THE EFFECT OF BEETROOT JUICE ON EXERCISE PERFORMANCE IN NORMOXIA AND MODERATE HYPOXIA

by

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Abstract

The effect of beetroot juice on exercise tolerance in normoxia and moderate hypoxia

AIMS:
Beetroot juice (BR) has been shown to lower the oxygen cost of exercise in normoxia, and may also be beneficial to performance in hypoxia. We investigated the effect of BR on steady state economy and 10-km time trial (TT) performance in normoxia and in hypoxia (simulated altitude of 2500 m).

METHODS:
Twelve trained male cyclists ($VO_{2max} \geq 60$ ml·kg^{-1}·min^{-1}) completed four exercise trials. Two hours prior to exercise, subjects consumed 70 mL BR (~6 mmol nitrate) or 70 mL placebo (nitrate-depleted BR) in a randomized, double-blind manner. Subjects then completed a 15-min self-selected cycling warm-up followed by a 15-min steady-state exercise bout at 50% of maximum power output and a 10-km time trial (TT) in either normoxia (~21% O_2) or hypoxia (~16% O_2). Environmental conditions were randomized and single blind.

RESULTS:
Economy at 50% power output was similar in hypoxic and normoxic conditions ($p > 0.05$), but subjects had a significantly higher mean power output in the normoxic TT relative to the hypoxic TT ($p < 0.05$). BR did not affect economy, mean power output, or time to complete the 10-km TT relative to placebo in normoxia ($p > 0.05$ in all comparisons). Similarly, in hypoxia, BR did not affect economy, steady state SpO_2, mean power output or time to complete the 10-km TT relative to placebo ($p > 0.05$ in all comparisons). BR supplementation resulted in a significantly greater fraction of expired nitric oxide relative to PL (28 [12] *vs*. 22 [9] ppb).

CONCLUSIONS:
In a small sample of well-trained male cyclists, BR did not improve exercise performance in normoxia or hypoxia. This finding is possibly related to BR dosage, although it is congruent with previous evidence showing that BR may be less effective in trained populations in normoxia. The lack of an effect in hypoxia was contrary to our hypothesis.

Preface

Collaborators and co-authors of this thesis study are as follows:

- Dr. James Rupert, PhD, supervised this project, assisted in the ethics application, helped with the funding application to Own the Podium (OTP), and provided guidance during sample analysis and writing.
- Dr. Michael Koehle, MD, PhD, aided in the development of the research design and assisted with equipment trouble-shooting and data analysis.
- Dr. Benjamin Sporer, PhD, assisted in development of the research design, provided insight into the practical application of research and helped with the application for funding through OTP.
- Dr. Susan Barr, PhD, assisted in the development of the research design from a sport nutrition standpoint.
- Martin MacInnis, BSc, assisted in the development of the research design, ethics approval, chamber reconfiguration, data collection, data analysis, and provided feedback during the writing process.
- Sean Nugent assisted in data collection.
- Kirsten Hogg, PhD, assisted with plasma nitrate and nitrite assays for plasma sample analysis
- Kristin MacLeod, BSc, developed the research design, obtained funding from OTP and the UBC Faculty of Education, completed the application for ethics approval, recruited subjects, performed data collection, assisted with assay analysis, analyzed data and wrote the final thesis document.

No manuscripts resulting from the work presented in this thesis have been published to date. The abstract and poster attached in Appendix A were accepted for a poster presentation at the Sport Innovation (SPIN) Summit organized by OTP in September 2013, in Calgary, Canada.

This study involved human subjects and was granted full board approval from the University of British Columbia Clinical Research Ethics Board (H12-01832).

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List of abbreviations

ADP: adenosine diphosphate

ATP: adenosine triphosphate

BP: blood pressure

-DBP: diastolic blood pressure

-SBP: systolic blood pressure

BR: beetroot juice

cGMP: cyclic guanosine monophosphate

CREB: Clinical Review Ethics Board

FENO: fraction of expired nitric oxide

HR: heart rate

L-NAME: L-NG nitroarginine methyl ester

NIRS: near-infrared spectroscopy

NO: nitric oxide

NO_2^-: nitrite

NO_3^-: nitrate

NOS: nitric oxide synthase (pathway)

PCr: phosphocreatine

P_i: inorganic phosphate

PL: placebo

PO_2: partial pressure of oxygen

SD: standard deviation

SpO_2: pulse oxygen saturation

TOI: tissue oxygenation index

TT: time trial

UBC: University of British Columbia

V_E: pulmonary ventilation

$\dot{V}O_2$: oxygen uptake

$\dot{V}O_{2max}$: maximal oxygen uptake

W: watts

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1. Introduction

1.1 Overview

At altitude, decreases in the barometric pressure and the alveolar partial pressure of oxygen result in less oxygen reaching the tissues (reviewed in Sheel *et al*., 2010). The drop in available oxygen detrimentally affects aerobic capacity. For example, $\dot{V}O_{2max}$ decreases by ~6%/1000 m between 300 m and 2800 m above sea level (Wehrlin and Hallén 2006). These factors combine to reduce overall training quality, particularly with acute hypoxic exposure in absence of acclimatization. As athletes often train and compete at moderate altitude (i.e., 1500-2500 m), the ability to maintain high exercise intensity is important for performance.

Beetroot juice (BR), a supplement with a naturally high concentration of nitrate (NO_3^-) has gained popularity with endurance athletes aiming to improve performance. There is evidence to suggest that dietary NO_3^- improves oxygen delivery (Masschelein 2012), lowers blood pressure during exercise recovery (Larsen *et al*., 2010; Kenjale *et al*., 2011), and increases mitochondrial efficiency through an improved oxidative phosphorylation ratio (Larsen *et al*., 2011). When oxygen is less available from the environment (e.g. at altitude), BR may have even greater physiological effects (Dauncey 2012); however, the efficacy of acute BR supplementation for exercise performance in moderate hypoxia requires further research. As hypoxia limits aerobic capacity, the potential benefits of BR are particularly relevant to aerobic exercise.

1.2 Aerobic exercise

Aerobic exercise, which involves continuous whole body or large muscle mass exercise, stresses the cardiorespiratory system. Generally, moderate intensity constant-load exercise can be sustained for relatively long periods of time due to the predominant use of fatigue resistant slow-twitch muscle fibres. These fibres have a slower contraction speed, meaning they utilize ATP at a slower rate; therefore, sufficient ATP production can be maintained through aerobic metabolism (McArdle *et al*., 2007). Over time, the

result of endurance training is increased aerobic capacity, or an improved ability to maintain a given power output or velocity (Jones and Carter 2000). Improved aerobic capacity is indicative of better endurance performance (reviewed in Jones and Carter 2000), making improved aerobic capacity a central goal of many training programs.

Exercise causes an acute stress response throughout the body. At rest, energy production requires oxygen, which is brought in through the lungs and diffuses to the blood within the pulmonary vasculature. The oxygenated blood flows back to the heart where it is pumped into the systemic circulation (McArdle *et al.,* 2007) where the oxygen diffuses into the tissue. Exercise increases the demand for oxygen, and the body responds with an increase in tidal volume, breathing frequency, heart rate and stroke volume, all of which help to maintain sufficient blood flow and oxygen delivery to support the working muscles (West 2007). Improved oxygen delivery is supported by increased extraction at the tissue level, which also facilitates the maintenance of elevated energy production. During aerobic exercise the main energy pathway is oxidative phosphorylation. At low, continuous exercise intensities, fat is the primary fuel for energy production, while higher intensities rely predominantly on carbohydrate metabolism (McArdle *et al.,* 2007). Over time, these acute responses to exercise result in chronic training adaptations that improve the body's ability to maintain a work rate substantially above resting rates over prolonged periods.

1.2.1 Adaptations to endurance exercise

Aerobic training causes both peripheral and central adaptations that act to enhance oxygen delivery to the mitochondria as well as enhance the ability of muscles to use the available oxygen (McArdle *et al.,* 2007). The training-induced increase in plasma volume and red blood cell mass increases the volume of blood passing through the heart, which results in cardiac remodeling—most notably physiological hypertrophy of the left ventricle—to help reduce strain on the myocardium during exercise (Ellison *et al.* 2011). In trained athletes, increased stroke volume accounts for most of the increase in cardiac output, as maximal heart rate is often unchanged or lower in comparison to untrained individuals (Wilmore *et al.,* 2008). The increased cardiac output allows for increased perfusion of exercising muscles. At the muscle level, there is increased capillarization to accommodate greater blood flow and increased myoglobin content to improve oxygen

delivery to the mitochondria. Greater energy demand on muscles requires a commensurate increase in energy production. The increase in energy production is facilitated by greater mitochondrial density and size (Wilmore *et al.,* 2008). Chronic endurance exercise has also been found to cause vascular remodeling, resulting in widened vessels and increased vascular tone (Niebauer and Cooke 1996). High shear stress promotes secretion of vasodilators (Paszkowiak and Dardik 2003) that are important for maintaining blood pressure. Highly trained athletes repeatedly experience high shear stress due to the increased cardiac output and hyperemic response associated with exercise. Increased shear may result in improved bioactivity of nitric oxide (Green *et al.*, 2004). Changes in pulmonary function are important contributors to adaptations elsewhere in the body. Trained individuals have a lower ventilation at a given submaximal exercise intensity, coupled with a much greater maximal ventilation than untrained individuals (McArdle *et al.,* 2007). Pulmonary blood flow increases during high intensity exercise. Together, the increase in blood flow and ventilation improve pulmonary diffusion of oxygen (Wilmore *et al.,* 2008). Once oxygen is carried to the muscle, there is greater oxygen extraction at the tissue level in trained individuals, a result of increased tissue perfusion (Bassett and Howley 2000).

Aerobic exercise causes adaptation across all body systems. These changes improve the body's ability to maintain continuous exercise at a given intensity and increase the workload the body can produce for extended durations by increasing oxygen delivery and extraction. Overall, these adaptations contribute greatly to cardiorespiratory health and improve endurance performance.

1.2.3 Determinants of performance

For high-level athletes, the main goals of endurance training are to improve aerobic capacity and performance. Performance may be measured in many ways, including competition results, time to complete a given task, and mean power output during an event. Endurance performance *potential* is often predicted from physiological tests that provide insight into an athlete's aerobic capacity (e.g., exercise economy, and

maximal oxygen uptake [$\dot{V}O_{2max}$]). $\dot{V}O_{2max}$ and exercise economy are described in more detail below.

Maximal oxygen uptake ($\dot{V}O_{2\,max}$)

$\dot{V}O_{2max}$ is the highest rate at which an individual can use oxygen during maximal exercise (Jones and Carter 2000). Both athletes and exercise physiologists consider $\dot{V}O_{2max}$ the "gold-standard" measurement of aerobic capacity. Generally, individuals who train in endurance events will have a higher $\dot{V}O_{2max}$ than their anaerobic sport or untrained counterparts. Elite athletes will also generally have a higher $\dot{V}O_{2max}$ than sub-elite athletes. While a good measurement of aerobic fitness and a general marker of performance potential (Levine 2008), $\dot{V}O_{2max}$ is not necessarily a predictor of performance, particularly among training-matched athletes. In other words, a high $\dot{V}O_{2max}$ is necessary to perform well, but in a group of individuals with similar $\dot{V}O_{2max}$ values, there can be a large variation in performance, suggesting that there are many additional factors (both physiological and psychological) that affect performance.

Exercise economy

Exercise economy (often grouped with mechanical *efficiency)* is a measure of the oxygen cost for a given amount of physiological work. During exercise on a cycle ergometer, $\dot{V}O_2$ increases by ~10 mL·min^{-1} for each additional watt of external power output (Jones *et al*., 2013). While economy improves with training (i.e., lowered oxygen cost of exercise), often as a result of training adaptations and improved biomechanical efficiency, it does not change greatly over time in trained individuals.

Training may increase mitochondrial density and therefore energy production. For example, the *vastus lateralis* muscle of highly trained cyclists typically has a higher percentage of Type I muscle fibres (Coyle *et al*., 1992), which have a greater mitochondrial density than Type II fibres and are more efficient for continuous energy production (Joyner and Coyle 2008). Irrespective of mitochondrial density, energy required for a given workload should be fairly consistent within an individual, and independent of how efficiently the energy is produced. In trained athletes, economy will

not differ greatly over time (assuming training is maintained), although economy may improve as $\dot{V}O_{2max}$ declines with age (Jones 1998). Exercise economy may be a better predictor of performance in athletes closely matched in training load and $\dot{V}O_{2max}$: $\dot{V}O_{2max}$ is a ceiling value for the rate of oxygen use, but economy determines how much work can be performed with that oxygen.

1.2.4 Limitations to aerobic capacity and performance

Aerobic capacity is limited by anything that negatively affects oxygen delivery to, or oxygen extraction by, exercising muscles. These factors may include anything that detrimentally affects pulmonary diffusion capacity (e.g., ventilation/perfusion mismatch or shunts), cardiac output (e.g., decreased stroke volume), oxygen carrying capacity (e.g., low hemoglobin mass), or exercising muscles (e.g., decreased mitochondrial enzyme activity) (Bassett and Howley 2000). A gas exchange limitation will likely impact highly trained athletes to a greater extent than recreationally active individuals (potentially due to a greater total blood volume and cardiac output), resulting in the muscular demand for oxygen outstripping delivery at high exercise intensities. For instance, hypoxic exposure negatively affects gas exchange and aerobic performance. A drop in the partial pressure of oxygen decreases availability of oxygen to the body. By extension, hypoxia causes a resultant decline in aerobic capacity that limits endurance performance. This drop is particularly relevant to athletes who attend training camps or competitions at moderate altitude, where training intensity and recovery will likely differ from sea level.

1.2.5 Aerobic exercise in hypoxia

A drop in PO_2 decreases the body's ability to deliver saturated hemoglobin to the tissues and the capacity for oxygen to diffuse to the mitochondria (Storz *et al.,* 2011). The body exhibits a number of physiological adjustments in response to hypoxia in an attempt to maintain oxygen supply. The immediate response to hypoxia is increased ventilation to maintain oxygen saturation, resulting in an increase in exhaled CO_2, and subsequent respiratory alkalosis (Bärtsch and Saltin 2008). This ventilatory response shifts the oxygen dissociation curve to the left, which better allows for oxygen to bind to hemoglobin due to an increased blood-O_2 affinity (West 2007). Cardiac output also increases (via elevated heart rate) to balance oxygen delivery with demand (Casey and

Joyner 2012); however, maximal heart rate may be reduced in hypoxia, as is maximal cardiac output (Bärtsch and Saltin 2008). Furthermore, an attenuated sympathetic response in contracting muscle, despite an increase in vasoconstrictor signals, results in compensatory vasodilation (Casey and Joyner 2012). Despite compensations, if oxygen saturation is decreased, blood flow during maximal large-muscle mass exercise is lower than in normoxic exercise (Calbet 2002). Reduced blood flow limits oxygen delivery, and $\dot{V}O_{2max}$ decreases by ~6%/1000 m altitude (to 3000 m, after which the rate of decrease may increase), due to the drop in oxygen driving pressure (Wherlin and Hallén 2006). Even with the reduced blood flow, there is evidence that pulmonary and muscle diffusion are the main components affecting this decrease in aerobic capacity (Wagner 2010). In the practical sense, physiological adaptations that allow an individual to maintain a high aerobic capacity will benefit endurance exercise at altitude.

