (*). (Lansley et al., 2010)

Mounting evidence shows that nitrate supplementation improves exercise efficiency over a range of exercise intensities, from ~40% to 100% VO$_2$ peak, and with many exercise modalities (cycling, walking, running) in recreationally active men. Considering that the O$_2$ cost of exercise is thought to be independent of age, health status, physical fitness, training, breathing O$_2$ enriched gas, and EPO administration (Bassett and Howley, 1999; Jones and Poole, 2005) it is quite remarkable that an acute dietary intervention can have such effects. In addition, the increased exercise efficiency following nitrate supplementation has been shown to translate to improved exercise performance in recreationally active men. The effect of nitrate supplementation on performance will be discussed in the next section. Furthermore, the mechanistic basis for the observed improvement in exercise efficiency will be discussed in detail in the following sections.
Reports show no change in the rate of carbon dioxide production (VCO₂), respiratory exchange ratio (RER), ventilation (VE), heart rate (HR) or plasma [lactate], in spite of the significant change in VO₂ kinetics. In eight studies (Larsen et al., 2007, 2009, 2011; Bailey et al., 2009, 2010; Vanhatalo et al., 2010; Lansley et al., 2010; Wylie et al., 2013) of recreationally active men provided with a dose of 5 – 7 mmol NO₃⁻ as either sodium nitrate dissolved in water or 500 ml of nitrate rich BR, only one (Larsen et al., 2011) observed a slight increase in RER. Although carbohydrate is a more efficient fuel, the authors reported that the 0.03 increase in RER could account for no more than a 1% change in efficiency and could not completely account for the 3% reduction in the oxygen cost of exercise (Larsen et al., 2011). In fact, even a large change in RER of 0.1 for a particular cycling power output could not account for the ~15% reduction in VO₂ typically observed following nitrate supplementation. Hypothetically, VO₂ would only decrease 2.4% if RER increased from 0.8 to 0.9 at a cycling PO that elicits an oxygen uptake of 2.5 L/min. However, logically if VO₂ is reduced and RER is unchanged then VCO₂ must decrease because RER = VCO₂/VO₂. A possible explanation is that VCO₂ is too variable to detect a change following nitrate supplementation with any statistical certainty.

**Effect of Nitrate Supplementation on Exercise Performance**

This section will review the performance effects of nitrate supplementation in recreationally active and/or moderately trained subjects who had VO₂ peaks < 60
ml/kg/min and exercised for recreation rather than training specifically for competition. In subsequent sections the effect of BR supplementation on performance in well-trained individuals who training for competition will be addressed.

The capacity to tolerate high intensity exercise is related to the maximal oxygen uptake and exercise efficiency of the individual (Coyle, 1999). Accordingly, studies reporting increased exercise efficiency have also shown that time to exhaustion was extended 3 – 25% following dietary nitrate supplementation in the form of BR during severe intensity cycling (Bailey et al., 2009; Wylie et al., 2013b; Kelly et al., 2013), knee extensor exercise (Bailey et al., 2010), and running (Lansley et al., 2010) in recreationally active subjects. Interestingly, during severe intensity running VO₂ at task failure was reduced following nitrate supplementation. However, the increase in exercise efficiency was enough to offset the reduction in VO₂ such that time to exhaustion was extended 16% (Figure 3).

Although time to exhaustion testing can assess exercise tolerance and allows for direct comparison of physiological parameters over time, performance in actual cycling and running competitions is not measured by the time an individual can sustain a particular power output but rather the time to complete a given distance. According to Hopkins and colleagues (1999), a 15 – 20% improvement in time to exhaustion tests of exercise tolerance correspond to a 1 – 2% improvement in time trial performance. Accordingly, cycling time trial performance at 4, 10 and 16.1 km
was improved 1.2 – 2.8% following BR supplementation in moderately trained subjects (Lansley et al., 2011; Cermak et al., 2012a). PO was increased during the time trial but VO₂ was unchanged between BR and placebo supplementation. This resulted in an 11 and 7% increases in the PO:VO₂ ratio following BR ingestion, for 4 and 16.1 km time trials, respectively (Lansley et al., 2011). So, improved exercise efficiency following BR supplementation improved performance in simulated competition by increasing the external power output for a given achievable metabolic rate (Lansley et al., 2011). In addition, Kelly and authors (2013) used the calculated critical power and curvature constant (W’) of the power-duration relationship in a model to predict time trial performances at increasing “work done” targets. Time to complete 75 – 225 kJ of work was reduced by 2 – 3% with BR supplementation compared to placebo.

In addition to endurance exercise, BR ingestion also improved repeat sprint performance as assessed by the Yo-Yo IR1 test in recreational team sport players (Wylie et al., 2013a). The Yo-Yo IR1 test is a repeated 20 m sprint test run at increasing speeds. Performance during the test is measured by the distance run before subjects can no longer match the required speed. Test performance correlates with high intensity running during soccer games which is a key determinant of soccer performance. Therefore, nitrate supplementation may improve team sport specific performance by improving high intensity exercise performance during games (Wylie et al., 2013a). BR supplementation has also been shown to improve time to complete 6 x 500 m rowing ergometry intervals in trained
rowers (Bond et al., 2012). These observations show that nitrate supplementation may be a practical ergogenic aid in a variety of sport and athletic competitions.

In summary, nitrate supplementation in the form of BR has repeatedly been shown to improve exercise tolerance, time to exhaustion, and performance in more practical time trial tests in recreationally active and moderately trained subjects. The typical ~ 15% increase in exercise capacity during severe intensity fixed work rate cycling and running following nitrate supplementation corresponds to a 1.2 – 2.8% improvement in the time to complete 4 – 16.1 km cycling time trials. By increasing power output at a given achievable metabolic rate, nitrate supplementation may improve performance by attenuating the development of critical levels of muscle metabolites implicated in fatigue such as [ADP], [Pi] and (H⁺) or the reduction in [PCr] and [glycogen] (Allen et al., 2008). This delay following nitrate supplementation could be mediated by decreased energy cost of muscle force production and/or greater ATP production per unit oxygen consumed. BR supplementation has been shown to enhance performance for exercise at 80 – 100% of Wmax. However, evidence suggests the magnitude of this effect may diminish at exercise intensities of 100% of Wmax and possibly above (Kelly et al., 2013). More research is needed to establish the relationship between competition duration and the improvement in performance following nitrate supplementation. Interestingly, more recent studies in well-trained subjects have failed to show an improvement in exercise efficiency and/or performance. The relationship between
training status and nitrate supplementation efficacy will be discussed in subsequent sections of this review.

**Mechanisms Conferring Improved Whole Body Exercise Efficiency and Performance**

Several potential mechanisms have been proposed to explain the improved exercise efficiency and performance observed following nitrate supplementation. In general, the decreased energy cost of exercise at any given fixed power output could result from I) a reduction in the ATP utilization of ATPases associated with muscle force production (acto-myosin ATPase, sarco-endoplasmic Ca$^{2+}$ -ATPase (SERCA) and/or Na$^{+}$/K$^{+}$ -ATPase), II) a reduction in the O$_2$ cost of mitochondrial ATP resynthesis and/or, III) compensatory increase in the ATP provision from substrate phosphorylation (Bailey et al., 2010). However, it is not possible for a compensatory increase in PCr breakdown and anaerobic glycolysis to completely augment ATP production because the magnitude of the reduction in oxygen consumption during exercise following nitrate supplementation would far exceed the capacity for anaerobic energy production. Furthermore, a compensatory increase in substrate level phosphorylation is not supported in the literature. Plasma [lactate] has consistently been shown not to increase following nitrate supplementation compared to placebo during moderate and high intensity fixed work rate exercise or during time trial cycling at self selected power outputs (Larsen et al., 2007, 2009, 2011; Bailey et al., 2009, 2010; Vanhatalo et al., 2010; Lansley et al., 2010; Peacock et al., 2012; Cermak et al., 2012a, 2012b; Wilkerson et al., 2012; Bescos et al., 2012;
Wylie et al., 2013). In addition, muscle pH measured by P-MRS and estimates of
glycolytic ATP contribution are the same between nitrate and placebo
supplementation (Bailey et al., 2010; Fulford et al., 2013). Therefore, it is proposed
that nitrate supplementation improves exercise economy by reducing the ATP cost
of contraction and/or improving mitochondrial respiratory efficiency.

**Reduction in the ATP Cost of Muscle Force Production**

Skeletal muscle ATP turnover during contraction is predominantly
determined by the activity of the actomyosin ATPase and the sarcoendoplasmic
reticulum calcium ATPase (SERCA), with a smaller contribution from the Na+/K+
ATPase (Bailey et al., 2010). NO may improve contractile efficiency by modulating
the activity of these enzymes in a way that reduces the hydrolysis of ATP while
maintaining force production. Interestingly, NO has been shown to slow cross-
bridge cycling kinetics and increase the force per power stroke *in vitro* (Evangelista
et al., 2010). Extending on this finding, PCr degradation following nitrate
supplementation is decreased in contracting human skeletal muscle (Bailey et al.,
2010; Vanhatalo et al., 2011; Fulford et al., 2013). Furthermore, the reduction in PCr
breakdown is proportional to the reduction in oxygen consumption. In addition, the
calculated estimate of total ATP degradation was reduced following BR ingestion
indicating that the ATP required to produce a given muscular force was reduced
(Bailey et al., 2010). Unfortunately, the methodology of this study was limited in
that total ATP breakdown cannot be directly measured and with any estimate there
is potential for error due to assumptions. Also, the source of the reduction in ATP turnover, whether by reduced ATP breakdown by the actomyosin ATPase, SERCA and/or Na\(^+\)/K\(^-\) ATPase, cannot be distinguished. If total ATP breakdown is reduced for a given muscular force production it is possible that a reduction in [ADP] would reduce oxygen consumption by diminishing the stimulus for mitochondrial respiration (Bailey et al., 2010). However, a reduction in the ATP cost of muscle force production cannot exclude an effect of NO on mitochondrial ATP resynthesis efficiency. In any case, there appears to be a reduction in the metabolic perturbations associated with fatigue as nitrate supplementation reduced the accumulation of [ADP] and [Pi] and diminished the reduction in [PCr]. In the context of a time trial, nitrate supplementation could afford a greater external power output for the same level of metabolic perturbation. This could lead to decreasing the time to complete a given amount of work before the attainment of critical levels of [ADP], [Pi] and the reduction of [PCr] were reached.

Further research has revealed that nitrate supplementation increased force production at low stimulation frequencies and the onset of force production at high frequencies in isolated rat extensor digitorum longus (EDL), but not soleus muscles (Hernandez et al., 2012). This effect was attributed to increased [Ca\(^{2+}\)] at any given stimulation frequency. Calcium release was increased by upregulation of calsequesterin and the resulting increase in SR calcium stores (Hernandez et al., 2012). The implications of these findings on exercise efficiency are not clear. Although force production was increased at low stimulation frequencies an increase
in calcium release would be expected to increase ATP turnover since Ca\(^{2+}\) re-uptake and SERCA activity would increase. Hernandez and colleagues (2012) speculated that for any given external force production the number of motor units recruited could be reduced. The net result could be a decrease in the overall O\(_2\) cost of force production but this is speculation and further research is required. In addition, this would also indicate that nitrate supplementation affects motor unit recruitment patterns and could have implications for muscle fatigue. It should be noted that changes in calcium handling properties following nitrate supplementation have not been investigated in human skeletal muscle.

**Reduction in the O\(_2\) Cost of Mitochondrial ATP Resynthesis**

Mitochondrial ATP resynthesis from ADP and Pi is driven by the development of an electrochemical proton gradient. Electrons from NADH and FADH\(_2\) are passed through the ETC to pump protons into the inner mitochondrial space. Slippage occurs when electrons are passed without proton pumping, thereby reducing the generation of membrane potential (Murphy, 1987). Membrane potential is the driving force for proton flux back into the inner mitochondrial space and can be coupled with ATP resynthesis when hydrogen ions pass through complex 5 or independent of ATP resynthesis when H\(^+\) pass through the inner mitochondrial membrane via Adenosine Nucleotide Transferase (ANT) or uncoupling protein (UCP) -3. ANT is a mitochondrial protein responsible for transferring ADP across the inner mitochondrial membrane while UCP-3 reduces reactive oxygen species
(ROS) production by dissipating membrane potential (Brand, 2000). Therefore, the rate by which ATP is resynthesized from the generation and utilization of membrane potential is affected by slippage, where electrons are passed without the pumping of protons, and leak, where the proton gradient is consumed via pathways that do not contribute to ATP resynthesis (Murphy, 1989). Mitochondrial respiratory efficiency (P/O ratio) can be calculated as the rate of ATP synthesis divided by the rate of oxygen consumption (Hinkle, 2005). Nitrate supplementation may improve the rate of ATP resynthesis by NO mediated effects on slippage and leak (Clerc et al., 2007; Larsen et al., 2011). In a separate way, NO can also competitively inhibit O₂ binding at complex IV (Basu et al., 2008). Therefore, increased NO availability could directly reduce the rate of oxygen consumption by replacing O₂ as the terminal electron acceptor. Any combination of these effects would improve the mitochondrial P/O ratio. Importantly, proteins of the ETC can reduce nitrite to NO providing temporal evidence for a role for nitrite in the regulation of mitochondrial respiration (Nohl et al., 2000).

Evidence shows nitrate supplementation increases the ATP yield of mitochondrial oxidative phosphorylation by decreasing proton leak across the inner mitochondrial membrane. Larsen and colleagues (2011) observed a 19% increase in the P/O ratio during sub maximal ADP stimulation in isolated mitochondria from subjects supplementing with sodium nitrate. Furthermore, the increased P/O ratio was observed with an unchanged state 3 and reduced “Leak” and state 4 respiration. State 3 respiration is the rate of oxygen consumption in the presence of ADP and
substrate while state 4 and Leak respiration are the rate of oxygen consumption when all ADP has been rephosphorylated to ATP. Leak respiration can be distinguished from state 4 in that the small amount of ADP regeneration in state 4 is blocked during leak respiration. In this way Leak respiration closely approximates oxygen consumption from proton leak back across the inner mitochondrial membrane. Therefore, reduced leak and state 4 respiration indicates that a greater proportion of the membrane potential was used for ATP resynthesis and less was wasted by leak. This reduction in leak was attributed to the reduction in ANT protein content and nearly statistically significant reduction in UCP-3. Importantly, the increase in mitochondrial P/O ratio was correlated with the decrease in VO₂ observed for fixed work rate sub maximal cycling following nitrate supplementation (Figure 4). This evidence suggests that the improvement in whole body exercise economy following nitrate supplementation is, at least in part, caused by increase mitochondrial respiration efficiency (Larsen et al., 2011).
Figure 4 - The correlation between change in whole body exercise efficiency and mitochondrial respiratory efficiency. (Larsen et al., 2011)

Increased NO bioavailability may also improve exercise efficiency by improving the intrinsic coupling of electron transfer and proton pumping. Reduced electron slippage has been reported with the addition of NO to isolated rat liver mitochondria when FADH$_2$ linked substrates were oxidized (Clerc et al., 2007). FADH$_2$ linked substrates are less efficient than NADH linked substrates because FADH$_2$ donates electrons at complex II and by-passes proton pumping at complex I. As a result more protons are pumped per electron transferred with NADH than FADH$_2$. The addition of NO to isolated mitochondria changed the relationship between oxygen consumption and membrane potential when FADH$_2$ linked substrates were oxidized to match the relationship observed when NADH linked substrates were oxidized. This finding indicates that the intrinsic coupling of electron transfer and proton pumping had improved when FADH$_2$ substrates were
oxidized. It was also noted that cytochromes a and a3 had become reduced during the oxidation of FADH$_2$ linked substrates compared to NADH linked substrates. So, when cytochrome IV becomes reduced it was suggested that it is prone to slippage. Thus, it was proposed that NO binding to cytochrome IV reduced slippage in this situation and ultimately improves respiratory efficiency.

**Improved Blood Flow and Oxygen Distribution**

Nitrate supplementation has also been shown to increase skeletal muscle blood volume in exercising humans, as assessed by near infrared spectroscopy (NIRS) (Bailey et al., 2009), and blood flow in rat hind limbs (Ferguson et al., 2012, 2013). Increased blood flow could improve oxygen delivery and/or carbon dioxide release. In addition, NO has also been shown to competitively inhibit oxygen binding to complex IV of the ETC and replace oxygen as the terminal electron acceptor (Basu et al., 2008). So, NO could further improve O$_2$ distribution by inhibiting the O$_2$ consumption of mitochondria closest to the blood vessels creating an increased pO$_2$ gradient that drives O$_2$ delivery to mitochondria further from the blood vessel (Bailey et al., 2010). Ultimately, distributing O$_2$ over a greater population of mitochondria could cause a net reduction in membrane potential, reducing leak and/or slippage and increasing mitochondrial P/O (Larsen et al., 2009).
Although speculative, a potential NO mediated reduction in membrane potential could also reduce ROS generation and fatigue. ROS thionylation of the ryanodine receptor (RyR) has been implicated in the development of fatigue (Westerbald and Allen, 2011). Therefore, another possible effect of NO on exercise performance would be a reduction in fatigue caused by ROS mediated thionylation of the RyR. However, further research is required to investigate this possibility. In addition, there is no clearly established link between RyR thionylation and contractile efficiency.