Training intensity and endurance sport performance are both affected by hypoxia, which has prompted athletes to try different strategies to attenuate the decline in physiological function. It has been shown repeatedly that individuals with higher baseline fitness tend to have a greater decline in aerobic performance than the untrained (Fulco *et al.,* 1998). Even for athletes who are well matched for fitness at sea level, there is great inter-individual variation for response to hypoxia.

Although $\dot{V}O_{2max}$ drops significantly, economy remains consistent after hypoxic acclimatization at moderate altitudes, although there may be modest improvements (Lundby *et al.,* 2006). This is potentially because a given workload requires an absolute $\dot{V}O_2$ that remains constant. However, it is more difficult (i.e. requires more work) to maintain a given workload at altitude–for example, pulmonary ventilation increases–so the body must work comparatively harder in acute hypoxia than at sea level.

1.2.6 Effects of altitude training

Although altitude reduces aerobic capacity, acclimatization improves performance at altitude and may have beneficial effects on sea-level performance. However, there is great individual variation in the extent of these responses to both acute and longer-term exposures (Martin *et al.,* 2010). A number of different strategies are used to elicit this

benefit. For instance, living "high" and training "low" is a popular approach, combining the benefit of training at sea-level with the physiological responses to prolonged altitude exposure during rest that could benefit endurance exercise (Millet *et al.,* 2010). The efficacy of such strategies is highly variable and seems to depend on each individual's ability to acclimatize.

Ideally, endurance athletes competing at altitude will be able to acclimatize fully to the conditions in which they will compete (a recommended two weeks for venues up to 3000 m) (Bergeron *et al.,* 2012); however, even with acclimatization, performance at altitude will not equal sea-level capabilities in endurance sport (if aerodynamic drag is not factored in). Moreover, acclimatization is not always feasible, nor optimal for training, as athletes are unable to maintain training intensities comparable to sea level at altitude (Millet *et al.,* 2010) and may have busy competition schedules, restricting the time they could spend at altitude before a single event.

To augment training efforts, athletes often supplement with dietary or pharmaceutical aids. Various treatments have been investigated to alleviate the strain of exercise in hypoxia. For example, sildenafil results in reduced hypoxia-induced pulmonary hypertension (Zhao *et al.*, 2001; Ghofrani *et al.,* 2004), while improving exercise capacity at altitude through improved oxygen saturation and increased maximal workload and cardiac output (Ghofrani *et al.,* 2004). There are concerns regarding the use of sildenafil at altitude, as it may increase the severity of headaches, a well-documented symptom of acute mountain sickness (Hackett and Roach 2001). Athletes could also experience other undesirable side effects (e.g., blue tinted vision (Marmor and Kessler 1999)).

1.3 Exercise and nitrate

In sport culture, supplementation is a common tactic to improve performance. In recent years, research into nitrate (NO_3^-) and nitrite (NO_2^-) supplementation has become more popular, particularly involving the use of beetroot juice (BR) as a source of NO_3^- leading to nitric oxide (NO) production. In an applied setting, it has become common for athletes to use BR for training and competition. These supplements contain juice from beets (often exclusively), and vary in volume and NO_3^- content, depending on whether

the supplement is 'concentrated' or not. For example, a BR 'sport shot' used in studies contains 70 mL of concentrated BR and ~4.2 mmol of NO_3^- (Wylie *et al.,* 2013). Some studies, however, supplement with up to 500 mL of BR a day, with the maximum reported dose being 500 mL BR (containing ~11 mmol NO_3^-) administered daily over a period of six days (Bailey *et al.,* 2009). While studies of BR and exercise capacity in both clinical populations and averagely active individuals are promising (e.g., Kenjale *et al.,* 2011), the few studies conducted in highly trained athletes in normoxia have returned mixed results (reviewed in Hoon *et al.,* 2013b). The few studies of BR in hypoxia have been unanimous in demonstrating positive effects (Vanhatalo *et al.,* 2011; Masschelein *et al.,* 2012; Muggeridge *et al.,* 2014), although only one of these studies (Muggeridge *et al.,* 2014) has been completed with well trained athletes. Current evidence suggests that training and health status, sex (Kapil *et al.,* 2010), dosage (Wylie *et al.,* 2013a) and altitude (Dauncey 2012) may all play a large role in the efficacy of BR as a supplement. Therefore, it is important to test the usefulness of BR in highly trained endurance athletes and to determine the conditions in which it is most effective.

In trained endurance athletes, there is evidence for responders and non-responders to BR supplementation (e.g., Christensen *et al.*, 2012). Varied 'response' between individuals may be related to differences in adaptations to the vasculature, endothelial function, and aerobic system. Physiological adaptations of endurance exercise should result in excellent oxygen delivery, potentially masking or making redundant any BR benefit. This possibility is reflected in a study finding no benefit of a six-day BR supplementation for a 400-kcal (~18 minute) time trial (TT) in elite male cyclists ($\dot{V}O_{2max}$ 72.1 [4.5]) in normoxia (Christensen *et al.*, 2012). This finding is contrasted by data from Lansley *et al.* (2011a), which demonstrated a significant improvement in 4- and 16.1-km TT performance in club-level males ($\dot{V}O_{2max}$ 56 [5.7] ml·kg^{-1}·min^{-1}) following acute BR supplementation. A meta-analysis by Hoon *et al.* (2013b) indicated greater performance improvements from BR have been found in inactive or recreationally active individuals. As yet, there are no studies to indicate that so-called "responders" would continue to respond repeatedly.

Despite differences in the effect of BR among athletes, some of the factors contributing to aerobic performance are limited in acute hypoxia, making it plausible that

BR may enhance exercise tolerance even in those individuals who do not respond to BR supplementation at sea level; however, this may be confounded by the variable response to hypoxia alone. The background physiology and research are presented below in more detail. Studies conducted to date assessing the efficacy of BR supplementation are summarized in Table 8 in Appendix B.

1.3.1 Nitric oxide pathways

Nitric oxide (NO) is a signaling molecule and potent vasodilator formed by nitric oxide synthase (NOS) enzymes (constitutive, inducible or neuronal) in response to chemical and mechanical stimuli (MacMicking *et al*. 1997; Bogdan 2001) through the oxidation of L-arginine (Palmer *et al*., 1988). This complex reaction is catalyzed by the NOS enzymes and requires oxygen and other substrates (Lundberg and Weitzberg 2010; Bailey *et al*., 2012).

A secondary pathway for the formation of NO has been suggested, in which dietary nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by anaerobic bacteria in the mouth (reviewed in Lundberg *et al*., 2008). Some of this NO_2^- is further reduced to NO in the acidic environment of the stomach (Lundberg and Govoni 2004; Lundberg *et al*., 2008), while other NO_2^- molecules continue to circulate in the blood and may form NO systemically, catalyzed by hemoglobin, myoglobin, and xanthine oxidoreductase among other species (Lundberg *et al*., 2011b; Lundberg *et al*., 2008). When the resultant NO from either pathway diffuses into surrounding smooth muscle cells, it activates guanylyl cyclase, which in turn stimulates cyclic guanosine monophosphate (cGMP) formation and smooth muscle cell relaxation (Bredt and Snyder 1994). See Figure 1.

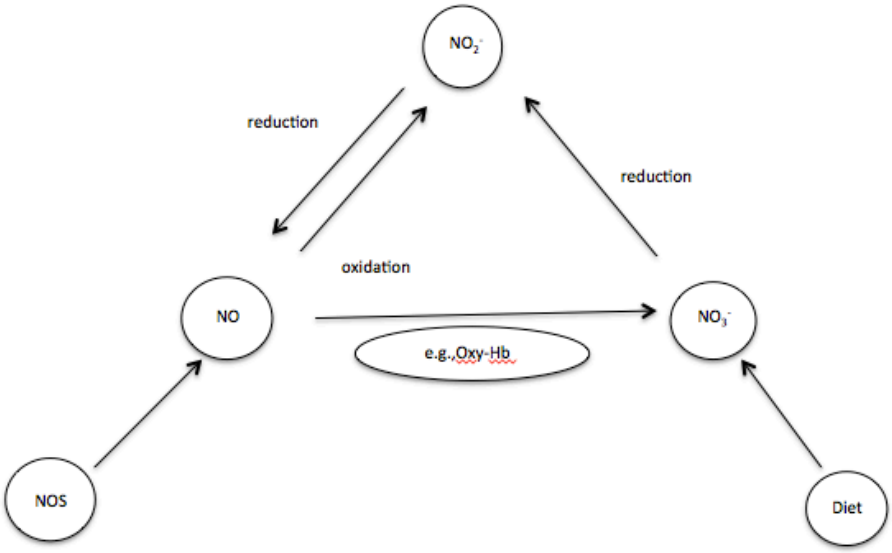

Fig 1: Sources of nitric oxide (NO) in the human body. NO can form in the body through the nitric oxide synthase pathway (NOS) or through nitrate (NO_3^-) in the diet. NO_3^- may be reduced to nitrite (NO_2^-) and subsequently NO. NO may also be oxidized to form NO_2^- and NO_3^-.

NO is known to be particularly important for health. In addition to being a vasomodulator, NO plays roles in cellular respiration and inflammation (Lundberg *et al.*, 2008). For example, in male Munich Wistar rats, chronic (two month) blocking of the NOS pathway through the use of L-NG nitroarginine methyl ester (L-NAME) resulted in systemic hypertension and glomerular injury (Baylis *et al.*, 1992). Jones *et al.* (2004) further reported that in humans, acute L-NAME infusion increased mean arterial pressure, and decreased $\dot{V}O_{2max}$ and heart rate.

Inorganic NO_3^- and BR supplementation have been found to decrease blood pressure (BP) (Siervo *et al.*, 2013). Specifically, BR supplementation has been shown to lower diastolic blood pressure (DBP), systolic blood pressure (SBP), and mean arterial pressure (e.g., Webb *et al.*, 2008). These results coincide with greater plasma [NO_2^-] following supplementation. An increase in plasma [NO_2^-] may be the result of NO_3^- reduction, indicating that NO_3^- supplementation is responsible for subsequent BP reductions, possibly mediated by increased NO. Chronic intake of a nitrate-rich diet (e.g., the "Mediterranean Diet") may have a cardioprotective effect, suggesting long-term

benefit (Hu 2003; Lundberg *et al*., 2006; Lidder and Webb 2012). Decreased BP is maintained for at least 15 days with chronic BR supplementation (Vanhatalo *et al*., 2010), but there are no investigations of longer duration. There may be a dose-dependent relationship between NO_3^- intake and change in BP (2010) (Kapil *et al*., 2010). An increase in BP occurred when oral microflora were disrupted with the use of antibacterial mouthwash (Kapil *et al*., 2012), further demonstrating the significance of the oral pathway in generating NO.

1.3.2 Sex differences in response to nitrate supplementation

Sex differences in the effect of NO_3^- supplementation on BP may exist. Kapil *et al*., (2012) reported that premenopausal females had higher basal [NO_2^-] coupled with lower BP than age-matched males and, following supplementation with potassium nitrate males had a greater reduction in BP than females. Thoonen *et al*. (2012), suggested that nitrate supplementation may be less effective in women, due to possible differences in sex-specific NO-cGMP signaling. The reason for the discrepancy is unknown but may be caused by differences in vasodilation signals. For example, it has been suggested that in female mice the main endothelial-derived relaxing factor is endothelial-derived hyperpolarizing factor, while in males it is NO and prostacyclin (Scotland *et al*., 2005). The possibility that supplement efficacy varies between sexes warrants further research, as data demonstrating such a dichotomy would be of interest to athletes interested in nitrate supplementation,

1.3.3 Effect of beetroot juice on exercise

Bailey *et al*. (2010) demonstrated that BR supplementation before small-muscle exercise significantly altered the intramuscular environment (demonstrated by decreased ADP and P_i accumulation and decreased PCr depletion) and increased exercise tolerance in normoxia, relative to placebo. In this study, subjects completed incremental double-legged knee extension exercise in a step-wise manner until task failure. There was a 25% increase in time to task failure with BR, with each of the seven subjects performing better in the BR condition. The authors suggest that this finding may be the result of a

decreased total ATP cost for muscle force production, as reduced ADP and P_i accumulation may be due to coupling between ATP hydrolysis and the energy required for force production at a given work rate with BR supplementation. If ADP and P_i are reduced, oxidative phosphorylation would likely also be reduced (Brown 1992). Results from this study indicated improved matching of energy produced for energy required (Bailey *et al*., 2010).

The majority of BR studies in humans have investigated performance outcomes rather than the effect's mechanism. Studies in animal models have allowed for a more in depth understanding of how BR and NO_3^- supplementation may affect muscle function. For example, fast-twitch muscle fibres from C57bl/6 male mice supplemented with 1 mM sodium nitrate in water for seven days had increased Ca^{2+} handling proteins and myoplasmic free [Ca^{2+}], as well as increased contractile force and a faster rate of force development compared to untreated mice (Hernández *et al*., 2012). Relatedly, Ferguson *et al*. (2012), demonstrated improved skeletal muscle blood flow and vascular conductance in male Sprague-Dawley rats supplemented with 1 mmol $NO_3^- \cdot kg^{-1} \cdot day^{-1}$ in BR during treadmill running, primarily in Type IIB+d/x fibres. Together, these studies indicate a greater role for NO in oxygen matching in fast-twitch fibres. Previous findings that NO directly inhibits force-generating proteins in skeletal muscle myofibrils (Galler *et al*., 1997) may seem counterintuitive considering conclusions by both Hernández *et al*., (2012) and Ferguson *et al*., (2012). However, this study used muscle fibres from rat leg muscles such as the soleus and vastus lateralis, which, in endurance trained humans, are likely composed of a high proportion of Type 1 fibres (Coyle *et al*., 1992). These results as a whole seem to be supported in humans by Bailey *et al*. (2010), as discussed above.

It has also been demonstrated in male Sprague-Dawley rats that renal injury was attenuated after a medium-to-high dose of dietary NO_2^- was provided in drinking water concomitantly to chronic L-NAME infusion (Kanematsu *et al*., 2008). This provides evidence that the NOS and $NO_3^- - NO_2^- - NO$ systems are independent, and one can act to maintain NO production should the other be compromised. This has important clinical applications, but also strengthens the rationale for BR as a supplement in hypoxia.