**Glycogen Sparing**

NO mediated vasodilation has been proposed to increase skeletal muscle glucose uptake (Baron, 1996). During repeated sprint exercise plasma [glucose] was decreased following nitrate supplementation compared to placebo (Wylie et al., 2013a). Although not directly measured, it is conceivable that nitrate supplementation increased plasma glucose use during exercise. However, this possibility is unlikely because skeletal muscle relies very little on plasma glucose during repeated sprint exercise. During sprint exercise, where large amounts of ATP are required for a short duration, glycogen is preferentially oxidized rather than plasma glucose because exogeneous glucose must be phosphorylated and would reduce the ATP contribution to muscle force production. If true, this finding presents the possibility that nitrate supplementation could also improve
performance by sparing glycogen. Further research is required to establish the effects of nitrate supplementation on fuel selection and use during exercise.

**Muscle Excitability**

In the same study of repeat sprint performance, Wylie and colleagues (2013a) detected a reduction in plasma [K+] following nitrate supplementation, indicating a reduction in potassium efflux. An increase in plasma [K+] is associated with fatigue (Allen et al., 2008). Therefore, this finding gives the possibility that nitrate supplementation could improve performance by better maintaining muscle excitability. However, more research is needed to corroborate this finding. It is also unclear whether potassium homeostasis affects exercise economy.

**Mitochondrial Biogenesis**

Increased mitochondrial biogenesis following nitrate supplementation is another proposed mechanism for improved performance. NO exposure results in cGMP mediated activation of SIRT1 which upregulates transcriptional factors and nuclear respiratory factors involved in mitochondrial biogenesis (Nisoli et al., 2004). However, the evidence to support an increase in mitochondrial biogenesis following nitrate supplementation is equivocal. No change in mitochondrial density was observed following 3 days of sodium nitrate supplementation (Larsen et al., 2011). However, this result is not surprising as increases in mitochondrial proteins following intense exercise, which is likely a much more potent stimulus for
mitochondrial biogenesis than nitrate supplementation, were not observed until after 5 days of training (Perry et al., 2010). In addition, following 15 days of BR supplementation no change was observed in the PCr recovery time constant which is an indication of maximal oxidative capacity (Fulford et al., 2013). However, increased peak power at VO$_2$ max and power output at the gas exchange threshold was observed following 15 days of BR ingestion compared to 1 and 5 days (Vanhatalo et al., 2010). These observations typically accompany increases in mitochondrial content following endurance training (Vanhatalo et al., 2010). Unfortunately, mitochondrial content was not measured directly so any potential effect of prolonged nitrate supplementation on mitochondrial content could not be confirmed. Also, considering VO$_2$ peak was not increased it is unlikely there was a change in mitochondrial content. Therefore, it is doubtful that long-term nitrate supplementation can increase mitochondrial content.

In summary, increasing evidence suggests that nitrate supplementation improves skeletal muscle contractile efficiency by reducing the ATP cost of contraction and/or improving mitochondrial respiratory efficiency. During exercise actomyosin ATPase and SERCA activity largely determine the rate of ATP turnover. A reduction in the ATP cost of contraction following nitrate supplementation could be due to NO mediated decrease in the rate of ATP hydrolysis of one or both of these enzymes (Bailey et al., 2010; Fulford et al., 2013). Evidence also shows that improved oxidative phosphorylation efficiency is caused by a decrease in leak (Larsen et al., 2011) and improved intrinsic coupling of the respiratory chain (Clerc
et al., 2007). Importantly, changes in skeletal muscle energetics correlate to whole body changes in exercise efficiency (Bailey et al., 2010; Larsen et al., 2011).

**Nitrate Supplementation Pharmacokinetics**

From a practical standpoint understanding the pharmacokinetic response to nitrate supplementation will allow practitioners to make informed recommendations on the timing, dose, and duration of supplementation. In this way, individuals can optimize the beneficial effects of nitrate supplementation on health or exercise performance.

**Time Course**

Following a dose of 4 – 24 mmol NO$_3^-$, provided as sodium nitrate or BR, plasma [NO$_3^-$] peaks at ~1.5 h post ingestion while plasma [NO$_2^-$] peaks at ~2.5 h post ingestion (Webb et al., 2008; Wylie et al., 2013b; Kapil et al 2010). The delay in time to peak for plasma [NO$_2^-$] compared to plasma [NO$_3^-$] reflects the time for nitrate to be taken up in the entero-salivary circulation and reduced to nitrite by commensal bacteria in the mouth before being swallowed and absorbed into the systemic circulation (Webb et al., 2008). Furthermore, the increase in plasma [NO$_2^-$] is ablated when the entero-salivary circulation is interrupted by spitting (Webb et al., 2008) or by the use of antibacterial mouthwash that eliminates the nitrate reducing bacteria in the mouth (Govoni et al., 2008). Not surprisingly, peak reductions in resting BP (systolic and diastolic BP and MAP) occur at 2 – 4 h post...
ingestion and coincide with peak increases in plasma [NO$_2^-$] (Kapil et al., 2013). The change in resting BP is correlated to changes in plasma [NO$_2^-$] (Wylie et al., 2013).

At 24 h post ingestion, plasma [NO$_3^-$], [NO$_2^-$] and BP return to baseline levels following smaller doses of nitrate (4.2 – 8.4 mmol). However, following a large dose of NO$_3^-$ (16.8 – 24 mmol) plasma [NO$_3^-$] and [NO$_2^-$] remain elevated compared to baseline and systolic BP (but not diastolic BP and MAP) is significantly reduced (Kapil et al., 2010; Wylie et al., 2013b).

**Dose Response**

In eleven studies of recreationally active men provided with a dose of 5 – 7 mmol NO$_3^-$ as either sodium nitrate dissolved in water or 500 ml of nitrate rich BR, average baseline plasma [NO$_2^-$] was 206 ± 137 nM (Bailey et al., 2009, 2010; Lansley et al., 2010, 2011; Larsen et al., 2007, 2009, 2011; Govoni et al., 2008; Webb et al., 2008; Vanhatalo et al., 2010; Fulford et al., 2013). At 2.5 h post ingestion, plasma [NO$_2^-$] increases 85% to 382 ± 178 nM. Few studies have actually investigated the dose-response relationship for nitrate supplementation. Where multiple doses have been administered, plasma [NO$_2^-$] increased in a dose dependent manner (Kapil et al., 2013; Wylie et al., 2013). Plasma [NO$_2^-$] increased 2.5, 4 and 8 fold following doses of 4.2, 8.4 and 16.8 mmol NO$_3^-$ provided as 70, 140 and 280 ml of concentrated BR, respectively (Wylie et al., 2013b). Furthermore, the dose response was similar whether sodium nitrate (Kapil et al., 2013) or concentrated BR (Wylie et al., 2013b) was administered. A dose dependent decrease in resting BP was also
observed following nitrate supplementation (Kapil et al., 2013; Wylie et al., 2013b). In addition, improvements in exercise efficiency increased in a dose dependent fashion. The reduction of end exercise VO$_2$ correlated to the change in plasma [NO$_2^-$] following doses of 4.2, 8.4 and 16.8 mmol NO$_3^-$ (Wylie et al., 2013b). Interestingly, tolerance to severe intensity exercise was increased following 8.4 mmol compared to 4.2 mmol NO$_3^-$ but no further increase was observed when 16.8 mmol was ingested (Wylie et al., 2013b). This finding suggests that there is a threshold to the magnitude of improvement following nitrate supplementation and the optimal dose for acute supplementation may be 8.4 mmol.

**Duration of Supplementation**

Few studies have directly compared the effects of the length of supplement duration on the efficacy of nitrate supplementation. Plasma [NO$_2^-$] was similarly elevated at 2.5 h post ingestion following ~5.5 mmol NO$_3^-$ provided as 500 ml of BR when subjects supplemented for 1 (acute), 5 or 15 (chronic) days (Vanhatalo et al., 2010; Fulford et al., 2013). There was also no difference in the reduction in VO$_2$ (Vanhatalo et al., 2010) or muscle contraction force (Fulford et al., 2013) at 1, 5 or 15 days of supplementation. Interestingly, ramp test peak power and the work rate at the gas exchange threshold were increased at 15 days of BR supplementation compared to 1 and 5 d (Vanhatalo et al., 2010). The significance of this finding is questionable because VO$_2$ was not increased. The effect of prolonged BR supplementation on VO$_2$ max and performance requires further research. In any
case, it appears that intolerance to nitrate supplementation does not develop for up to 15 days. Similarly, no time effect was reported for plasma [NO$_2$] or tolerance to severe intensity cycling over 5 laboratory sessions during a supplementation period of 7 – 12 days (Kelly et al., 2013). When comparing studies that employ an acute dosing protocol to those supplementing for 3 – 6 days, some have reported that the magnitude of reduction in the O$_2$ cost of exercise is similar (Lansley et al., 2011) while others have reported that it is diminished (Wylie et al., 2013). The exact effect of supplementation duration on the response to nitrate ingestion remains to be established. However, it seems logical that where protein modifications have been implicated in the underlying mechanism for improved exercise economy or performance, that these adaptations would not be present following an acute dose.