1.3.4 Nitrate supplementation and normoxic exercise performance

Although most human research has been conducted using untrained populations, a small, but highly relevant, number of studies have investigated the effect of BR on endurance performance in highly trained athletes. Studies of highly trained or elite cyclists in normoxia have found improvement in time trial performance following BR supplementation in a 4-km TT (Lansley *et al*., 2011a), a 10-km TT (Cermak *et al*., 2012a), and a 16.1-km TT (Lansley *et al*., 2011a) but not in a 50-mile TT (Wilkerson *et al*., 2012). Lansley *et al*. (2011a) concluded that mean power output increased for both 4-km and 16.1-km TT distances with BR supplementation, although VO_2 values did not differ between BR and placebo (PL). Conversely, Wilkerson *et al*. (2012) demonstrated that neither power output nor mean $\dot{V}O_2$ differed significantly between BR and PL trials. This study reported a lower mean $\dot{V}O_2$ with BR compared to placebo over the 50-mile TT that approached statistical significance ($p = 0.06$), which may be practically meaningful. Wilkerson *et al*. (2012) also demonstrated that individuals with higher plasma [NO_2^-] following supplementation performed better in the time trial. While the 50-mile TT completed in this study was of a much longer distance than those used in other studies (e.g., Lansley *et al*. (2011a); Cermak *et al*. (2012a)), the exercise intensity was fairly low. This may have limited the efficacy of the $NO_3^- - NO_2^- - NO$ pathway, which is potentiated in hypoxic and acidic conditions (Dauncey 2012; Modin *et al*., 2001).

BR supplementation may be more effective for improving performance over moderate distances and/or higher intensity exercise. In a study by Christensen *et al*. (2012), a six-day BR loading protocol did not improve endurance capacity or repeated sprint performance in elite male cyclists. However, two of the ten subjects were identified as "responders" who demonstrated decreased $\dot{V}O_2$ at stages of 50% and 70% of aerobic peak power with BR supplementation. Furthermore, studies have concluded that an improvement in economy (reflected in decreased VO_2 for a given power output) is likely due to changes in skeletal muscle rather than cardiopulmonary variables (Bailey *et al*., 2009; Bailey *et al*., 2010).

1.3.5 Beetroot juice and hypoxic performance studies

As of 2013, data has been published from three studies that examined BR supplementation in normobaric hypoxia. Masschelein *et al*. (2012) used a cycling protocol consisting of 20-minute steady state exercise at 45% of maximum power output, followed by an incremental test to exhaustion. It was demonstrated that, in hypoxia (11% O_2; equivalent to ~5000 m), BR increased time to exhaustion by ~5%, and improved muscle oxygenation relative to placebo. Two-legged knee extension exercise to exhaustion in hypoxia (14.5% O_2) when supplemented with BR, was also demonstrated to be comparable to normoxic trials (Vanhatalo *et al*., 2011). Another recent study examined economy and TT performance at a simulated altitude of 2500 m (~15% O_2) (Muggeridge *et al*., 2014) and showed that steady-state economy at 60% of maximum work rate and 16.1 km TT performance were both improved with BR, relative to placebo. This finding strengthens the rationale for BR being effective in hypoxia for highly trained individuals. In a related study, patients with peripheral arterial disease (a model of pathological ischemia that models poor oxygen delivery) demonstrated that BR supplementation increased time to onset of claudication pain (caused by too little blood flow during exercise), indicating improved perfusion in hypoxic tissue (Kenjale *et al*., 2011). Together, these studies suggest that exercise performance in moderate hypoxia may benefit from BR supplementation.

1.3.6 Nitric oxide in hypoxia

During submaximal exercise in hypoxia, changes in oxygen saturation are compensated for by changes in cardiac output and muscle blood flow to maintain oxygen delivery (Calbet *et al*., 2000). However, as intensity increases during large-muscle exercise, such as cycling, the body cannot appropriately compensate for the lack of inspired oxygen. There is also a right shift of the oxygen dissociation curve that limits oxygen unloading in the muscle and decreases total oxygen consumption (Calbet *et al*., 2009). If cardiac output is reduced, as in maximal exercise in moderate hypoxia (Calbet *et al*., 2009), muscle blood flow is decreased, reducing oxygen delivery. Vasodilation may work to better match O_2 requirements to delivery, regulated by oxygen sensing (Calbet *et al*., 2002). In hypoxia, the production of NO is up-regulated by the NOS

system, contributing to compensatory vasodilation and improved oxygen delivery. This up-regulation is likely related to increased shear stress and blunting of sympathetic vasoconstrictor signals (Casey and Joyner 2012). NO is also formed preferentially from NO_2^- as oxygen pressure falls (Cosby *et al*., 2003; Lundberg and Weitzberg 2005; Lundberg *et al*., 2008) mainly through anaerobic pathways. The reduction of NO_2^- is potentiated as oxygen tension falls, and the rate of conversion of NO_2^- to NO by hemoglobin is highest when oxygen saturation approaches 50%, due to available binding sites on hemoglobin and a peak in nitrite reductase activity (this occurs with desaturation at the tissue level) (Lundberg *et al*., 2008). Furthermore, this reaction benefits from a drop in pH to that expected during increased metabolic activity or hypoxia (Modin *et al*., 2001). Therefore, it seems possible that this secondary pathway of NO production could also improve compensatory vasodilation in hypoxia (Joyner and Casey 2013).

One of the main physiological roles played by NO is the regulation of mitochondrial respiration, making NO particularly relevant to aerobic exercise performance. For example, NO, carried by myoglobin, competes with O_2 for binding sites on cytochrome c oxidase, effectively inhibiting cytochrome C oxidase and sparing O_2. This results in less ATP generation in mitochondria close to the feeder artery (Dauncey 2012); however, due to a change in the inner mitochondrial membrane potential, there is less proton leak back into the mitochondrial matrix, leading to greater efficiency in cellular respiration. The O_2 gradient seems to be extended, and O_2 molecules spared from proximal mitochondria are used to generate ATP more distally (Dauncey 2012). Improved mitochondrial efficiency could make exercise more economical and would be particularly important in hypoxia, where matching oxygen delivery to consumption becomes more difficult. Supplementing with dietary NO_3^- could benefit exercise in hypoxia in a few ways: 1) improved mitochondrial efficiency (Larsen *et al*., 2011), meaning less O_2 used for a given energy output; 2) improved muscle contractile efficiency (Bailey *et al*., 2010), and therefore fewer ATP used for a given force production (Jones *et al*., 2013); and 3) increased vasodilation due to increased NO availability, allowing for improved oxygen delivery (Siervo *et al*., 2013).

1.4 Summary and potential significance

Athletes may travel to altitude for training camps or competitions. There are currently many strategies to enhance training and performance under hypoxia, although research shows inconsistent results, due to differences in length of hypoxic exposures and dose. It is also likely there is considerable variation between individuals in the physiological response to hypoxia. Dietary NO_3^- in the form of BR has been demonstrated to improve normoxic exercise tolerance in clinical populations and recreational athletes. There is further evidence that exercise tolerance in simulated hypoxia may benefit from BR. Comparing recreationally active individuals and elite athletes, greater performance improvements have been demonstrated in less fit populations. In the few studies that have assessed the efficacy of BR in highly trained individuals, there has been no obvious effect of BR, although some individuals seem to be 'responders.' Highly trained endurance athletes benefit from training volume and duration that results in physiological improvements specific to continuous exercise. In this case, the increased endogenous production of NO (Green *et al.,* 2004) coupled with other training adaptations (Joyner and Coyle 2008) may mask the effect of BR. However, these adaptations do not explain differences in training matched, fit individuals, and little is known about why these responses would differ. Therefore, the specific sport, intensity and duration of exercise may also alter the efficacy of BR. Whether effective in normoxia or not, the decreased PO_2 in hypoxia may benefit BR-related performance enhancement in highly trained athletes. BR could potentially ameliorate training intensity and recovery through improved oxygen saturation, economy and blood pressure, which are impacted at altitude. Theoretically, these improvements would benefit both repeated training bouts (such as during a training camp) and competitive performance in hypoxia.

2. Effect of beetroot juice on exercise performance in normoxia and moderate hypoxia

2.1 Introduction

At altitude, decreases in the barometric pressure ultimately result in less oxygen reaching the tissues (reviewed in Sheel *et al.,* 2010). $\dot{V}O_{2max}$ decreases almost linearly with oxygen saturation (Wagner 2010), limiting intense endurance exercise as a given submaximal workload has approximately the same oxygen cost at altitude as at sea level. Relying on the aerobic energy system to maintain a given workload in hypoxia requires that the individual increase ventilation and cardiac output to improve diffusion and maintain oxygen delivery. Thus, ventilation and cardiac output are higher for a given submaximal workload. The combined effect of increasing cardiopulmonary workloads while lowering maximal aerobic capacity reduces overall training quality, particularly with acute hypoxic exposure in absence of acclimatization. As athletes often train and compete at moderate altitude (i.e., 1500-2500 m), anything that would ameliorate these limiting factors would help to maintain high exercise intensity and performance.

Dietary NO_3^- lowers blood pressure during exercise recovery in individuals of average fitness (Larsen *et al.,* 2010) and clinical populations (Kenjale *et al.,* 2011). Dietary NO_3^- also increases mitochondrial efficiency through an improved oxidative phosphorylation ratio (Larsen *et al.,* 2011) in normoxia. Beetroot juice (BR), a supplement with a naturally high concentration of NO_3^- that may have ergogenic effects for athletes due to subsequent increases in circulating NO_3^- and NO_2^- that increase NO availability, has gained popularity with endurance athletes aiming to improve performance; however, normoxic studies in highly trained athletes supplemented with BR indicate that it may be less effective in this population (*e.g.,* Cermak *et al.,* 2012b). Despite evidence that trained athletes may not benefit from BR at sea level, when oxygen is limited in the environment (e.g. at altitude) BR may have even greater physiological effects Muggeridge *et al.,* 2014; Dauncey 2012; Masschelein *et al.,* 2012). Therefore, BR may positively affect trained athletes in hypoxic environments. While Muggeridge *et al.* (2014) have previously investigated the effect of BR in trained athletes in moderate

hypoxia, a comparison between the efficacy of BR supplementation in normoxia and moderate hypoxia for trained athletes has not been done. This may be useful for understanding under which conditions BR is most effective for athletes.

2.2 Purpose, objectives and hypotheses

The primary purpose of this project is to assess whether an acute dose of BR can improve indices of exercise performance in trained men, and, if so, whether these effects are due to the available [NO_3^-] in the supplement. There is evidence to suggest that highly trained athletes do not benefit from BR supplementation in normoxia to the same extent as less fit individuals; however, there may be a greater ergogenic effect on performance in hypoxia because it is more difficult to maintain endogenous NO production. To address this question, I examined the efficacy of acute BR supplementation for improved exercise performance in both normoxia and moderate hypoxia in trained male cyclists. Through the course of my thesis, I aimed to address the following four questions by testing the associated hypotheses:

Question 1: Do highly trained athletes experience an ergogenic benefit from BR[1] under normoxic conditions?

Question 2: Do highly trained athletes benefit from BR supplementation in hypoxia?

Question 3: Does resting BP decrease following BR supplementation?

Question 4: Does BR supplementation increase fraction of exhaled nitric oxide (FENO)?

Hypothesis 1: BR will have no effect on exercise performance in normoxia (*i.e.*, no change in economy, or TT performance), relative to placebo.

Hypothesis 2: BR will improve indices of exercise performance in hypoxia (i.e., improved economy, increased oxygen saturation, improved TT performance), relative to placebo.

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[1] For the purpose of this thesis, "BR" will indicate beetroot juice containing NO_3^-, while "PL" will indicate beetroot juice that has been NO_3^- depleted. The protocol described in this thesis specifically tests whether the NO_3^- in beetroot juice is effective as an ergogenic aid.

Hypothesis 3: Acute BR supplementation will decrease resting blood pressure in highly trained men, relative to placebo.

Hypothesis 4: FENO will increase with BR juice supplementation.

2.3 Methods

2.3.1 Participant information

Between April and August 2013, 12 trained male cyclists were recruited to participate in this study. Subjects were recruited through poster advertisements at the University of British Columbia (UBC) campus, in cycling shops and through email contact with cycling clubs in Vancouver. Initially, 15 individuals expressed interest in participating; however, two were excluded during screening, as they did not meet all of the inclusion criteria. Of the remaining 13 subjects, one individual did meet the inclusion criteria but withdrew from the study due to personal reasons.

Subjects were given $20.00 per visit to the lab ($100.00 total for all five visits). Informed written consent was obtained from all subjects. The UBC clinical research ethics board (CREB) approved this study.

Table 1: Inclusion and exclusion criteria

Inclusion Criteria	Exclusion Criteria
Males aged 19 (18 for UBC students*) to 40 years	Females
Self-reported trained cyclists	History of pulmonary or heart disease
Maximal oxygen uptake of at least 60 $mL \cdot kg^{-1} \cdot min^{-1}$ or 5 $L \cdot min^{-1}$	Diabetes
English speaking	Smoking
	Recent (within 2 months) visit to 2500 m or above
	Inability to communicate in English

Note. UBC CREB approved treating 18-year-old UBC students as emancipated for the purpose of this study.

2.3.2 Experimental design

A randomized, placebo-controlled, crossover study design was used for this experiment (see Figure 2). The 12 subjects that qualified for the study visited the

laboratory five times in total. Following an initial screening visit, there were four randomly assigned treatment days: 1) normoxic trial supplemented with BR; 2) normoxic trial supplemented with placebo (PL); 3) hypoxic trial supplemented with BR; and 4) hypoxic trial supplemented with PL. Each visit was separated by at least 96 hours to allow for washout and recovery. All cycling tests were completed on a fully adjustable Velotron cycle ergometer (Racermate Dynafit Pro, Racermate Inc., Seattle WA) to mimic real road cycling as closely as possible. Data collection took place in the Environmental Physiology Laboratory and the Gene, RNA, Informatics and Proteins Laboratory at the University of British Columbia, Vancouver, Canada. The lab is located approximately 75 m above sea level.

Individual subjects were tested at approximately the same time of day on each visit. Supplementation was administered in a double-blind manner, whereas only the subjects were blinded to the environmental condition. Informing the researcher of the environmental condition was necessary to allow oxygen saturation to be monitored during the exercise protocol, which was required by CREB. Subjects were given either 70 mL BR or 70 mL PL, and completed the testing protocol in either moderate hypoxia (normobaric hypoxia chamber set to 2500 m) or normoxia. The BR supplement (Beet It Sport, James White Drinks Ltd., Suffolk, UK), which contained beetroot juice and lemon juice had 72 kcal and 0.4 g of NO_3^- (6.5 mmol NO_3^-) in 70 mL. The PL product was identical to the BR supplement, except that it was NO_3^- depleted. This allowed for any differences in the experimental conditions to be explained by the available NO_3^- content. The supplement was Informed-Sport tested and both the BR and PL were consumed at room temperature.

Subjects were asked to record their food intake for the 24-hour period leading up to the initial test and to then replicate this diet as closely as possible over the 24 hours leading up to each subsequent test day. They were also asked to arrive at the lab fully hydrated (*i.e.,* at least 500 mL of water consumed in the preceding hour) but to avoid alcohol and caffeine consumption for 24 and 6 hours, respectively, before laboratory visits. As well, subjects were asked not to use anti-bacterial mouthwashes or chew anti-bacterial gum for the duration of the study. Other than the treatment, subjects did not eat or drink anything during the testing with the exception of water, which was permitted *ad*

libitum between supplementation and exercise testing. Finally, subjects did not exercise on test days and remained in the laboratory between supplementation and exercise testing.

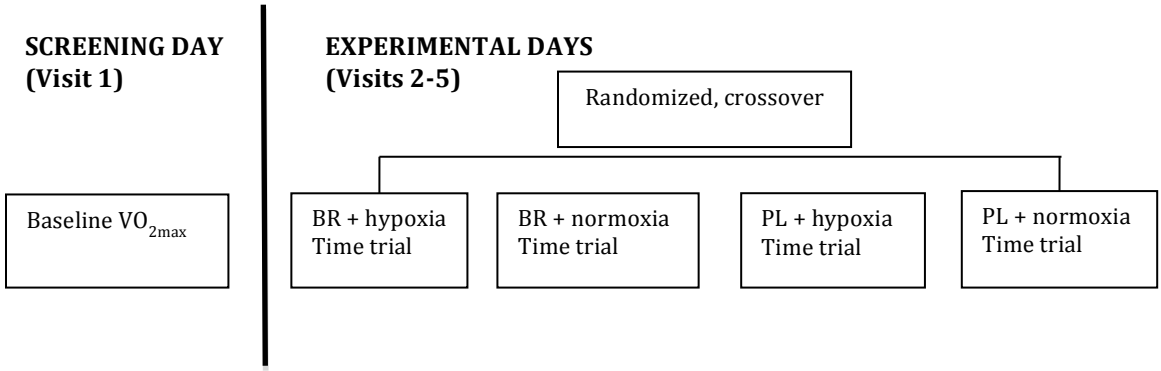

Figure 2: General study overview. Subjects came to the laboratory on five separate occasions. The first visit included a maximal exercise test and familiarization with the exercise protocol to be completed on subsequent days. During data collection days, subjects underwent one of four protocols: supplementation with either beetroot juice (BR) in hypoxia or normoxia, or supplementation with nitrate-depleted beetroot juice (PL) in either normoxia or hypoxia (see text for details).

2.3.2.1 Visit 1: Fitness screening

The first visit consisted of anthropometric measurements, baseline fitness testing and familiarization with the lab, the equipment to be used on test days, and the experimental day protocol (see Appendix D for subject information sheets). Subjects were weighed and measured. A venous blood sample was taken to measure baseline plasma [NO_3^-] and [NO_2^-], and a finger prick sample was drawn into a capillary tube to determine hematocrit using a CMH30 Micro Hematocrit Centrifuge (UNICO, Dayton NJ, USA). To determine whether prospective subjects met the inclusion criteria for fitness, each individual completed an incremental $\dot{V}O_{2max}$ test to volitional exhaustion. A mouthpiece was fitted to each subject's face fixed with a two-way, non-rebreathing valve. Subjects inhaled room air and exhaled to a metabolic cart via a breathing tube. Subjects completed a self-selected, five-minute warm-up on the Velotron cycle ergometer. The test started immediately afterwards at a work rate of 0 W. The work rate increased by 1 W every 2 seconds. $\dot{V}O_{2max}$ was met when the subject met at least three of the following criteria: 1) a plateau in oxygen uptake despite an increase in work rate; 2) a respiratory

exchange ratio >1.15; 3) heart rate > 90% of the age predicted max (220 bpm – age in years); 4) volitional exhaustion. The subject then completed a self-selected cool down. For those subjects who achieved a $\dot{V}O_{2max}$ value ≥60 mL·kg^{-1}·min^{-1} (or 5 L·min^{-1}) this ramp test was also used to determine their 50% maximal power outputs (in Watts) for subsequent test days. Each subject's 50% power output was calculated from power output at $\dot{V}O_{2max}$. After a rest period, subjects completed the TT protocol in normoxia (described in detail below), used for subsequent testing days, to familiarize them to the ergometer's gearing and the intensity and duration of the TT protocol.

2.3.2.2 Visits 2, 3, 4, 5: Data collection

On arrival to the lab, resting blood pressure (BP) was assessed manually with a sphygmomanometer and a stethoscope. Subjects sat quietly for five-minutes and kept both feet on the floor during BP assessment. BP was measured in duplicate, and the second measurement was used for analysis. Fraction of expired nitric oxide (FENO) was measured following the American Thoracic Society and European Respiratory Society guidelines for measurement of exhaled lower respiratory nitric oxide using offline measurements (ATS and ERS 2005). While in a seated position, subjects held the NIOX MINO handheld electrochemical analyzer (Aerocrine AB, Solna, Sweden). Subjects exhaled completely, inhaled NO-free air through the mouthpiece until reaching vital capacity, and immediately exhaled for 10 seconds at a rate of 50 mL·s^{-1} to complete the test. FENO was measured once.

Immediately following these tests, subjects drank 70 mL of either BR supplement or PL (distributed using a double blind protocol). Regardless of treatment subjects waited in the laboratory for two hours, to allow time for any increase in plasma [NO_3^-] and [NO_2^-]. After two hours, post-supplementation blood pressure and FENO were assessed and a second venous blood sample was taken.

Following post- supplementation tests, subjects were fitted with a heart rate monitor (Polar T31 Transmitter, Polar; Kempele, Finland), an earlobe pulse oximeter (Avant 9600, Nonin Instruments, Plymouth, MN), and a facemask (Hans Rudolph, Oro-Nasal 7450 V2 Mask, Shawnee, KS) connected to a two-way non-rebreathing valve

(2700 T-shape, Hans Rudolph, Shawnee, KS). The valve was attached on one side to a normobaric hypoxia chamber (Colorado Altitude Training; Louisville, CO) with a breathing tube, and to a metabolic cart (Parvo Medics; Sandy, UT) on the other side with another breathing tube (see Figure 3).

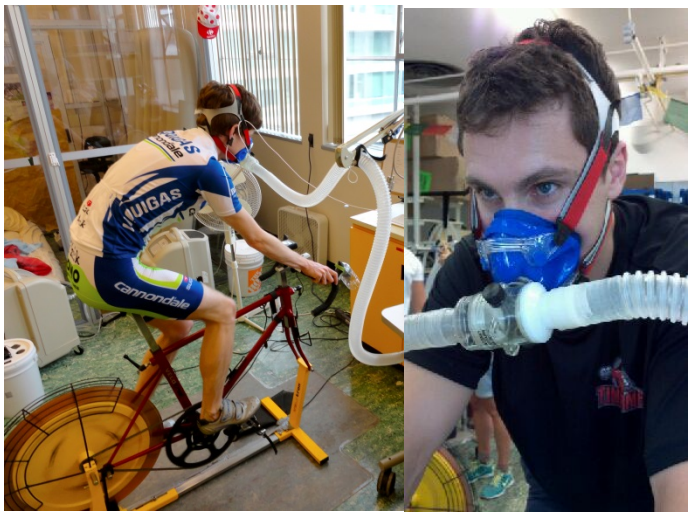

Fig 3: Breathing set-up. Subjects breathed either normoxic or hypoxic air from the normobaric hypoxia chamber through a breathing tube, and exhaled through a second tube to a metabolic cart.

Prior to warming up on the cycle ergometer, emission and detection fibre optic probes connected to a near infrared spectroscopy (NIRS) system (NIRO-200 Oxygenation Monitor, Hamamatsu, Japan) were taped to the subject's upper thigh (over the *vastus lateralis*) to assess tissue oxygenation index (TOI). The probes were covered with a tensor bandage, which lay under the subjects' cycling shorts. The site of probe placement was marked on the skin in permanent marker, so probe position could be replicated for subsequent test days. Muscle oxygenation data was collected during the steady state and TT portions of the cycling protocol.

Subjects were seated on the cycle ergometer, facing a computer screen, breathing either ~21% or ~16% O_2 from the normobaric hypoxia chamber. A 15-minute self-selected warm-up on the cycle ergometer was then completed followed by 15 minutes at 50% of each subject's specific maximal power output (as pre-determined by their initial $\dot{V}O_{2max}$ test). During the steady state portion of the test, power output was fixed, and subjects were asked to maintain a seated position and a consistent cycling cadence.

Metabolic data was measured over the final five minutes of this task. This data was used to assess exercise economy (mL $O_2 \cdot min^{-1} \cdot W^{-1}$), the main outcome variable for the steady state portion of the test. Subjects were not given any feedback regarding gearing or cadence during the warm-up to prevent subjective comparison with future and past testing days. Following the steady state exercise, subjects completed a 10-km TT on a flat and straight course (RacerMate Interactive 3D Software, Seattle, WA) (see Figure 4 and Table 3). During the TT, subjects were able to change gears and position if desired. Only feedback on distance covered and cadence was provided to them via the computer screen. Metabolic data was collected for the duration of the TT. The main outcome variable for the TT was mean power output relative to body weight ($W \cdot kg^{-1}$).

Following completion of testing protocols, subjects were asked whether they thought they had experienced hypoxia or normoxia and whether they consumed BR or PL. This was used to assess the efficacy of the single- and double- blinding. See Appendix C for a more detailed explanation of the normoxic and hypoxic breathing protocol, as well as metabolic calculations.

Table 2: Summary of procedures for all subjects on laboratory visits.

Appointment 1	Appointments 2, 3, 4, 5
-Anthropometric measures (height, weight)	-BP and FENO
-Hematocrit measurement	-Supplemented with either 70 mL BR or PL
-Incremental maximal oxygen uptake test ($\dot{V}O_{2max}$)	-2 hours post-supplementation, second BP and FENO
	-15 minute self-selected warm up, breathing either normoxic (~21% O_2) or hypoxic air (~16% O_2), followed by 15 minutes at 50% max power (HR, SpO_2, TOI, $\dot{V}O_2$, power output)
	-10-km time trial (SpO_2, TOI, $\dot{V}O_2$, power output)

Note. NO_3^-=nitrate; NO_2^-=nitrite; $\dot{V}O_{2max}$ =maximal oxygen uptake; FENO=fraction of expired nitric oxide; HR=heart rate; BP=blood pressure; SpO2=pulse oxygen saturation; $\dot{V}O_{2max}$ =oxygen uptake; TOI=tissue oxygenation index.

2.3.3 Statistical analysis

A power calculation (G*Power 3.1, Heinrich Heine, Universität Düsseldorf) indicated that the required sample size for this study was 12 subjects. This was based on submaximal cycling economy data from Bailey *et al*. (2009). This study showed that the mean cycling economy of the placebo condition was 11.6 (SD = 0.9) and the mean of the nitrate-supplementation condition was 10.8 (SD = 0.8). This power calculation was based on an alpha level of 0.05 and a power of 0.84.

Descriptive analyses of all variables are presented as mean (SD). A two-way repeated measures ANOVA was used to analyze differences between the four conditions for BP and FENO variables. The independent variables for these tests were time (pre- and post- supplementation) and treatment (PL or BR). Dependent variables were systolic BP, diastolic BP and FENO. Paired samples t-tests were used to compare the average difference in FENO between pre- and post- supplementation with BR or PL. For these analyses, averages of the two BR and two PL test days were used.

A two-way repeated-measures ANOVA was used to analyze differences between the four conditions for dependent variables relating to steady state and TT performance. The independent variables for these tests were condition (hypoxia, normoxia) and supplement treatment (BR, PL). For the steady state portion of the protocol, dependent variables were cycling economy (mL $O_2 \cdot min^{-1} \cdot W^{-1}$), SpO_2, TOI, HR, BP, and V_E. In the TT, dependent variables were time to complete 10-km, mean power output per kg, and mean $\dot{V}O_2$. For all tests, the alpha-level was set at $p = 0.05$.

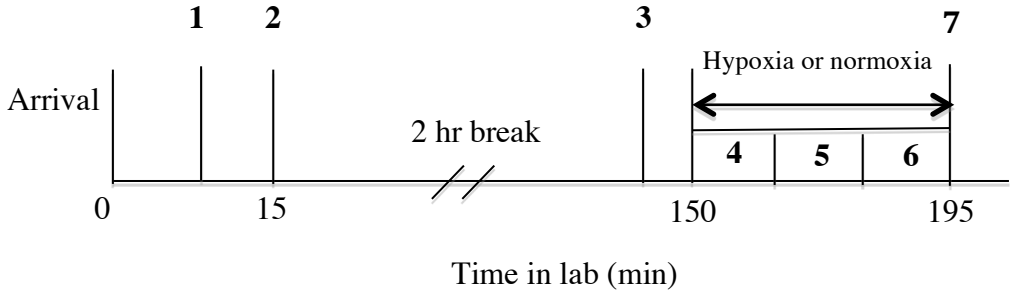

Fig. 4: Timeline of data collection (sessions 2-5). After pre-supplementation blood sampling and tests for expired nitric oxide (FENO) and blood pressure (BP)(1), subjects consumed either 70 mL beetroot juice (BR) or placebo (PL)(2). Following a two-hour break, blood sampling and FENO and BP tests were completed again (3). Subjects were then exposed to either normoxia or hypoxia, and completed a cycling exercise protocol consisting of a 15-minute self-selected warm-up (4), 15 minutes at 50% maximum power output (5) and a 10-km time trial. Each session concluded with a cool-down.

2.4 Results

2.4.1 Subject characteristics

Twelve healthy, trained male cyclists and triathletes volunteered to participate in this study. Subject anthropometric data is presented in Table 4.

Table 3: Subject anthropometric, fitness, and power data.

Statistic	Age (yrs)	Height (cm)	Weight (kg)	$\dot{V}O_{2max}$ (ml·kg^{-1}·min^{-1})	50% max power (W)	Hematocrit (%)
Mean	29.3	181	75.9	67.5	211	45
SD	5.1	5.2	6.5	5.8	13.1	3
Min	21	173.5	65.4	60.9	190	38
Max	40	187.5	86.8	81.2	230	50

Note. n = 12, except for hematocrit where n = 10. $\dot{V}O_{2max}$, maximal oxygen consumption (mL·kg^{-1}·min^{-1}).

2.4.2 Hypoxic and normoxic exposures

The PO_2 did not differ between PL and BR supplementation days in hypoxia (117 [1] and 118 [1] mmHg, respectively. t[11] = -1.659; p = 0.125), or normoxia (155 [1] and 155 [2], respectively. t[11] = -0.312; p = 0.761). The average PO_2 for hypoxic exposures corresponded to an altitude of 2440 m (~8000 feet). Subjects correctly identified the environmental condition on 56% of trials, while 21% of assumptions were incorrect and 23% did not know.