**Individual Variability in Nitrate Supplementation**

Individual variability in the physiological response to nitrate supplementation is affected by multiple factors. The magnitude of the improvement in exercise efficiency and performance is likely related to the ability of supplementation to adapt the underlying mechanisms that confer the response. Specifically, where nitrate supplementation can modulate contractile efficiency, mitochondrial respiratory efficiency and/or blood flow and oxygen distribution an effect is more likely to be observed. “Non-responders” are characterized as individuals whom do not have an improvement in exercise efficiency or performance after nitrate ingestion. Within recreationally active men, non-
responders have been reported (Larsen et al., 2011; Wylie et al., 2013b). Larsen and colleagues (2011) revealed a significant correlation between the reduction in whole body VO$_2$ during sub maximal cycling and increased mitochondrial P/O ratio. This finding indicates that individual variability in the whole body response to nitrate supplementation may be in part due to changes in respiratory efficiency at the level of the mitochondria. As expected with human subjects, there is a continuum of responses to nitrate supplementation. Interestingly, one subject clearly did not have an increase in plasma [NO$_2$-] and at least one subject did not have a reduction in UCP-3 or ANT (Larsen et al., 2011). Unfortunately, the authors did not make it clear if the same subject that showed no response to whole body exercise economy and mitochondrial respiratory efficiency also had no increase in plasma [NO$_2$-] and/or decrease in mitochondrial proteins associated with leak. Therefore, it is not certain from this report if there was a correlation between plasma [NO$_2$-] response and changes in the protein levels of UCP-3 and/or ANT.

**Training Status**

Recent evidence shows that more highly trained individuals are less likely to positively respond to nitrate supplementation. In a study of moderately trained but non-competitive men (VO$_2$ peak = 56 ml/kg/min) nine of nine subjects showed improved time to complete 4 and 16.1 km time trials following an acute dose of BR. Similarly, Cermak and colleagues (2012a) reported that eleven of twelve moderately trained males (VO$_2$ peak = 58 ml/kg/min) completed a 10 km cycling time trial
faster following 6 days of concentrated BR supplementation. Conversely, no studies in well trained subjects, where the VO$_2$ peak of the subjects was $\geq$ 60 ml/kg/min and subjects train for competition rather than recreation, have reported a significant improvement in mean time trial performance following nitrate supplementation (Cermak et al., 2012b; Bescos et al., 2012; Wilkerson et al., 2012; Peacock et al., 2012; Christensen et al., 2012). In addition, in studies with well-trained subjects, all have reported no changes in the PO:VO$_2$ ratio (Bescos et al., 2012; Peacock et al., 2012; Christensen et al., 2012) or a diminished increase ($\leq$5%) (Bescos et al., 2011; Wilkerson et al., 2012) compared to that seen in moderately trained subjects (11%) (Lansley et al., 2011). This finding provides further support that the mechanisms underpinning improved exercise efficiency also enhance performance. Although mean time trial performance was unaffected by nitrate supplementation individual “responders” have been identified in well-trained subjects. Five of eight well-trained subjects (VO$_2$ peak = 63 ml/kg/min) had a $\sim$2% improvement in 50 mile time trial completion time following acute BR supplementation (Wilkerson et al., 2012). In highly trained cyclists (VO$_2$ peak = 72 ml/kg/min) only 2 of 8 individuals improved their time to complete 400 kcal of work (Christensen et al., 2012). It appears that as the training status of the subject population under investigation increases the likelihood of observing a response in any individual decreases.

Logically, you would not expect to observe any physiological response following nitrate supplementation if plasma [NO$_2^-$] was unchanged in a particular individual. Evidence shows a significant correlation between individual
improvement in performance and increased plasma $[\text{NO}_2^-]$ in recreationally trained people (Figure 5) (Wilkerson et al., 2012). Specifically, two of the three subjects who had less than a 30% increase in plasma $[\text{NO}_2^-]$ did not show an improvement in performance. This finding suggests that a lack of change in plasma $[\text{NO}_2^-]$ at the start of exercise may preclude individuals from enhanced performance. A lack of response in plasma $[\text{NO}_2^-]$ could result from individual differences in commensal nitrate reductase bacteria in the mouth or other factors but the exact reason is not certain (Bescos et al., 2012). Indeed, the time to peak for plasma nitrite can range from 130 – 360 min (Wylie et al., 2013b). So, individual variability in the time to peak $[\text{NO}_2^-]$ could account for the lack of change in plasma $[\text{NO}_2^-]$ and resulting lack of enhancement in exercise efficiency and performance. This factor would be independent of training status because it is unlikely that there are differences in oral bacteria between trained and untrained subjects (Bescos et al., 2012). It should be noted that triathletes who regularly engaged in swim training failed to show an increase in plasma $[\text{NO}_2^-]$ following nitrate supplementation. This finding may be due to the elimination of nitrate reductase bacteria from chlorine in the pool (Bescos et al., 2012). In any case, individual variability in the time to peak plasma $[\text{NO}_2^-]$ cannot completely explain the lack of effect seen in well-trained individuals as even subjects who display a ≥50% increase in plasma $[\text{NO}_2^-]$ have failed to show an improvement in performance (Bescos et al., 2012).
In general, one would expect that nitrate supplementation fails to improve exercise efficiency or performance in well-trained subjects because supplementation doesn’t change any of the underlying mechanisms that confer the response in recreationally active individuals. It is possible that nitrate supplementation fails to elicit a response in well-trained subjects because supplementation does not augment the contribution of endogenous NO$_2^-$ to NO production during exercise. There are two possibilities to explain a failure of nitrate supplementation to significantly contribute to the endogenous NO$_2^-$ to NO conversion during exercise. Most likely the training adaptations that accompany endurance exercise have afforded well-trained individuals sufficient endogenous nitrite to meet their demands. If this is the case there may exist a threshold where further increases in NO$_2^-$ via nitrate supplementation will not confer additional...
improvements. Conversely, well-trained subject may require greater nitrate exposure, via a higher dose and/or increase supplement duration, to elicit an improvement in efficiency and performance.

In well-trained subjects additional nitrite provided through supplementation may not contribute to NO production because of a combined effect of increased endogenous plasma [NO₂⁻] and decreased reliance on the production of NO from nitrite during exercise. Evidence shows that exercise training increases eNOS expression and activity leading to increased plasma [NO₃⁻] and [NO₂⁻] (Green et al., 2004). Also, trained individuals exhibit higher baseline plasma [NO₃⁻] compared to untrained individuals (Poveda et al., 1997). In addition, well-trained individuals may rely less on the nitrate – nitrite – NO pathway during exercise because the development of hypoxic loci within the skeletal muscle may be reduced (Wilkerson et al., 2012). Endurance trained individuals have increased skeletal muscle capillary density so blood flow and O₂ distribution may already be optimal in well-trained individuals (Brodal et al., 1977). Therefore, potential improvements in muscle perfusion and O₂ distribution afforded by nitrate supplementation may not be observed in well-trained subjects. In addition, any improvement in blood flow following nitrate supplementation may be specific to type II fibers (Ferguson et al., 2012). Therefore, the shift to a higher proportion of type I fibers that accompanies endurance training may diminish the capacity to utilize nitrite in trained individuals (Wilkerson et al., 2012). However, studies citing preferential response in type II fibers following nitrate supplementation are investigations of rodent skeletal
muscle (Hernandez et al., 2012; Ferguson et al., 2012, 2013). It is unclear if nitrate supplementation affects human type I and II fibers differently. Unfortunately, determining nitrite utilization is difficult considering that plasma [NO$_2^-$] is a product of nitrate reduction and NO oxidation from the classical NOS pathway (Wylie et al., 2013b). In any case, the capacity to utilize nitrite for the production of NO may be met by an endogenous supply in well-trained individuals. In this way, training status could directly reduce the efficacy of nitrate supplementation from a combination of high baseline plasma [NO$_2^-$] and decreased reliance on the production of NO from nitrite. This is supported by evidence showing baseline plasma [NO$_2^-$] could not distinguish between responders and non-responders in well-trained subjects (Bescos et al., 2012; Christensen et al., 2012; Cermak et al., 2012).