2.4.3 Resting blood pressure and FENO

Resting SBP and DBP were similar before and after BR and PL supplementation (see Figure 5). Mean FENO was similar before (25 [11] *vs*. 23 [8] ppb) supplementation with either BR or PL. There was a significant interaction effect for time x treatment, and follow up paired samples t-tests indicate FENO post-BR was significantly greater than post-PL (28 [12] *vs*. 22 [9] ppb) (see Tables 4 and 5). Subjects correctly identified the supplement 8% of the time and incorrectly identified the supplement 11% of the time (81% did not know.) Descriptive data and average difference data for BP and FENO variables are reported in Tables 5 and 6.

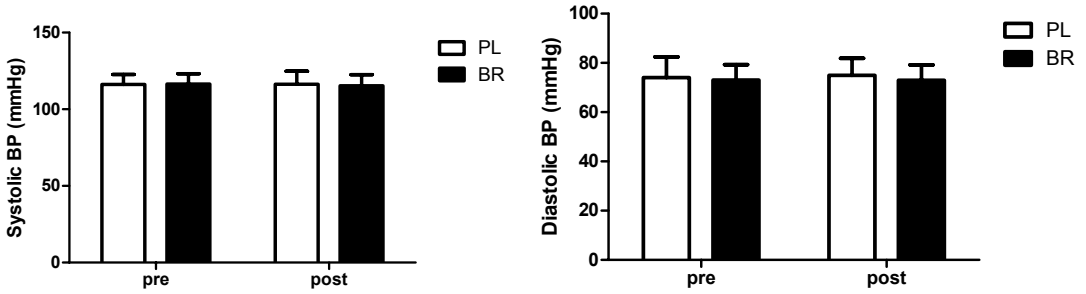

Fig 5: Systolic and diastolic blood pressure (mmHg) pre- and post-supplementation with either placebo (PL) or beetroot juice (BR). Error bars represent standard deviation.

Table 4: Summary data for blood pressure and fraction of expired nitric oxide measurements pre- and post-supplementation.

Variable	Time	Supplement	N	Mean [SD]	Paired samples t-test and p-value
SBP (mmHg)	Pre-supplement	BR	12	116 [7]	t[11]=0.114; p=0.911
		PL	12	116 [7]	
DBP (mmHg)	Pre-supplement	BR	12	73 [6]	t[11]=-0.435; p=0.672
		PL	12	74 [8]	
SBP (mmHg)	Post-supplement	BR	12	115 [7]	t[11]=-0.543; p=0.598
		PL	12	116 [8]	
DBP (mmHg)	Post-supplement	BR	12	73 [6]	t[11]=-0.758; p=0.464
		PL	12	75 [7]	
FENO (ppb)	Pre-supplement	BR	12	25 [11]	t[11]=1.484; p=0.166
		PL	12	23 [9]	
FENO (ppb)	Post-supplement	BR	12	28 [12]*	t[11]=5.016; p<0.001
		PL	12	22 [9]	

Note: BR, beetroot juice; PL, placebo; SBP, systolic blood pressure; DBP, diastolic blood pressure; FENO, fraction of exhaled nitric oxide.
(*) Indicates a significant difference from the PL treatment.

2.4.4 Steady-state cycling

Variables measured during the 50% power output trial are presented in Table 7. Relative to normoxia, hypoxic exposure increased V_E and HR and decreased SpO_2 and TOI ($p < 0.05$ for all variables). There was no difference in $\dot{V}O_2$ or exercise economy ($p > 0.05$ for both variables) between normoxic and hypoxic conditions. In normoxia, there was no effect of BR on V_E, HR, $\dot{V}O_2$, or exercise economy between BR and PL trials. In hypoxia, there was no effect of BR on V_E, HR, SpO_2, TOI, $\dot{V}O_2$, or economy ($p > 0.05$ for all variables.) See Figure 7 below and Figures 8-11 in Appendix E.

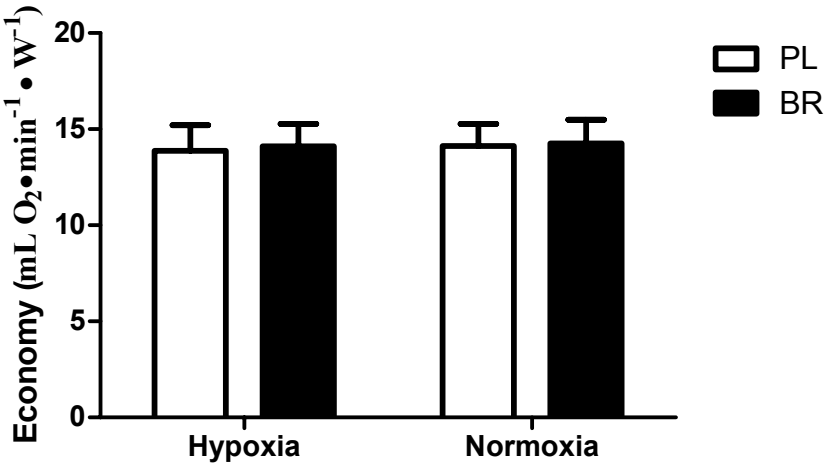

Fig 6: Economy (mL $O_2 \cdot min^{-1} \cdot W^{-1}$) during steady state cycling at 50% maximum power output in normoxia or hypoxia when supplemented with either placebo (PL) or beetroot juice (BR). Error bars represent standard deviation.

2.4.5 10-km time trial

Relative to normoxia, TT performance was significantly decreased in hypoxia. Time to complete the 10-km TT and increased, and mean power output decreased in hypoxia relative to normoxia ($p < 0.05$ for both variables). Mean $\dot{V}O_2$ and SpO_2 were also significantly lower in hypoxia (see Table 8). TOI was similar between both conditions (see Table 8).

In normoxia, there was no improvement in performance with BR. There was no difference in time to complete the 10-km TT, mean power output, or mean $\dot{V}O_2$ over 10 km ($p > 0.05$ in all variables). In hypoxia, there was also no improvement in performance with BR. Mean $\dot{V}O_2$ over 10 km, time to complete the 10-km TT, mean power output,

SpO_2 and TOI did not differ between BR and PL trials. See Table 8 for summary statistics of variables measured during the 10-km TT. See also Figure 8 below and 14-18 in Appendix F.

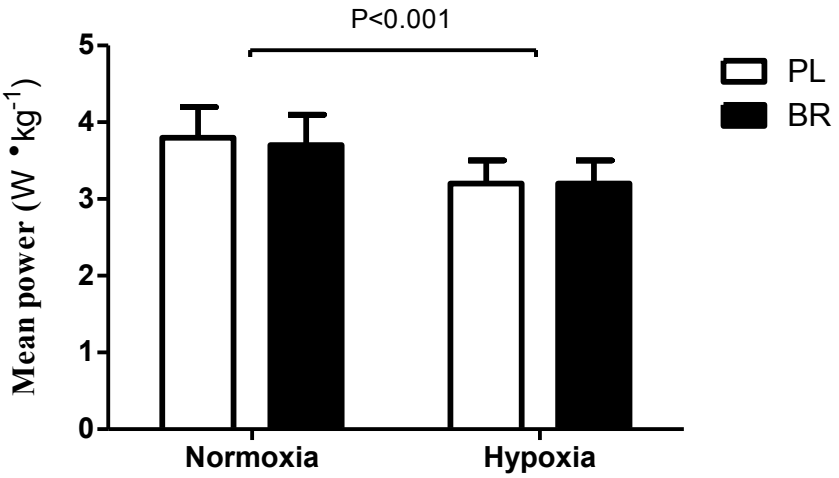

Fig 7: Mean power output ($W \cdot kg^{-1}$) during the 10-km time trial in either normoxia or hypoxia when supplemented with placebo (PL) or beetroot juice (BR). Error bars represent standard deviation.

Table 5: Summary statistics for systolic and diastolic blood pressures and fraction of expired nitric oxide pre- and post- supplementation with BR or PL.

Variable	n	Pre-PL	Pre-BR	Post-PL	Post-BR	ANOVA Results		
						Time	Treatment	Interaction
SBP (mmHg)	12	116 [7]	116 [7]	116 [8]	115 [7]	F(1,11)=0.178 P=0.681	F(1,11)=0.077 P=0.786	F(1,11)=0.843 P=0.378
DBP (mmHg)	12	74 [8]	73 [6]	75 [7]	73 [6]	F(1,11)=0.254 P=0.625	F(1,11)=0.460 P=0.512	F(1,11)=0.176 P=0.683
FENO (ppb)	12	23 [8]	25 [11]	22 [9]	28 [12]	F(1,11)=0.946 P=0.352	F(1,11)=11.219 P=0.006*	F(1,11)=15.621 P=0.002*

Note. BR, beetroot juice; PL, placebo; SBP, systolic blood pressure; DBP, diastolic blood pressure; FENO, fraction of expired nitric oxide. Data presented as mean [SD].

Table 6: Summary statistics for physiological responses to cycling in normoxia and hypoxia at 50% of maximum power following supplementation with BR or PL

Variable	n	NPL	NBR	HPL	HBR	ANOVA Results		
						Treatment	Condition	Interaction
$\dot{V}O_2$ (mL·kg^{-1}·min^{-1})	12	39.3 [3.6]	39.7 [3.8]	38.5 [5]	39.1 [3.6]	F(1,11)=0.412; p=0.534	F(1,11)=0.785; p=0.395	F(1,11)=0.012 p=0.914
Economy (mL O_2·min^{-1}·W^{-1})	12	14.2 [1.1]	14.3 [1.2]	13.9 [1.3]	14.1 [1.2]	F(1,11)=0.248 p=0.682	F(1,11)=0.670 p=0.430	F(1,11)=0.057 p=0.816
V_E (L·min^{-1})	12	80.7 [17]	76.5 [10]	88.1 [9.9]	91.2 [13.9]	F(1,11)=0.097 p=0.761	F(1,11)=31.272 p<0.001*	F(1,11)=1.949 p=0.190
HR (bpm)	12	150 [13]	149 [11]	156 [9]	156 [12]	F(1,11)=0.218 p=0.650	F(1,11)=34.726 p<0.001*	F(1,11)=0.061 p=0.810
SpO_2 (%)	12	98 [1]	98 [2]	87 [4]	88 [3]	F(1,11)=2.926 p=0.115	F(1,11)=156.708 p<0.001*	F(1,11)=1.347 p=0.270
TOI (%)	7	51 [12]	52 [13]	45 [12]	46 [10]	F(1,6)=0.161 p=0.702	F(1,6)=13.785 p=0.01*	F(1,6)=0.023 p=0.884

Note. BR, beetroot juice; PL, placebo; $\dot{V}O_2$ (mL·kg^{-1}·min^{-1}), oxygen uptake; V_E, ventilation; HR, heart rate; SpO_2, pulse oxygen saturation; TOI, tissue oxygenation index. Data presented as mean [SD].

Table 7: Summary statistics for physiological responses to a 10-km cycling time trial in normoxia and hypoxia following supplementation with BR or PL.

Variable	n	NPL	NBR	HPL	HBR	ANOVA Results		
						Treatment	Condition	Interaction
Mean $\dot{V}O_2$ (mL·kg^{-1}·min^{-1})	12	50.5 [5.5]	51.2 [5.3]	43.3 [7.4]	44.4 [5.4]	F(1,11)=0.681 p=0.427	F(1,11)=29.513 p<0.001*	F(1,11)=0.009 p=0.925
Mean power output (W·kg^{-1})	12	3.8 [0.4]	3.7 [0.4]	3.2 [0.3]	3.2 [0.3]	F(1,11)=0.379 p=0.550	F(1,11)=161.971 p<0.001*	F(1,11)=2.505 p=0.142
Time (s)	12	950 [47]	959 [53]	1023 [47]	1015 [51]	F(1,11)=0.006 p=0.939	F(1,11)=143.784 p<0.001*	F(1,11)=2.385 p=0.151
SpO_2 (%)	12	97 [2]	97 [3]	87 [4]	87 [4]	F(1,11)=0.363 p=0.559	F(1,11)=365.943 p<0.001*	F(1,11)=0.156 p=0.692
TOI (%)	7	48 [12]	49 [13]	46 [10]	45 [11]	F(1,6)=0.013 p=0.913	F(1,6)=5.848 p=0.06	F(1,6)=0.177 p=0.692

Note. BR, beetroot juice; PL, placebo; Mean $\dot{V}O_2$, mean oxygen uptake over 10-km; SpO_2, pulse oxygen saturation; TOI, tissue oxygenation index. Data presented as mean [SD].

2.5 Discussion

2.5.1 Layout of discussion

The purpose of this study was to assess the effects of BR supplementation on exercise performance in trained male cyclists in normoxia and moderate hypoxia. The discussion is divided into three parts: 1) the effects of BR on pre- and post-supplementation variables; 2) the effects of BR on exercise economy; and 3) the effects of BR on 10-km TT performance.

2.5.2 Pre- and post-supplementation variables

The supplement used in this study is sold as an ergogenic aid, and was selected as appropriate for practical application to the target athlete group as it is commercially available and inexpensive. Furthermore, the NO_3^- content of the supplement that we used (400 mg) falls within the suggested "safe" limits of NO_3^- intake of 300-500 mg (Lundberg *et al.,* 2011a). Despite the suggested benefits of NO_3^-, little is known of the long-term effects and safe amounts for consistent use. In the past, there have been concerns regarding high concentrations of NO_3^- and NO_2^- in drinking water. If NO_2^- binds to hemoglobin and oxidizes the heme group to its ferric state (Fe^{3+}), the reaction forms methemoglobin, which cannot bind with O_2 (Lundberg *et al.,* 2008). Infants under six months have a smaller amount of the enzyme that converts methemoglobin back to hemoglobin, making them vulnerable to methemoglobinemia ("blue baby syndrome") (Avery 1999). Methemoglobinemia is very unlikely in adults without underlying pathology, but it remains a worthwhile consideration. Consumption of meat products preserved with NO_2^- may be linked to cancer; however, epidemiological studies have not demonstrated conclusive evidence of this belief (Mensinga *et al*., 2003). Nonetheless, uncontrolled intake of NO_3^- supplements could result in NO_3^- toxicity (Lundberg *et al.,* 2011a). Anecdotally, some people find that foods containing dietary NO_3^- or NO_2^- trigger headaches, and migraines. To date, there have been no reported headaches in BR studies, although some individuals may be susceptible to this effect. This would be particularly relevant in hypoxia, where headache is a symptom of acute mountain

sickness (Hackett and Roach 2001). However, it is obvious from other studies that continuous supplementation with BR over multiple days (*e.g.,* Vanhatalo *et al.,* 2010) or a larger acute dose (*e.g.*, Wylie *et al.,* 2013a) causes greater increases in plasma NO_3^- and NO_2^- than a single concentrated dose (*e.g.,* Wylie *et al.,* 2013a).

Our study is the first to measure the effect of BR on FENO, and our results show that an acute dose of ~6.5 mmol [NO_3^-] in 70 mL BR is sufficient to increase FENO. Previously, a meal rich in NO_3^- has been demonstrated to increase FENO in healthy individuals (Olin *et al.,* 2001). Dietary NO_3^- and NO_2^- that are not reduced in the acidic stomach may be absorbed from the intestine to the blood. As blood passes through the lungs, it is possible that some NO_3^- or NO_2^- carried in the blood is reduced to NO.

2.5.3 Blood pressure

Supplementation with BR did not decrease resting BP in this study. BR supplementation has demonstrated a small but consistent drop in systolic blood pressure (Siervo *et al.,* 2013), and a recent study by Wylie *et al.* (2013a) indicated that systolic BP significantly decreased following acute supplementation with 70 mL, 140 mL or 280 mL of BR containing 4.2, 8.4 and 16.8 mmol NO_3^-, respectively. Therefore, the dose of BR used in our study should have been sufficient to decrease BP; however, these authors also indicated that the peak reduction in BP following the smallest dose of BR was at 4 hours post-supplementation. In the present study, subjects' BP was assessed two hours post-supplementation, which may not have allowed enough time for BP to decrease. Metadata presented by Siervo *et al.,* (2013) included data from individuals with health comorbidities, where BP may be more responsive to NO_3^- or BR supplementation. Subjects in our study had fairly low BP and perhaps BR would reduce BP more in individuals with higher baseline BP. It is also possible that physiological arousal was high immediately prior to completing the TT protocol, which could counteract any lowering effects of the BR. It is worth noting that this study was not specifically powered to detect a change in blood pressure.

2.5.4 Exercise economy

In our study, economy was similar between BR and PL trials in normoxic and hypoxic steady-state trials. Economy also did not differ significantly between hypoxia and normoxia. Any improvement in economy would likely be indicative of alterations in oxidative phosphorylation, as a given amount of work should have a consistent energy cost (Jones n.d). Our data is inconsistent with previous evidence demonstrating measurable improvements in exercise economy with BR supplementation.

Previous studies investigating the effect of BR on economy reported improvements with BR supplementation in normoxia relative to PL. Lansley *et al*. (2011b) concluded that economy was significantly improved following BR (0.5 L (~5 mmol NO_3^-) per day for 15 days prior to exercise) during both moderate- and severe-intensity treadmill exercise. Using a similar six-day loading protocol to Lansley *et al*. (2011b), Bailey *et al*. (2009) also demonstrated lowered oxygen cost at the end of exercise for moderate intensity cycling; however, the provided dose of NO_3^- in the BR supplement was greater in these studies than the supplement used in the present study. It seems promising that a greater acute NO_3^- dose will result in greater benefit to performance (Hoon *et al*., 2013a), although smaller doses of BR over a few days would likely be more palatable to athletes.

One of the discrepancies between this study and those previously published is the fitness of subjects. The majority of studies have used recreationally active subjects, while our study used well-trained subjects; however, Cermak *et al*. (2012a) demonstrated a lower $\dot{V}O_2$ in trained cyclists during 30-minute steady state cycling at 45% and 65% of maximum power output, when supplementing with 140 mL BR per day (~8 mmol NO_3^-) over six days. The daily dose in that study was greater than the acute dose provided in our study. Data from Cermak *et al*. (2012a) indicated that BR may be effective in trained athletes, but athletes may require a larger dose than untrained individuals to see a benefit in performance. Furthermore, highly trained athletes routinely cause sufficient shear to their vessels to up-regulate NO production through the endogenous NOS system. Training adaptations specifically affecting NO production may mask the effect of a small, acute, dose of BR on economy in the trained athletes in this study. Studies showing an improvement in economy indicate that loading protocols

are a viable option to improve economy; however, large acute doses may be more practical for athletes. Different loading and acute supplementation protocols, as well as fitness, are therefore important considerations for athletes competing in normoxia. The NO_3^- dose per kg of body weight could also be an important mediator of the effects. The average weight of the subjects in our study was 75.9 [6.5], ranging from 65-87 kg; however, each individual was provided with the same dose of BR. It is possible that the NO_3^- dose per kg was not sufficient to cause a measurable effect on economy in the heavier subjects. However, there was no change in economy in our study and no correlation between dose by weight and economy.

The acute dose of BR used in this study was not expected to have an effect in normoxia in our subject population due to their baseline fitness (*i.e.* subjects were well trained). While the NOS system is upregulated in hypoxia (Casey and Joyner 2012), the lack of available oxygen inhibits oxidation of L-arginine, which we hypothesized would increase the efficacy of BR; however, steady-state economy was similar between BR and PL in hypoxia. The NO_3^- – NO_2^- – NO system is potentiated by hypoxic environments (Dauncey 2012), and the conversion of NO_3^- to NO_2^- is maximized as oxygen saturation approaches 50% (Lundberg *et al.,* 2008). Furthermore, this system is primarily anaerobic. Hypoxic exposure affects aerobic performance to a greater extent in fit individuals compared to individuals of lower baseline fitness (Fulco 1998). If highly trained individuals are affected more by hypoxia (and if they become more hypoxemic than less fit individuals (Dempsey and Wagner 1999); it follows that they will have less oxygen available for their NOS systems, making the anaerobic NO pathway particularly useful to fit individuals in hypoxia. Our normobaric hypoxic exposure was equivalent to moderate altitude (~2440 m), which may not be sufficient to negatively impact NOS production in the trained subjects. Another possibility is that the BR dose we used was not great enough to enhance the effects of endogenous NO production to a degree that would be measurable within the normal day-to-day variation of metabolic data.

Our results disagree with those of Muggeridge *et al*. (2014), which indicated a measurable benefit to performance in trained athletes supplemented with BR in moderate hypoxia. Muggeridge *et al*. (2014) had a very similar protocol to our study: trained cyclists completed a 15-minute steady-state cycling bout at 60% of hypoxic

maximum power output at a simulated 2500 m. The authors concluded that $\dot{V}O_2$ was lower in the BR trial compared to PL (2542 [114] mL.min^{-1} *vs*. 2727 [85] ml·min^{-1} p = 0.049). The acute BR supplementation protocol in this study was similar to ours, although it had a lower NO_3^- content (70 mL containing ~5 mmol NO_3^-). Muggeridge *et al*. (2013b) provided the BR supplement three hours before performance testing as opposed to our two hour interval, which may not have allowed enough time for plasma [NO_3^-] and [NO_2^-] to peak, and reduced the likelihood of BR benefitting performance. Muggeridge *et al*. (2013b) also based their hypoxic steady state intensity on a hypoxic $\dot{V}O_{2max}$ test. In our study, 50% of maximum power output was based on a normoxic $\dot{V}O_{2max}$ test. It is likely that in our study, 50% power in normoxia was equivalent to a greater percentage of maximal workload in hypoxia, making it more difficult to maintain metabolic steady state. However, while the exercise intensity had a greater physiological cost (*e.g*., V_E and HR were higher) in hypoxia compared to normoxia, hypoxic exposure for both BR and PL trials was consistent, allowing for direct comparison between hypoxic steady state trials. Furthermore, measured economy and $\dot{V}O_2$ did not differ between normoxic and hypoxic steady state trials in our study. While subjects may have been slightly less economical in hypoxia, it was not obvious from our collected metabolic data that the chosen intensity was too high to maintain steady state, despite subjects working harder in hypoxic than in normoxic trials.

The HR and V_E were significantly increased and SpO_2 and TOI significantly decreased during the hypoxic PL economy trial compared to the normoxic PL trial in our study; however, these variables were unchanged between BR and PL trials in both normoxia and hypoxia. Previously, Masschelein *et al*. (2012), demonstrated improvement in SpO_2 as well as TOI in the *vastus lateralis* during steady state cycling at 45% maximum power after supplementing with a BR dose of 0.07 mmol NO_3^-·kg^{-1}·day^{-1} for six days prior to testing (equating to ~5.25 mmol NO_3^- in a 75 kg individual). In that study, hypoxic exercise trials were completed at a simulated altitude of 5000 m, making the exposure more severe than ours, and mean SpO_2 during steady-state exercise was below 70%. On average, SpO_2 during hypoxic steady-state cycling in our study was 88% with BR and 87% with PL; given that we did not find a difference in saturation between

the supplement trials, it is possible that there needs to be a greater degree of hypoxemia for BR to have an effect on blood oxygen saturation. Similar to our study, Muggeridge *et al*. (2013b) demonstrated no difference in steady-state SpO_2 between BR and PL trials at a simulated altitude of 2500 m. At an oxygen saturation of ~88%, oxygen concentration remains on the relatively flat portion of the oxygen dissociation curve. It is possible that if BR attenuated a decrease in saturation through NO availability, it would be more obvious at higher altitudes, because the conversion of NO_2^- to NO is potentiated as oxygen tension falls and the oxygen dissociation curve becomes much steeper (i.e., small effects would cause a greater shift in SpO_2). If this were correct, the efficacy of BR supplementation would correlate with severity of hypoxic exposure.

2.5.5 10-km TT performance

Acute BR supplementation did not improve 10-km TT performance relative to PL in either normoxia or hypoxia in our study: there was not a significant difference between time to complete the TT, mean power output or mean $\dot{V}O_2$. TOI and SpO_2 did not differ between BR and PL supplementation. As expected, hypoxia impaired TT performance: SpO_2 and mean $\dot{V}O_2$ were significantly lower, time to complete 10-km significantly increased, and mean power output was decreased by ~17% in hypoxia relative to normoxia.

Data from Muggeridge *et al*. (2013b) indicated an improvement in 16.1-km TT performance following acute BR supplementation at a simulated altitude of 2500 m (70 mL; ~5 mmol NO_3^-); however, as discussed earlier, these authors tested their subjects three hours post-supplementation, potentially allowing more time for NO_3^- and NO_3^- to peak.

Previous studies testing trained cyclists suggested that some individuals are "responders" to BR supplementation. Christensen *et al*., (2012) supplemented with 500 mL (0.5 g NO_3^-, ~8 mmol NO_3^-) BR per day for six days and had subjects complete a two-hour cycling warm-up consisting of moderate- to high-intensity blocks, followed by a 400 kcal TT. Although there was no significant improvement with BR, these authors identified two responders ($\dot{V}O_{2max}$ of 71 and 74 ml·kg$^-$1·min^{-1}) as individuals who had a

2.5% and 8% improvement, respectively, in the 400 kcal TT. These individuals did not differ from the rest of the subjects in baseline plasma [NO_3^-]. Wilkerson *et al*. (2012) also did not report an overall significant improvement in trained cyclists over a 50-mile TT with an acute 500 mL dose of BR containing 6.2 mmol NO_3^-; however, six of eight subjects demonstrated a performance improvement, and $\dot{V}O_2$ tended to be lower, resulting in an improved economy.

Unlike the two studies of highly trained cyclists discussed above (Christensen *et al*., 2012; Wilkerson *et al*., 2012), individual subjects were not identified as responders in our study. Subjects were not tested repeatedly in each environmental condition and we therefore could not conclude whether any differences in performance between BR and PL were due to chance or the effect of supplementation, on an individual basis (i.e. when the overall result is no effect, some subjects will inevitably demonstrate an effect ("responders") and some subjects will not ("non-responders"), as data will be dispersed around the mean). Mean $\dot{V}O_{2max}$ (67.5 [5.8] ml·kg^{-1}·min^{-1}) in our study was slightly higher than the average in the Wilkerson *et al*. (2012) (63 [8] ml·kg^{-1}·min^{-1}). The NO_3^- dose in the BR supplement was similar between the two studies, but differences in results are unlikely due to a difference in fitness. However, subjects tested by Christensen *et al*. (2012) had a mean $\dot{V}O_{2max}$ of 72.1 [4.5] ml·kg^{-1}·min^{-1}, indicating that while fitness likely affects the efficacy of BR, it may not fully explain the discrepancy between our study and previous research. If BR does have an effect, the most likely reason for this discrepancy could be a combination of subject weight and acute standardized dosage. However, on average, our subjects were similar to subjects in these studies (75.9 [6.5] kg in our study vs. 69 [8] kg (Christensen *et al*., 2012) and 79 [9] kg (Wilkerson *et al*., 2012), but were provided with a small, acute dose of NO_3^- (i.e., 6.5 mmol or ~0.09 mmol·kg^{-1}). While this dose was nearly equivalent by weight to the Wilkerson *et al*., (2012) study (0.08 mmol·kg^{-1}) where most subjects improved with BR, Christensen *et al*., (2012) provided subjects with a dose of ~0.11 mmol·kg^{-1} over six days and identified only two subjects as benefitting from BR. The relative dose-by-weight of the subjects with the greatest improvement with BR in these studies are unknown. Cycling protocols in these studies were also different from the present study, with both protocols having a longer duration warm-up and TT relative to our study. As

mean power output remained high in these other studies, it is possible that the effects of BR may be more evident in longer-duration exercise as [NO_3^-] and [NO_2^-] peak; however, it should be noted that Cermak *et al*. (2012b) reported that acute BR supplementation with ~8.7 mmol NO_3^- did not improve performance of a 1073 [21] kJ (~1-hour) TT. Average power output during that trial was reasonably high (275 [7] W with BR vs. 278 [7] W with PL), corresponding to ~70% maximal power output. Finding a balance between dose-response and exercise duration and intensity should be a primary goal for individual athletes wishing to improve performance with BR.

Improvement of shorter TT distances has also been reported with BR supplementation. Cermak *et al*. (2012a) demonstrated an improvement in a 10-km TT in trained cyclists supplemented with 140 mL BR (~8 mmol NO_3^-) over 6 days; however, their study involved a supplement loading protocol and a longer warm-up (allowing more time for plasma NO_2^- or NO_3^- to peak) than our study, both factors that may play a role in enhancing BR-related performance gains.