On the other hand, the possibility that well-trained individuals may require greater nitrate exposure to improve efficiency and performance cannot be ruled out. Evidence shows that the reduction in the O$_2$ cost of exercise following BR supplementation is affected by dose in recreationally active men. When subjects received 4.2, 8.4 and 16.8 mmol NO$_3^-$ in the form of concentrate BR, three individuals had no reduction in VO$_2$ during sub maximal cycling following ingestion of 4.2 mmol and there where two non-responders at 8.4 mmol and one at 16.8 mmol. Importantly, subjects who showed no reduction in VO$_2$ at lower doses responded at higher doses. Interestingly, increasing the nitrate dose eliminated non-responders in a manner that was independent of baseline or change in plasma [NO$_2^-$] as both measures were similar for responders and non-responders (Wylie et al.,
2013b). The reason for the lack of response in some subjects is not clear but this finding indicates that dose can explain some of the variability in individual response in a way that is independent of [NO$_2$] bioavailability. The dose response in well-trained subjects has not been investigated and it is uncertain what effect training status would have on the dose-response relationship. It is possible that well-trained subjects could increase the likelihood of responding positively to nitrate supplementation by increasing the dose. It has also been suggested that well-trained athletes could increase the likelihood of eliciting a response if a longer duration of supplementation is administered (Cermak et al., 2012b). However, the evidence to support this is equivocal and confounded by the different length of time trials used between studies (Lansley et al., 2011; Cermak et al., 2012a, 2012b; Wilkerson et al., 2012). In any case, some underlying mechanisms shown to enhance exercise efficiency and performance require protein modifications. Logically, an extended supplementation protocol would be more likely to produce these changes than an acute dose only 2.5 h prior to exercise. Further research is required to determine if well-trained individuals can benefit from nitrate supplementation if nitrate exposure is increased above what has currently been tested.

Furthermore, the possibility that training status could affect the range of time trial distances where nitrate supplementation could enhance performance cannot be ignored. Nitrate supplementation has failed to improve cycling and running performance in well-trained subjects where the time to complete a given distance is
in the range of ~15 min – 2 h (Christensen et al., 2012; Peacock et al., 2012; Bescos et al., 2012; Cermak et al., 2012; Wilkerson et al., 2012). Moderately trained subjects have shown improvements in the time to complete time trials lasting 6 – 30 min (Lansley et al., 2011; Cermak et al., 2012a). However, a direct assessment of the effect of time trial length on performance following nitrate supplementation has not been investigated. Furthermore, it is impossible to draw certain conclusions about the effect of time trial distance by comparing these studies because they are confounded by differences in training status and supplementation protocol. Nitrate supplementation could be most effective in time trial distances completed at higher intensities resulting in greater recruitment of type II fibers considering that nitrate supplementation has been shown to preferentially improve blood flow (Ferguson et al., 2012, 2013) and contractility (Hernandez et al., 2012) in type II fibers. In addition, the NO$_3^-$, NO$_2^-$, NO pathway contributes to NO production in acidic and hypoxic conditions. Therefore, the greatest benefit following nitrate supplementation may be for shorter duration endurance competitions that elicit acidic and hypoxic local skeletal muscle environments. In fact, the greatest improvements in cycling time trial performance following BR supplementation are predicted to be for events lasting ~4 min (Kelly et al., 2013).

A better understanding of the underlying mechanisms that confer improved exercise efficiency and performance following nitrate supplementation will improve the understanding of individual variability in response. Interestingly, out of 8 highly trained cyclists (mean VO$_2$ peak = 72 ml/kg/min), the same 2 subjects (VO$_2$ peak =
71 and 74 ml/kg/min) who improved their time to complete 400 kcal of work were also the only two subjects to have a reduction in VO₂ while cycling at 50 and 70% of Wmax (Christensen et al., 2012). This finding reveals two important facts. First, some highly trained subjects can improve performance with BR supplementation. Second, improved performance is seen in individuals who have increased submaximal cycling economy indicating that the underlying mechanisms responsible for improved efficiency lead to improved performance.

Chapter 2: Statement of the Problem

Rationale

Nitrate supplementation, in the form of nitrate-rich BR, has received considerable attention in the literature and become increasingly popular among athletes. BR ingestion could represent a legal and healthy way to improve performance in elite athletes. Therefore, the purpose of the current study was to determine the effects of acute and chronic BR supplementation on submaximal running and 1500 m time trial performance in elite middle distance runners. On testing days subjects of this study received a ~19.5 mmol dose of nitrate, in the form of 210 ml of concentrated nitrate rich BR juice. Subjects supplemented with ~13 mmol NO₃⁻ (140 ml of BR) on non-testing days. The dose given on testing days was higher than that given in any previous study in trained individuals. A higher dose was given because previous evidence showed a lack of effect of nitrate supplementation in trained individuals given 5 – 8 mmol NO₃⁻ (Cermak et al., 2012b;
Wilkerson et al., 2012; Bescos et al., 2012; Christensen et al., 2012; Peacock et al., 2012). In addition, increasing the dose eliminated non-responders to performance in recreationally active subjects (Wylie et al., 2013b).

An acute and chronic supplementation protocol was prescribed to establish the effects of supplement duration. Acute BR supplementation has been shown to improve performance in moderately trained individuals (Lansley et al., 2011). It was important to test the acute effects of BR with elite athletes because if acute BR supplementation could confer the same enhancement as chronic that would be practically meaningful. It was also important to include a chronic supplementation protocol since acute supplementation had failed to elicit an improvement in performance in well-trained cyclists (Cermak et al., 2012b; Wilkerson et al., 2012; Peacock et al., 2012). In addition, certain underlying mechanisms postulated to confer benefit rely on protein modifications. Supplementation would be more likely to produce these changes over a longer duration than 2.5 h after an acute dosage. Therefore, to increase the likelihood of detecting an effect of BR a chronic supplementation protocol was also used.

Subjects supplemented with BR and placebo in a double blind, randomized, cross over design. The placebo beverage was a nitrate-depleted beetroot juice supplied in identical packaging and indistinguishable by taste, smell or appearance. In this way the subjects and experimenters were truly blinded to the conditions. BR was selected rather than sodium nitrate because BR is the form that athletes are
currently using to supplement in the field. In this way the study provided the most practically relevant results for athletes. Although the efficacy of BR and sodium nitrate has never been compared directly, the physiological responses following BR supplementation are similar if not better when compared to sodium nitrate (Kapil et al., 2010; Wylie et al., 2013b).

The subjects recruited to participate in this study were all current elite level track runners. These individuals currently competed at the provincial, national and/or international level in athletics. This population was selected because the effects of supplements on performance are most practically important to elite athletes. Despite recent null findings of the effect of BR supplementation with well-trained cyclists it was important to determine the effects on running. Presently, no study has investigated the effects of BR supplementation on running specific performance in elite runners. In addition, evidence suggested that some highly trained athletes do respond to BR supplementation (Christensen et al., 2012). In a practical sense identifying responders is also meaningful. To test the effects of BR supplementation in elite trained runners, subjects completed a submaximal treadmill run at three running speeds and a 1500 m individual time trial on an indoor 200 m track. During the submaximal run oxygen consumption was measured to determine if BR supplementation reduced the oxygen cost of running. Importantly, in elite cyclists, only subjects who had improved exercise efficiency at submaximal work loads improved time trial performance (Christensen et al., 2012).
Thus, to identify any potential responders it was important to assess exercise efficiency.

An individual 1500 m time trial was chosen to test the effects of BR supplementation on performance. This distance was selected because previous research suggests that the potential ergogenic effects might be limited to exercise that recruits fast twitch muscle fibers (Ferguson et al., 2012; Hernandez et al., 2012). In addition, exercise that creates an acidic and/or hypoxic local muscle environment is proposed to increase the contribution of the nitrate – nitrite – NO pathway to NO production (Lundberg et al., 2008). Finally, Kelly and colleagues (2013) predicted that the greatest improvement following BR supplementation would be for time trial performances lasting ~4 min. Furthermore, a time trial was chosen to assess performance because it has higher ecological validity compared to time to exhaustion tests (Currell and Jeukendrup, 2008). Overall, the testing protocol and subjects were selected to provide the most practically relevant evidence of the effect of BR supplementation on middle distance running performance.
Hypotheses

1) Plasma [NO$_3^-$] will be significantly increased compared to basal concentrations following dietary nitrate supplementation in the form of concentrated nitrate-rich BR (providing 19.5 mmol NO$_3^-$) in elite distance runners.

2) Neither acute or chronic BR supplementation will improve the time to complete a 1500 m individual time trial in elite distance runners.