Overall, results from studies in the literature indicated that the effect of BR is smaller in trained athletes than in untrained individuals, and that the effect is nearly undetectable for TT performance (e.g., G = 0.11, 95% CI = -0.16, 0.37) (Hoon *et al*., 2013b). While these were expected outcomes in our study in normoxia, our hypothesis that any effect of BR would be more obvious in hypoxia was not supported by our data, in contrast to results from Muggeridge *et al*. (2013b). As the most directly comparable study, these authors demonstrated a significant performance improvement in time to complete a 16.1-km TT, with nine subjects and a calculated effect size of D = 0.67. Both our study and Muggeridge *et al*. (2013b) had small sample sizes (twelve and nine subjects, respectively). Therefore any differences in our conclusions are most likely the result of a lack of statistical power due to small sample size resulting in high sampling variability. Considering the conservative effect size of BR (G = 0.11) suggested by Hoon *et al*. (2013b), and assuming an alpha level of 0.05, with the 12 subjects tested in this study, we would have a 6% chance of detecting an effect with BR. Using the same assumptions, Muggeridge *et al*. (2013b) would have a 5% chance of finding the effect of BR that they reported. If we assume that BR would be more effective in hypoxia than in normoxia, and assume the upper limit effect size calculated by Hoon *et al*. (2013b), G =

0.37, we would have a 22% chance of demonstrating an effect of BR, and Muggeridge *et al*. (2013b) would have a 16% chance. If we test the effect size suggested by Muggeridge *et al*., (2014) in hypoxia, D = 0.67, these authors would have a 42% chance of finding their original result in a follow-up experiment (with their sample size of nine subjects). Our study would have a 56% chance of detecting a difference with BR with the same assumptions (n=12). Therefore, it is likely that the effect of BR falls somewhere between the two studies, although the main difference is likely sampling variability. With a calculated Cohen's D of 0.18 in our study, and of 0.67 in the study by Muggeridge *et al*. (2013b), the weighted mean of the effect of BR in hypoxia is 0.39. For 80% power, we would require 43 subjects to see an effect of BR. While the data in our study do not directly indicate that BR would be effective for improving TT performance, the results are in the direction of our hypothesis. Thus, in the future studies investigating BR for TT performance in hypoxia would need to increase sample size, or change other variables (e.g., increase altitude, increase supplement) to increase statistical power. While the sample size could be increased in order to show a "statistically significant finding," it is also worth considering that the effect appears to be small and highly variable. In this regard, BR is unlikely to negatively affect performance, but it may not be worthwhile for athletes to consume BR when competing in hypoxia in lieu of other methods to legally and safely enhance performance, whether currently known or yet to be discovered.

2.5.6 Summary

The primary outcome of this study was that cycling performance was not improved following supplementation with BR relative to PL, although performance was negatively impacted by hypoxic exposure. There were no differences in economy or time trial performance between BR and PL trials. Resting blood pressure was also similar pre- and post- supplementation with BR or PL trials and FENO did not increase post-BR supplemention, indicating that the supplement was not sufficient to increase NO production in the body.

3. Conclusion

BR supplementation has become popular with athletes, although the few studies investigating highly trained individuals in normoxia indicate that its efficacy may be limited in this population. Aerobic capacity is decreased in hypoxia and highly trained athletes experience greater decrements in endurance performance than untrained individuals in hypoxia. Previous research has indicated that even if athletes do not benefit from BR in normoxia, the supplements may be ergogenic at altitude. The proposed mechanisms of action behind BR supplementation may be particularly effective in hypoxia, where the NOS system and aerobic performance are both limited. Our data, however, does not support this postulate. In our study, twelve male subjects completed four cycling trials consisting of a 15-minute warm-up, 15-minute steady-state cycling at 50% of maximum power output and a 10-km TT in a randomized protocol, with single-blind environmental condition (sea level or 2500 m) and double-blind supplementation (BR or PL). Our BR supplementation protocol had no effect on FENO or resting BP. Hypoxic exposure increased HR and V_E and decreased steady-state SpO_2 and TOI relative to normoxia, but there was no difference in any variables between BR and PL supplementation. Performance in 10-km TT was negatively affected by hypoxia, but there was no difference in 10-km TT performance measures between BR and PL trials. Overall, the lack of effect of BR in our study is likely attributable to relatively high subject fitness, the moderate severity of our hypoxic exposure, and our dosing protocol.

3.1 Conclusions regarding thesis hypotheses

Hypothesis 1: I accept our hypothesis that BR would have no performance enhancing effect in normoxia. BR did not improve markers of exercise performance in normoxia.

Hypothesis 2: I reject our hypothesis that BR would improve these markers in hypoxia, relative to PL. BR did not improve markers of exercise performance in moderate hypoxia.

Hypothesis 3: I reject our hypothesis that BR would decrease resting blood pressure. BR did not lower resting systolic or diastolic BP relative to PL following acute BR supplementation.

Hypothesis 4: I accept our hypothesis that FENO would increase after consumption of BR. FENO was significantly increased with BR compared to PL.

3.2 Strengths, limitations, future directions

This is the first study to compare the effect of BR supplementation on exercise tolerance and performance in trained athletes in normoxia *and* hypoxia in a double blind, randomized, crossover design. The BR product is commercially available and the acute supplementation protocol used is representative of realistic practices for athletes in training or at competition. In designing the experiments, we chose to use a BR dose that falls within the recommended "safe" guidelines for NO_3^- supplementation (Lundberg *et al.,* 2011a). Anecdotally, subjects could not differentiate between the BR and PL supplements, confirming the strength of the double blind.

During the course of this project, data was published indicating that there is a dose-response effect with BR (Wylie *et al.,* 2013a) suggesting that the BR dose in this study was too small to have a significant effect on performance (although other studies have demonstrated performance improvement with a similar dose to ours, e.g., Muggeridge *et al.,* (2014)). As Wylie *et al.* (2013a) reported no adverse events in their study with increasing NO_3^- dose; it may be necessary to revisit guidelines for what is a "safe" acute dose. These authors also demonstrated that supplementing with BR containing 16.8 mmol NO_3^- resulted in no greater improvement to performance than BR containing 8.4 mmol NO_3^-. This conclusion is particularly relevant to athletes and warrants further investigation.

Although we did not report any significant performance related results, other studies have shown BR to have a positive effect on performance. Variables affecting the efficacy of BR should be investigated. Future studies should also investigate the best BR dose relative to fitness. Realistically, each athlete will have a different optimal dose, possibly based on fitness and weight. BR supplementation protocols should be

developed on a case-by-case basis to balance maximized performance with potential risks. To this end, general BR supplementation guidelines and recommendations should also be developed as a starting point for athletes and coaches. The effect of BR during acute hypoxic exposures (from low to high altitude) and chronic altitude acclimatization should also be further investigated. Despite the lack of effect on performance in this study, the increase in FENO following BR supplementation indicates increased NO production. NO production has previously been suggested to be important for functional adaptation to hypoxia (Erzurum *et al.,* 2007). Therefore, there may be beneficial effects at the molecular level that were not great enough to cause a statistically detectable change in exercise performance in this thesis study. Longitudinal studies assessing safety of long term NO_3^- supplementation are also important.

Part of the appeal of supplementing with BR products is that beets may be considered a "functional food" (Reid 2013) that could enhance sport performance through consumption of a healthy diet. While this study focused specifically on whether the $[NO_3^-]$ in our BR dose would improve performance, other components of BR juice have also been suggested as potential performance enhancers. For example, betaine is gaining interest in sport literature, and has been demonstrated to benefit repeated cycling sprint performance (Pryor *et al.,* 2012). Beets also contain antioxidant compounds (Kanner *et al.,* 2001), which may be helpful in recovery. It is possible that these components in beets work synergistically in the body, and are collectively involved in the overall performance enhancement that has been demonstrated with BR supplementation. As our BR and PL supplement were identical aside from NO_3^- concentration, this could have affected our results. It would be worthwhile to study these components separately to determine pathways that could be further manipulated to benefit sport performance, and to perhaps reveal other so-called functional foods.

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Appendix A

The effect of beetroot juice on exercise tolerance in moderate hypoxia

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AIMS: Athletes often train and compete at moderate altitudes where hypoxia decreases endurance performance. Beetroot juice (BR) has been shown to lower the oxygen cost of exercise in normoxia, and therefore may be beneficial to performance in hypoxia. We investigated the effect of BR on steady state economy and 10-km time trial (TT) performance in normoxia, and in hypoxia (simulated altitude of 2500 m).

METHODS: Trained male cyclists (VO_{2max} ≥ 60 ml·kg^{-1}·min^{-1}) completed four exercise trials. Two hours prior to exercise, subjects consumed 70 mL BR (~6 mmol nitrate) or 70 mL placebo (nitrate-depleted BR) in a randomized, double-blind manner. Subjects then completed a 15-min self-selected warm up followed by a 15-min steady-state bout at 50% max power output, and a 10-km time trial (TT) in either normoxia (~21% O_2) or hypoxia (~16% O_2). Environmental conditions were randomized and single-blind. Exercise economy (mL O_2·min^{-1}·W), oxygen saturation (SpO_2), mean power output (MPO) and time to complete the 10-km TT were measured.

RESUL TS: Economy at 50% power output was similar in hypoxic and normoxic conditions (mean [SD]: 14.5 [0.4] *vs*. 14.3 [1.2] mL O_2·min^{-1}·W; $p > 0.05$), but subjects had a significantly higher MPO in the normoxic TT (294 [44] W) relative to the hypoxic TT (246 [36] W; $p < 0.05$). BR did not affect economy (14.2 [1.4] *vs*. 14.3 [1.3] mL O_2·min^{-1}·W), SpO_2 (98 [1.7] *vs*. 98 [1.4 %]), MPO (295 [42] *vs*. 293 [46] W) or time to complete the 10-km TT (941 [48] *vs*. 951 [58] s) relative to placebo in normoxia ($p > 0.05$ in all comparisons). Similarly, in hypoxia, BR did not affect economy (14.6 [1.3] *vs*. 14.3 [1.2] mL O_2·min^{-1}·W), SpO_2 (87 [1.9] *vs*. 87 [2.1] %), MPO (244 [35] *vs*. 247 [37] W) or time to complete the 10-km TT (1014 [61] *vs*. 1010 [55] s) relative to placebo ($p > 0.05$ in all comparisons).

CONCLUSIONS: In a small sample of well-trained cyclists, BR did not improve exercise performance in normoxia or hypoxia; however, more subjects will need to be tested to confirm these conclusions if these data are to be applied to elite athletes.

The effect of beetroot juice on exercise tolerance in normoxia and moderate hypoxia

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INTRODUCTION

- High-level athletes often train and compete at moderate altitudes, where endurance performance is limited by hypoxia.
- Nitrate, found in beetroot juice (BR), is a precursor for nitric oxide (NO) [1].
- BR improves exercise tolerance in normoxia [2] and hypoxia [3] in moderately fit subjects; however, it is unclear if BR supplementation is effective in highly trained athletes [4].

OBJECTIVES

- To determine if BR improves economy in both normoxia (21% O_2) and moderate hypoxia (16% O_2).
- To determine if BR improves 10-km time trial (TT) performance in both normoxia and moderate hypoxia.

METHODS

Participants

- 8 male cyclists and triathletes (26-40 years of age, VO_{2max} 67 ± 6 mL/kg/min).

Test Days

- Subjects completed four test days in a randomized cross-over design.
- Subjects consumed either 70 mL of BR or 70 mL placebo (PL) two hours before exercise in either normoxia or hypoxia.
- Exercise economy (mL O_2/min/W) was assessed through a 15-minute cycling bout at 50% maximum power output.
- 10-km TT performance was assessed through mean power output.

Data Analysis

- Paired samples t-tests were used to assess differences in economy, SpO_2, heart rate, ventilation and TT performance between BR and PL trials in both conditions.

Figure 1. Subjects breathed hypoxic or normoxic air from an altitude chamber in a single-blind manner.

RESULTS

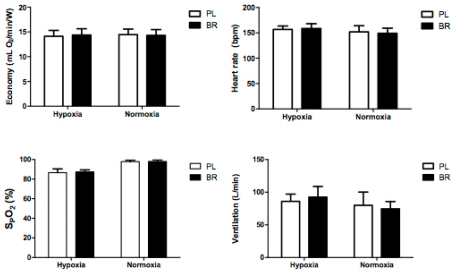

Figure 2. Mean (SD) data for economy, heart rate, SpO_2, and ventilation in all four conditions during 50% maximum power output cycling.

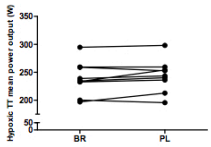

Figure 3. Individual subject data of mean power output over a 10-km TT in hypoxia after supplementation with BR or PL.

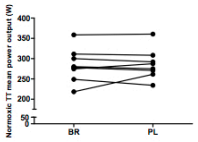

Figure 4. Individual subject data of mean power output over a 10-km TT in normoxia after supplementation with BR or PL.

CONCLUSIONS

- We are still collecting data; however, based on this sample size, it seems that exercise economy, SpO_2, heart rate and ventilation were unaffected by BR supplementation in both normoxia and moderate hypoxia.
- 10-km TT performance was not improved with BR supplementation in normoxia or moderate hypoxia.
- The lack of an effect may be due to the standard dose given to participants, or to the high fitness of the subjects.

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Appendix B

Table 8: Summary of studies using beetroot supplementation.

Author	Participants	BR protocol	Exercise Protocol	Conclusions
Bailey *et al*., 2009	8 healthy, recreationally active young men	500 ml per day BR (11.2±0.6 mmol NO_3^-) x 6 days. Double blind, cross-over with cordial placebo	Moderate- and severe-intensity exercise tests on last three days of supplementation (including TTE), cycling.	BR reduced oxygen cost of exercise in both moderate and severe intensity, possibly related to improved mitochondrial efficiency. (Assessed muscle oxygen uptake kinetics)
Bailey *et al*., 2010	7 young men	500 ml per day of either BR (5.1 mmol NO_3^-) or PL for 6 consecutive days. Double blind, crossover	Low- and high-intensity "step" exercise tests over last 3 days of supplementation (knee extensor exercise)	BR decreased: muscle ATP turnover rate, ADP accumulation, muscle P_i accumulation, PCr depletion, VO_2 amplitude during low-intensity exercise, VO_2 slow-component during high-intensity exercise-increased severe exercise limit of tolerance
Vanhatalo *et al*., 2010	8 healthy adults (5 males, 3 females) physically active	500 ml BR per day (5.2 mmol NO_3^-) or 500 ml low-calorie cordial placebo for 15 days	2 moderate-intensity step tests followed by ramp test repeated after 2.5 hrs following first ingestion, and after 5 and 15 days of BR and PL	Reduced BP, and O_2 cost of submaximal exercise, can be maintained for 15 days if BR supplementation is continued.
Kenjale *et al*., 2011	4 male and 4 female subjects; PAD patients	Consumed either 500 mL BR or 500 mL orange juice (matched for calories, sugar, carbohydrates and protein) 120 min before second tests. Cross over	Treadmill maximal cardiopulmonary test	With BR subjects walked 18% longer before claudication pain onset, and had a 17% longer peak walking time, reduction in fractional O_2 extraction at working tissue, decreased DBP at rest and SBP and HR during recovery from max exercise. No changes in endothelial function
Lansley *et al*., 2011a	9 club-level competitive male cyclists	500 ml BR (~6.2 mmol NO_3^-) or 500 ml PL (NO_3^- depleted BR) in a	4 km and 16.1 km TT	BR increased mean PO for both TT distances, although VO_2 values did not

		cross over design 2.5 hours before TT		differ (higher PO for same VO_2)
Lansley *et al*., 2011b	9 healthy, physically active males	500 ml BR per day, containing ~6.2 mmol NO_3^- or 500 ml placebo for 6 days (double blind, crossover)	Treadmill VO_{2max} Treadmill tests on days 4 (2x 6 min moderate-intensity running and one exhaustive bout of severe-intensity as a measure of exercise tolerance) & 5 (2x 6 min bouts moderate-intensity running and one 6 min bout of severe intensity running) for VO_2 responses Day 6, incremental single-legged knee-extension exercise test	BR reduced SBP, and O_2 cost of walking, mod-intensity and severe-int running. BR improved TTE during severe-intensity running. Mitochondrial oxidative capacity not different between BR and PL
Vanhatalo *et al*., 2011	7 males, 2 females, healthy and moderately trained in rec sport	750 mL BR (9.3 mmol NO_3^-) or 750 mL NO_3^- depleted BR (double blind, crossover) 24 hrs prior to hypoxia trials	Knee-extension exercise to limit of tolerance–once in normoxia, twice in hypoxia (14.5% O_2)	Limit of tolerance reduced with hypoxia, but no difference between normoxic control and hypoxia+BR. BR reduced muscle metabolic perturbation [PCr, Pi, pH]. PCr recovery time was higher in hypoxia+PL vs hypoxia+BR and normoxia+control. Exercise tolerance and oxidative function restored with BR in hypoxia ≅ normoxia+control
Bond *et al*., 2012	14 well-trained male junior rowers	500 ml BR or PL for 6 days (7 day washout)	6 maximal 500 m ergometer repetitions	Improved max rowing-ergometer reps (particularly reps 4-6).
Cermak *et al*., 2012a	12 male cyclists	6 days of 140 ml per day (8 mmol NO_3^-) BR or NO_3^- depleted BR (double blind, crossover) 14 day washout	60 min submaximal cycling (2x 30 min at 45% and 65% max watts) followed by 10 km TT	TT and PO improved with BR. Submaximal VO_2 was lower with BR. Whole body fuel selection, plasma lactate, glucose and insulin did not differ between conditions
Cermak *et al*., 2012b	20 trained male cyclists	140 mL concentrated BR or 140 mL PL 2.5 hrs before TT. Randomized, double blind	~1 hour TT	BR did not improve TT performance, or PO. Plasma NO_2^- higher following BR