3) Acute or chronic BR ingestion will not reduce VO$_2$ during submaximal treadmill running.
Chapter 3: Methods

Subjects

Ten elite male distance runners were recruited to participate in this study. One subject withdrew from the study after completing only 1 of 4 experimental trials due to injury. One other subject could only complete 2 of 4 experimental trials due to training and competition conflicts. Therefore, all data and analysis presented is for the eight subjects who completed all 4 experimental trials (mean ± SD: age = 23.8 ± 5 years old; weight = 65.7 ± 7 kg; VO₂ peak = 80 ± 5 ml/kg/min; 1500 m personal best (PB), 3:56 ± 9 s). Subjects were all club athletes competing in provincial, national or international caliber events and had experience competing in the 1500 m dash. Subjects completed 12.3 ± 4 h of training each week. All subjects were given an explanation of the requirements and potential risks of the study and written informed consent was obtained. The study was approved by the Research Ethics Board of the University of Guelph.

Pre-experimental Tests

Initially, each subject completed an incremental running test to exhaustion on a treadmill (Livestrong LS13.0T, Johnson Health Tech, USA) for determination of VO₂ peak and the running speed required to elicit 50, 65 and 80% of VO₂ peak. Subjects began running at 14.3 km/h and incline of 0% and the speed and incline
was increased incrementally until voluntary exhaustion. Ventilation and expired oxygen and carbon dioxide concentrations were measured (Moxus Modular VO₂ System, AEI Technologies, Pittsburgh, USA) for the duration of the treadmill run. VO₂ peak was determined as the greatest VO₂ averaged over 40 s. For the purpose of familiarization, subjects completed a practice exercise test consisting of a complete submaximal treadmill run and 1500 m time-trial (described in detail below).

**Study Design**

In a randomized, double-blind, crossover design, subjects supplemented with concentrated BR (0.4 g NO₃⁻ / 70 ml; Beet It Sport, James White Drinks, Ipswitch, UK) or a nitrate-free BR placebo (PL) (0.004 g NO₃⁻ / 70 ml, Beet It Sport) for eight days separated by 4 ± 4 weeks (Figure 6). The placebo drink was created by passing the juice through an ion exchange resin that selectively removed the nitrate ions (Lansley et al., 2011). The BR and PL drinks were supplied in identical packaging and indistinguishable by taste, smell or appearance. The supplementation order was counterbalanced such that 4 subjects began supplementing with BR and 4 began with PL. To test the acute and chronic effects of BR supplementation subjects completed a submaximal treadmill run and 1500 m time-trial on days 1 and 8 of each phase. On days 1 and 8 subjects consumed 210 ml of BR (1.2 g NO₃⁻) or PL, 2.5 hr prior to the 1500 m time-trial. Subjects were instructed to consume the drink within 20 min. On days 2 - 7 subjects consumed
140 ml of BR (0.8 g NO$_3^-$) or PL with lunch. Experimental trails were separated by at least 7 days to ensure that the subjects had adequate recovery and to minimize disturbances in the subjects training routines.

Figure 6 - Schematic overview of the experimental protocol. Subjects completed two eight day supplementation phases separated by a washout 4 ± 4 week washout period. On the first and eighth days of supplementation for each phase subjects completed a submaximal treadmill run and individual 1500 m running time trial (denoted by ↓)

**Dietary and Training Standardization**

Subjects were instructed to refrain from using antibacterial mouthwash and chewing gum during the supplementation period because these have been shown to disrupt nitrite bioavailability by killing the bacteria in the mouth required to convert nitrate to nitrite (Govoni et al., 2008; Webb et al., 2008). In addition, subjects were asked to abstain from using BR or other supplements during the course of the study. However, subjects were not instructed to reduce their intake of nitrate rich foods so that the study reflects the most practical application of BR supplementation. This is consistent with previous work (Lansley et al., 2010). Prior to exercise tests, subjects were advised to eat and drink as they normally would when preparing for a competition. Subjects recorded their food intake for the 36 h
preceding the practice trial and were asked to replicate this diet for all subsequent experimental trials. Subjects ingested a diet abundant in carbohydrate so that glycogen supply would not be limiting during exercise. Urine specific gravity was measured upon arrival at the lab and confirmed that subjects arrived similarly hydrated for all trials (1.014 ± 0.006). Training during the 3 days prior to the first exercise test was recorded and replicated as closely as possible with respect to intensity and volume for subsequent trials. In the 24 h prior to the exercise test, subjects were asked not to complete any exhaustive exercise and train as though preparing for a competition. Exercise tests were performed on the same day of the week and at the same time of day to maintain the subject’s normal training routine.

**Exercise Tests**

Subjects arrived at the lab (20.9 ± 0.4 °C, 22 ± 2% R.H.) 1.5 h after ingesting the BR or PL drink to begin the submaximal treadmill run (Figure 2). The run was designed to simulate a typical pre-competition warm-up and allow for VO₂ measurements at three different running speeds. The run consisted of 19 min of continuous treadmill running. Subjects ran for 7 min at 50% VO₂ peak followed without stopping by 7 min of running at 65% VO₂ peak and 5 min at 80% VO₂ peak. The running speed corresponding to 50, 65 and 80% VO₂ peak was the same in all four trials. Running speed was 10.7 ± 1.3, 14.2 ± 1.2 and 17.5 ± 1.3 km/h for 50, 65 and 80% VO₂ peak, respectively. The treadmill incline was set to 1% to simulate running on a level surface due to lack of air drag while running on a treadmill.
Ventilation and expired oxygen and carbon dioxide concentration were measured (Moxus Modular VO₂ System) for the duration of the treadmill run and measurements were averaged over 40 s intervals. Errant breaths caused by coughing or swallowing were adjusted following visual inspection. For each running intensity, VO₂, VCO₂ and RER were calculated as the average over the last 120 s at that running speed. HR was recorded with 60 s remaining at each running speed (RS4000sd, Polar, Kempele, Finland). Immediately following the treadmill run subjects walked from the lab to the indoor track (17.7 ± 1.9 °C, 27 ± 8% R.H.) and were given time to complete their warm-up routine consisting of dynamic stretching and strides. At 2.5 h post BR or PL ingestion, subjects began the individual 1500 m run time trial on an indoor 200 m track (Figure 7). Subjects were told the number of laps remaining in the run. To minimize the variability in pacing strategy subjects were given their elapsed time at 200, 400 and 600 m only (Peacock et al., 2012). No further feedback regarding performance was given. Subjects were not allowed to wear their own watches during the time trial. Subjects received standard encouragement to complete the time trial as quickly as possible and the time to complete the 1500 m distance was recorded. Following the time trial, subjects completed a questionnaire to determine if they were blinded to the supplementation condition.
Figure 7 – Schematic overview of the exercise test. On four separate occasions subjects complete a submaximal treadmill run at three increasing speeds corresponding to 50, 65 and 80% of maximal oxygen uptake followed by an individual 1500 m time trial on an indoor track.

**Blood Sampling**

Blood sampling was carried out at a separate time following the exercise testing. Subjects supplemented for 8 days as described above but only with BR. On days 1 and 8, subjects arrived at the lab and a ~4 ml baseline blood sample (t = 0) was collected from the antecubital vein into a sodium-heparinized tube. On day 8, subjects arrived 23.5 ± 1.5 h after supplementing with BR the day before. Following the initial blood sample, subjects were provided with 210 ml of BR to be ingested within 20 min. At 1.5 h a second blood sample was collected just prior to the subjects completing the submaximal treadmill run. A final blood sample was collected at 2.5 h post ingestion corresponding to the time at which the 1500 m run would be completed. However, for the blood sampling procedure subjects did not complete the 1500 m time trial. All blood samples were centrifuged for 4 min at
10,000 rpm. The plasma was collected and filtered using a centrifuge filter tube (Millipore, Amicon Bioseperations, Billerica, USA) with a molecular weight cut-off of 30 kD and spun for 10 min at 14,000 g. The filtered plasma was collected and frozen at -80°C for later analysis.

**Plasma Analysis**

Plasma samples were analyzed for nitrate + nitrite (NOₓ) concentrations. Nitrate concentrations in the blood are in the µM range, while nitrite concentrations are in the nM range. Therefore, plasma NOₓ very closely represents plasma nitrate levels. Filtered plasma samples were analyzed fluorometrically for NOₓ content using a commercially available Nitrate/Nitrite Assay Kit (Caymen Chemical, Item NO. 780051, Ann Arbor, USA). Briefly, plasma samples were appropriately diluted with assay buffer (20 mM KH₂PO₄, pH = 7.4) and incubated for 2.5 h with nitrate reductase and the enzyme cofactor to convert all plasma nitrate to nitrite. Following incubation, 2,3-Diaminonaphthalene was added for fluorometric detection of nitrite. A spectrofluorometer (SpectraMax M2e) was used to determine nitrite concentration at an excitation wavelength of 365 nm and emission wavelength of 430 nm. Plasma [NO₂] was also measured fluorometrically but reliable results could not be obtained with this technique and are not reported.
Statistical Analysis

Differences in plasma NO\textsubscript{x}, VO\textsubscript{2}, VCO\textsubscript{2}, RER and HR were analyzed using a two-way (supplement by time) ANOVA. Differences in the time to complete the 1500 m time trials were assessed using a one-way ANOVA. Statistical tests were performed using Stat-Plus (Analysoft, USA). Statistical significance was accepted when P < 0.05. All data are presented as mean ± SD.

Chapter 4: Results

Plasma [NO\textsubscript{3}]

Baseline plasma [NO\textsubscript{3}] on day 1 (acute) prior to any supplementation was 37 ± 15 µM (Figure 8). Following BR ingestion plasma [NO\textsubscript{3}] increased significantly compared to baseline at 90 and 150 min (615 ± 151 and 569 ± 64 µM, respectively). There was no significant difference in plasma [NO\textsubscript{3}] between 90 and 150 min. Following chronic BR supplementation (day 8) baseline plasma [NO\textsubscript{3}] was 270 ± 182 µM (Figure 8). Plasma [NO\textsubscript{3}] was significantly increased compared to baseline at 90 and 150 min post ingestion (870 ± 259 and 842 ± 243 µM, respectively). There was no significant difference in plasma [NO\textsubscript{3}] between 90 and 150 min. At all time points, plasma [NO\textsubscript{3}] was significantly greater for chronic compared to acute supplementation.
Figure 8 - Plasma [NO$_3^-$] at baseline (t = 0 min) and 90 and 150 min post ingestion of 210 ml concentrated BR for acute and chronic supplementation. Values are mean ± SD. For both acute and chronic supplementation plasma [NO$_3^-$] is significantly greater at 90 and 150 min compared to baseline (*a). At all time points plasma [NO$_3^-$] was significantly greater for chronic compared to acute (*b).

**1500 m Time Trial Performance**

The time to complete the 1500 m individual time trial was 250.7 ± 4.3 s following acute BR, 250.5 ± 6.2 s following chronic BR, 250.4 ± 7.0 s following acute PL and 251.4 ± 7.6 s following chronic placebo supplementation (Figure 9). There was no significant difference in the time to complete the 1500 m run between any of the four conditions. In addition, no order effect was observed in the time to complete the 1500 m run. There was no significant difference between time trial 1, 2, 3 and 4, regardless of the condition.
Submaximal Treadmill Run

There was no significant difference in VO$_2$ at any time between any of the four supplementation conditions during treadmill running at speeds corresponding to 50, 65 and 80% of VO$_2$ peak (Figures 10, 11 and 12). In addition, VCO$_2$, RER and HR were unchanged at all three running speeds between all four supplementation conditions (Table 1). Regardless of condition, VO$_2$, VCO$_2$ and HR were increased during running at 65% VO$_2$ peak compared to 50% and further increased for running at 80% compared to 50 and 65%. RER was significantly greater during running at 80% VO$_2$ peak compared to 50 and 65%. However, the increase in RER during running at 65% compared to 50% did not reach statistical significance.
Figure 10 - Oxygen uptake for subjects running at 50% VO₂ peak (10.6 ± 1.3 km/h) following acute and chronic PL and BR supplementation. Values are mean ± SD. No significant difference between any condition at any time.

Figure 11 - Oxygen uptake for subjects running at 65% VO₂ peak (14.0 ± 1.2 km/h) following acute and chronic PL and BR supplementation. Values are mean ± SD. No significant difference between any condition at any time.
Figure 12 - Oxygen uptake for subjects running at 80% VO₂ peak (17.5 ± 1.3 km/h) following acute and chronic PL and BR supplementation. Values are mean ± SD. No significant difference between any condition at any time.

**Supplementation Blinding**

Subjects could not consistently distinguish between the nitrate depleted PL drink and the nitrate rich BR drink. When asked which beverage they had consumed subjects responded that they were unsure 14 out of 32 times. Subjects correctly identified the drink they had received 9 times but responded incorrectly 9 times.
Table 1 – Physiological responses to running at 50, 65 and 80% of VO$_2$ peak following acute and chronic PL and BR supplementation. Values are mean ± SD. Measurements for VO$_2$, VCO$_2$ and RER are the average of the last 120 s at each running intensity. HR was taken with 60 s remaining at each intensity. There are no significant differences between any condition.

<table>
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<tr>
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<th>Intensity (% VO$_2$ peak)</th>
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<tr>
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<tr>
<td><strong>VO$_2$ (ml/min)</strong></td>
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<tr>
<td>Acute PL</td>
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<tr>
<td>Chronic PL</td>
<td>2596 ± 206</td>
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<tr>
<td>Acute BR</td>
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</tr>
<tr>
<td>Chronic BR</td>
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<tr>
<td><strong>VCO$_2$ (ml/min)</strong></td>
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<tr>
<td>Acute PL</td>
<td>2160 ± 257</td>
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<tr>
<td>Chronic PL</td>
<td>2257 ± 199</td>
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<tr>
<td>Acute BR</td>
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<tr>
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<tr>
<td><strong>RER</strong></td>
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<td><strong>HR (beats/min)</strong></td>
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<tr>
<td>Acute BR</td>
<td>129 ± 14</td>
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<td>Chronic BR</td>
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Chapter 5: Discussion

The principle finding of this study was that nitrate supplementation with NO₃⁻-rich BR did not improve submaximal exercise efficiency or 1500 m time trial performance in elite distance runners. This finding is novel because this is the first study to test the effects of BR supplementation in elite distance runners (VO₂ peak > 75 ml/kg/min) using a running specific test of performance. The lack of effect of BR supplementation on performance is consistent with recent publications in well-trained competitive cyclists and cross-country skiers (Wilkerson et al., 2012; Cermak et al, 2012b; Peacock et al., 2012; Bescos et al., 2012; Christensen et al., 2012).

In recreationally active and moderately trained subjects nitrate supplementation improved exercise efficiency and performance, potentially by reducing the ATP cost of contraction and/or improving mitochondrial respiratory efficiency via increased NO bioavailability (Bailey et al., 2010; Larsen et al., 2011). The findings of the current study support the idea that in well-trained individuals, the training adaptations that accompany endurance exercise have diminished the possible benefit of nitrate supplementation (Wilkerson et al., 2012; Cermak et al, 2012b; Peacock et al., 2012; Bescos et al., 2012; Christensen et al., 2012). It is possible that nitrate supplementation fails to elicit a response in well-trained subjects because supplementation does not augment the use of nitrite from endogenous sources for production of NO during exercise. In other words, there is a
threshold where further increases in NO$_2^-$ via dietary nitrate supplementation will not confer additional improvements.

**Nitrite Utilization**

Dietary sources of nitrite may not enhance NO bioavailability in well-trained individuals for two reasons. First, trained individuals exhibit higher baseline plasma [NO$_3^-$] compared to untrained individuals. Plasma nitrate was 41% higher in endurance athletes compared to untrained control subjects (23.2 and 16.4 µM for athletes and control, respectively) (Poveda et al., 1997). Also, exercise training has been shown to increase eNOS expression and activity leading to higher plasma [NO$_2^-$] and [NO$_3^-$] (Green et al., 2004). Second, endurance training may diminish the reliance on the nitrate – nitrite – NO pathway during exercise because the development of hypoxic loci within the skeletal muscle may be reduced (Wilkerson et al., 2012). Potential improvements in muscle perfusion and O$_2$ distribution afforded by nitrate supplementation may not be observed in well-trained subjects because endurance training increases skeletal muscle capillary density (Brodal et al., 1977). Therefore, blood flow and O$_2$ distribution may already be optimal in well-trained individuals. Unfortunately, determining an individual’s capacity to utilize nitrite is difficult considering that plasma [NO$_2^-$] is a product of nitrate reduction and NO oxidation from the classical NOS pathway (Wylie et al., 2013b). Regardless, high basal [nitrate] and [nitrite] and/or diminished reliance on nitrite during
exercise in well-trained individuals may reduce the efficacy of nitrate supplementation.

**Plasma [Nitrate]**

Previous studies in well-trained subjects have suggested that these individuals may require greater nitrate exposure via a higher dose and/or increase supplement duration in order to see a performance benefit (Wilkerson et al., 2012; Cermak et al., 2012b; Peacock et al., 2012; Bescos et al., 2012; Christensen et al., 2012). In the current study baseline plasma [NO$_3^-$] prior to acute supplementation was 37 ± 15 and similar to the average of five studies with other well-trained subjects (37 ± 6 µM) (Cermak et al., 2012b; Bescos et al., 2012a, 2012b; Peacock et al., 2012; Christensen et al., 2012). With supplementation of 5 – 11 mmol NO$_3^-$ in previous studies with well-trained subjects, plasma [NO$_3^-$] increased to 261 ± 66 at 2.5 h post ingestion (Δ[NO$_3^-$] from baseline = 224 µM). In the present study, subjects were provided a dose of 19.5 mmol NO$_3^-$, nearly double the highest dose reported in studies of well-trained subjects. Accordingly, the increase in plasma [NO$_3^-$] was more substantial as plasma [NO$_3^-$] increased to 569 ± 64 and 842 ± 243 µM at 2.5 h post ingestion for acute and chronic supplementation, respectively (Δ[NO$_3^-$] from baseline = 532 and 572 µM for acute and chronic, respectively). Despite the larger increase in plasma [NO$_3^-$], no improvement in exercise efficiency or performance was observed in the present study. In addition, we directly compared the effects of acute and chronic (8 days) nitrate supplementation. Interestingly, plasma [NO$_3^-$]
had not returned to pre-supplementation levels at 24 h after BR ingestion the day before subjects arrived at the lab for baseline blood sampling during chronic supplementation (37 ± 15 and 270 ± 182 µM for acute and chronic baseline, respectively). This finding indicates that plasma [NO$_3^-$] exposure was significantly increased for the duration of the chronic supplementation. However, our results show that increasing nitrate exposure by extending the duration of supplementation did not reduce the O$_2$ cost of submaximal treadmill running or improve the time to complete a 1500 m running time trial. This evidence further supports the idea that increased plasma [NO$_2^-$] via dietary supplementation cannot improve efficiency or performance because it does not augment nitrite use from endogenous sources in well-trained individuals.

**Time Trial Duration**

Evidence in recreationally active subjects predicts that the ergogenic effects of BR supplementation are maximized for time trials lasting ~4 min (Kelly et al., 2013). Despite this fact, our results show that the time to complete a 1500 m run, which took the subjects 4 min 10 s, was not improved following BR supplementation. In addition, other studies of well-trained subjects have failed to show improved performance during time trials lasting ~15, 20, 40, 60 minutes and 2 h 15 minutes following nitrate supplementation. These observations taken together suggest that the efficacy of nitrate supplementation in well-trained subjects is not dependent on time trial length because nitrate ingestion has failed to improve
performance over a wide range of time trial durations (Wilkerson et al., 2012; Cermak et al., 2012b; Peacock et al., 2012; Bescos et al., 2012; Christensen et al., 2012).

**Individual Responders**

Although mean VO$_2$ and time trial performance was unaffected by BR supplementation two potential "responders" were identified within the group of elite runners. These subjects were the only two subjects to show improved 1500 m run performance following both the acute and chronic BR compared to acute and chronic PL. Performance was improved by 5.8 and 7.0 s (acute and chronic) and 4.99 and 0.45 s (acute and chronic) for the two responders. The coefficient of variation for 1500 m running performance for elite males is 0.9% (Hopkins, 2005) and the smallest worthwhile enhancement in performance for elite 1500 m runners is 0.3 - 0.5 of the CV (Hopkins et al., 1999). This corresponds to a 0.68 – 1.13 s improvement in time to complete the 1500 m run in the current study. Therefore, the improvement in performance following BR supplementation could be practically meaningful during actual competition for the two responders.

VO$_2$ also tended to be decreased for the two responders. For one responder VO$_2$ was unchanged during running at 50% VO$_2$ peak but decreased 52 ml and 88 ml for running at 65% VO$_2$ peak and decreased 211 ml and increased 35 ml for running at 80% VO$_2$ peak following acute and chronic BR supplementation, respectively. The
other responder had 42 and 157 ml decreases in VO₂ for running at 50% VO₂ peak, 96 and 210 ml reductions at 65% and 25 and 66 ml decreases in VO₂ for running at 80% VO₂ peak following acute and chronic BR supplementation, respectively. In the current study the coefficient of variation for VO₂ between the acute and chronic PL trials, where presumably there was no effect of condition, was 3, 4 and 5% for running at 50, 65 and 80% of VO₂ peak, respectively. The corresponding measurement variability for VO₂ was 70, 163 and 164 ml at 50, 65 and 80%, respectively. For the two responders the magnitude of the reduction in VO₂ was greater than the measurement variability at some times but not always. Therefore, repeated testing of these two subjects could determine if a true reduction in VO₂ following BR supplementation occurs within these individuals.

These results support the findings of Christensen and colleagues (2012) who identified 2 "responders" out of a group of 8 highly trained cyclists. Similarly, the only two subjects to demonstrate improved exercise efficiency during submaximal cycling at 50 and 70% incremental test peak power were also the only two subjects who had improved time trial performances. Taken together these findings are important for two reasons. First, although the likelihood of seeing a response may be reduced, it is possible that elite runners and cyclists can respond to nitrate supplementation. Second, improved performance was seen in individuals who had increased submaximal exercise efficiency. In the current study, responders could not be distinguished by baseline or increase in plasma [NO₃⁻]. However, the two responders tended to have slower 1500 m personal bests (5th and 6th fastest out of
and lower VO\textsubscript{2} peaks (6\textsuperscript{th} and 8\textsuperscript{th} highest out of 8; 80.1 and 69.2 ml/kg/min, respectively) compared to other subjects of the study. In addition, the two responders had lower self-reported training volume for both h/week and number of years (6\textsuperscript{th} and 8\textsuperscript{th} highest out of 8). It is possible that BR ingestion improved performance in the two responders of the current study because they were less adapted to endurance training compared to the other subjects. Further research is required to fully explain the observed individual variability following nitrate supplementation.

**Limitations**

One limitation of the current study is that plasma [NO\textsubscript{X}], which closely approximates [NO\textsubscript{3}⁻], was measured rather than [NO\textsubscript{2}⁻]. It is possible that subjects who had increased plasma [NO\textsubscript{3}⁻] did not have a subsequent increase in plasma [NO\textsubscript{2}⁻]. However, the possibility of this occurrence was probably small given the large dose of nitrate ingested. In any case, this only inhibited our ability to determine the reason why subjects may not respond to nitrate supplementation. No change in plasma [NO\textsubscript{2}⁻] may preclude individuals from responding positively to BR ingestion (Wilkerson et al., 2012). However, this does not limit the ability to detect responders because baseline and increase in plasma nitrite cannot distinguish between responders and non-responders (Bescos et al., 2012; Christensen et al., 2012). Rather, improvement in submaximal exercise efficiency may be the best way to predict who will have improved time trial performance (Christensen et al., 2012).
In the current study the coefficient of variation for VO\textsubscript{2} between the acute and chronic PL trials, where presumably there was no effect of condition, was 3, 4 and 5% for running at 50, 65 and 80% of VO\textsubscript{2} peak, respectively. So, the change in VO\textsubscript{2} following BR supplementation would have to be greater than 70, 163 and 164 ml at 50, 65 and 80%, respectively, to detect an effect. Therefore, it is possible that the current methodology was not sufficiently sensitive to detect small reductions in VO\textsubscript{2} following nitrate supplementation.

The present study used a 1500 m individual time trial to assess performance which has been shown to have higher practical validity and reliability compared to time to exhaustion tests (Currell and Jeukendrup, 2008). The testing protocol was very repeatable for the elite trained subjects of the current study as the coefficient of variation for all trials was 0.97% and matches that reported for actual competition for elite male runners (0.9%) (Hopkins, 2005).

**Conclusion**

The principle finding of the current study is that supplementation with nitrate-rich BR did not improve submaximal treadmill running efficiency or 1500 m time trial performance in 8 elite male distance runners when examined on a group basis. This finding is novel because we are the first to investigate the effects of nitrate supplementation in elite runners using a running specific test of
The lack of improvement in exercise efficiency or performance observed in the current study corroborates the observations in well-trained cyclists and cross-country skiers (Wilkerson et al., 2012; Cermak et al., 2012b; Peacock et al., 2012; Bescos et al., 2012; Christensen et al., 2012). Furthermore, the lack of improvement in exercise efficiency or performance cannot be attributed to insufficient nitrate exposure as plasma $[\text{NO}_3^-]$ following BR ingestion in the current study was 2.2 and 3.3 fold greater for acute and chronic, respectively, than that previously reported in studies of well-trained subjects. Importantly, two responders in our group of elite runners were identified. This indicates that BR supplementation may be effective in a small proportion of elite athletes. However, the explanation for this individual variability is unclear. Although the likelihood of elite trained athletes responding to nitrate supplementation is reduced one cannot ignore the psychological importance of the placebo effect. So, considering that vegetables are an important part of a healthy diet there is no reason for an athlete to avoid BR consumption.
References