Engan *et al*., 2012	12 healthy men and women, trained breath-hold divers	70 ml BR or nitrate depleted BR 2.5 hrs prior to testing	Static apnea series, of two-min sub-max apneas separated by 3 min recovery, followed y 5 min recovery before final maximal effort apnea	BR increased maximal apneic duration by 11%, significantly higher SaO_2 for BR supplemented sub-max apneas, SaO_2 lower with BR after max apneas (but longer duration), reduced MAP with BR
Hobbs *et al*., 2012	Study 1: 18 normotensive, healthy males Study 2: 14 healthy males	Study 1: randomized to receive either 500 g H_20, 100 g BR with 400 g H_20, 250 g BR with 250 g H_20, or 500 g BR Study 2: randomized to receive either 200 g white bread, 200 g bread enriched with white beetroot (50% total weight), or 200 g bread enriched with red beetroot (50% total weight)	ABP measured every 15 minutes from 8 am-1 pm, every 30 minutes from 1-10 pm and every 60 minutes from 10 pm – 8 am. Urine collected at baseline, 2 and 4 hrs post intervention and 24 hrs post	Nearly dose-dependent decrease in ABP with BR enriched products.
Masschelein *et al*., 2012	15 young, physically active males	6-day supplementation with 500 ml BR (0.07 $mmol.kg^{-1}$ nitrate) or apple-blackcurrant juice in 5 equal doses throughout the day, and full dose 1-2 hrs pre-exercise tests on the final day (single-blind, cross over)	20 min submax constant load cycling at workload equal to 45% VO_{2peak}, and an maximal exercise test to volitional exhaustion (one normoxic control test, and hypoxic+PL and hypoxic+BR tests equivalent to 5500 m)	With BR, lower VO_2 at rest and during 45% ex test, SpO_2 higher. BR partly negated decreased TTE in hypoxia. BR improved muscle oxygenation but not cerebral oxygenation in severe hypoxia. BR improved exercise tolerance, but there were similar AMS symptoms with both BR and PL
Murphy *et al*., 2012	11 recreationally fit men and women	Randomized cross over, 200 g baked whole beetroot or cranberry relish 75 minutes pre-exercise trials	5-10 minutes self-paced treadmill warm up followed by 5 km running TT	With BR: 3% faster TT, RPE lower over 1^{st} 1.6 km
Wilkerson *et al*., 2012	8 well trained male cyclists	500 ml BR or 500 ml NO_3^- depleted BR 2.5 hrs before TT	50 mile TT	PO did not differ between conditions, but VO_2 tended to be lower in BR (p=0.06) resulting in a greater

				PO/VO_2 ratio. BR did not improve TT performance.
Bond *et al*., 2013a	12 healthy females	Randomized cross over supplementation with either 500 mL BR or 500 mL orange juice	Steady state submaximal cycling, with three successive stages of 5 min at 40%, 60% and 80% of VO_{2peak}	BR improved cerebral and systemic hemodynamics at rest and at 40% and 60% workloads
Bond *et al*., 2013b	12 healthy women	500 mL BR or orange juice placebo	Trials consisting of 40%, 60% and 80% of VO_{2peak} submaximal exercise	BR decreased VO_2, SBP and heart rate-systolic pressure product at rest, and at 40%, 60% and 80% of VO_{2peak}
Christensen *et al*., 2013	10 elite trained male cyclists	Cross over (with 14-day washout in between supplementation periods). 6-day supplement period of either 500 ml BR (~0.5% NO_3^-) or 500 ml isocaloric PL (apple & blackcurrant drink)	Baseline VO_{2max}. 4th day of supplementation: 2 x 6 min at 70 & iPPO separated by 60 min. Followed by RST of 6x20 s sprints separated by 100 s low-int cycling. 6th day suppl: 1 x 6 min at 70% iPPO, 5 min later an endurance tests consisting of 2-hr "pre-load" (20 min at 50% iPPO, 10 min variable int avg of ~53% iPPO 4 times) followed by 400 kcal TT	No significant changes in VO_2 kinetics, endurance capacity (no change in economy), and repeated sprint performance. Two responders benefitted from BR, with improved economy
Fulford *et al*., 2013	8 active males	Double blind, randomized cross over. Two-week washout. 15 days 500 mL per day BR or PL (NO_3^- depleted BR)	Short bouts of continuous exercise followed by a repetitive MVC protocol	BR supplementation reduced PCr cost of contraction while maintaining muscle-force generating capacity
Hobbs *et al*., 2013	23 healthy males	200 g bread containing 100 g BR or 200 g white bread, randomized, open-label, crossover	Postprandial microvascular vasodilation, arterial stiffness and ambulatory BP measured over 6 hours	BR bread increased endothelium-independent vasodilation and decreased DBP
Hoon *et al*., 2013a	10 highly-trained male rowers	Double blind crossover supplementation with PL (0 mmol NO_3^-), single BR (4.2 mmol), or double BR (8.4	2000 m rowing TT	Single BR demonstrated a negligible effect on TT performance. Double BR possibly beneficial to TT performance

		mmol)		
Kelly *et al*., 2013	6 male, 6 female healthy adults (60-70 yrs)	3 days with BR (2x70 ml) or PL (NO_3^- depleted BR) Double blind, randomized, cross over	BP, sub-maximal ramp incremental treadmill ex test, 2x 6 min walk tests (moderate intensity) followed by 10 min passive recovery. 3 cognitive tests	BR decreased resting BP (DBP/SBP/MAP), accelerated VO_2 kinetics during treadmill walking (though there was no improvement during the 6 minute walk tests). Cognitive performance unaltered.
Lanceley *et al*., 2013	11 trained females runners	Double-blind, repeated measures crossover with 14 day washout. Supplementation with either BR (~8 mmol NO_3^-) or PL for 4 days and 2.5 hrs pre exercise	5 km TT	No main effect for BP, although slightly lower SBP with BR. TT performance unchanged between trials, with a trend towards improvements with BR.
Lane *et al*., 2013	12 male and 12 female competitive cyclists	Double blind latin squares design. Supplementation with either caffeine (3 $mg \cdot kg^{-1}$), BR (8.4 mmol NO_3^-), BR + caffeine, or no supplementation (control)	Females: 29.35 km cycling TT Males: 43.83 km cycling TT	Power output significantly increased with caffeine and caffeine + BR compared to control. BR not ergogenic alone or when combined with caffeine, compared to the caffeine trial. BR not ergogenic in this trial.
Liu *et al*., 2013	26 participants aged 38-69	Randomized cross over, high (220 mg NO_3^- from spinach) or low nitrate energy-matched meals	Blood pressure and arterial stiffness measurements pre- and up to 210 min post meal	Spinach resulted in higher large artery elasticity index, lower pulse pressure, and lower systolic BP
Muggeridge *et al*., 2013	8 male kayakers	Randomized, cross over design. 70 mL concentrated BR or tomato juice PL	3 x trials consisting of 15 min paddling at 60% max work rate, five x ten-sec sprints and a 1 k TT (2^{nd} and 3^{rd} trials with BR or PL)	No difference in peak power or TT performance. VO_2 reduced. BR shows no effect with repeated supramaximal kayak sprints or 1 km TT performance.
Torre *et al*., 2013	7 healthy males	Randomized double-blind crossover supplementation with either 0.5 L/day spinach juice (5.5 mol/day NO_3^-)	Five "all out" 6 second sprints on a cycle ergometer, separated by 24 seconds passive recovery	Absolute peak power not different between spinach and PL sprints 1 and 2. Peak power significantly greater during sprints 3, 4 and 5 with spinach

		or PL		
Wylie *et al*., 2013a	14 male, recreational team-sport players	Double-blind, randomized crossover 490 mL BR or NO_3^- depleted PL over 30 hrs preceding test	Yo-Yo intermittent recovery level 1 test	Intermittent performance improved with BR. Lower blood glucose concentration and reduced plasma potassium increase with BR
Wylie *et al*., 2013b	10 healthy males	Randomized, cross over design. 70, 140 or 280 mL concentrated BR containing 4.2, 8.4 and 16.8 mmol NO_3^- respectively, or NO_3^- depleted PL	24 hr assessment of dose on plasma $[NO_3^-]$ and $[NO_2^-]$. On separate days, moderate- and severe- intensity cycle exercise tests	Plasma $[NO_2^-]$ increased in a dose-dependent matter. 140 mL and 280 mL BR reduced steady-state VO_2 by 1.7% and increased time to task failure significantly
Muggeridge *et al*., 2014	9 competitive male cyclists	70 mL concentrated BR or NO_3^- depleted PL	3 x trials consisting of 15 min steady state at 60% max work rate and a 16.1 km TT at simulated 2500 m	VO_2 lower during steady state exercise, and TT performance significantly faster with BR

Note. BR: beetroot juice, PL: placebo, SBP: systolic blood pressure, DBP: diastolic blood pressure, ABP: ambulatory blood pressure, MAP: mean arterial pressure, I/R: ischemia reperfusion, V_E: ventilation, RER: respiratory exchange ratio, PO: power output, RPE: rating of perceived exertion, PAD: peripheral arterial disease, iPPO: incremental test to peak power output, MVC: maximal voluntary contraction

Appendix C

Normoxic and hypoxic air:

Subjects breathed air from a normobaric hypoxia chamber while seated on the cycle ergometer outside of the chamber, through a breathing tube attached to the facemask. On hypoxic test days, subjects breathed ~16% O_2 from the chamber to simulate an altitude of ~2500 m. On normoxic control days, subjects breathed ~21% O_2, which corresponded to sea level. In either condition, the sounds and set-up of the exposure were indistinguishable, helping to enhance the quality of the environmental single-blind. With subjects sitting outside of the altitude chamber, low CO_2 concentrations could be maintained inside, as subjects exhaled to the metabolic cart outside the chamber. Analyzers were set up to continuously monitor F_IO_2 and F_ICO_2 from the chamber, and these values were later used to correct metabolic cart calculations.

To use the metabolic cart when subjects were breathing air from the hypoxia chamber, analyzers (17625 O_2 Analyzer and 17630 CO_2 Analyzer, Vacumed, Ventura, CA) were calibrated and set up to sample O_2 and CO_2 continuously from the chamber. Gas sampling data was collected with a Power Lab (PL3508 Powerlab, ADInstruments, Colorado Springs, CO), collected by a computer, and saved to a Lab Chart (LabChart V7, ADInstruments, Colorado Springs, CO) file. Temperature and humidity in the room were noted during calibration and were later used to calculate water vapour pressure to correct metabolic cart data.

Because the metabolic cart calculations data during exercise assumed subjects breathed normoxic air, the data was later corrected. Fifteen-second mean F_ICO_2 and F_IO_2 data from the hypoxia chamber was calculated to match with 15-second data intervals calculated by the metabolic cart. The metabolic cart calculates data based on dry air. Therefore, it was necessary to calculate (and remove) water vapour from our measured FIO_2.

Water vapour pressure during the test was calculated using a water vapour vs. temperature chart and the following calculations:

1. Water vapour pressure (P_{H2O} in mmHg) was calculated by multiplying the pressure of aqueous vapour over water with humidity measured in the laboratory, divided by 100.
   e.g., (adjusted temperature * humidity) /100 = P_{H2O}
2. The fraction of the barometric pressure made up of water vapour pressure was calculated by dividing the result of the above calculation by barometric pressure in the laboratory, multiplied by 100.
   e.g., (P_{H2O} / P_B) * 100 = X%
3. This fraction was then multiplied by the measured F_IO_2 in the chamber, divided by 100, to determine the fraction of water vapour in measured F_IO_2
   e.g., X * F_IO_2 / 100
4. This value was then subtracted from the measured F_IO_2 to determine actual F_IO_2

Once the corrected F_IO_2 was calculated, the data provided by the cart needed to be adjusted. The following calculations were used to determine final metabolic data:

Fraction of inspired nitrogen (F_IN_2): = 1 - F_IO_2 / 100 - 0.0005

Fraction of expired nitrogen (F_EN_2): = 1 - F_EO_2 / 100 - F_ECO_2 / 100

Ratio of inspired to expired nitrogen (F_IN_2/F_EN_2): = F_IN_2 / F_EN_2

Inspired volume (V_I): = V_E * F_EN_2 / F_IN_2

$\dot{V}O_2$ ($L \cdot min^{-1}$): = (V_I * F_IO_2 – V_E * F_EO_2) / 100

$\dot{V}O_2$ STPD: = calculated $\dot{V}O_2$ / BTPS to STPD conversion value (provided by cart)

$\dot{V}O_2$ ($ml \cdot kg^{-1} \cdot min^{-1}$): = $\dot{V}O_2$ STPD * 1000 / subject mass in kg

$\dot{V}CO_2$: = (V_E * F_ECO_2 – V_I * F_ICO_2) / 100

$\dot{V}CO_2$ STPD: = $\dot{V}CO_2$ / BTPS to STPD conversion value (provided by cart)

RER: = $\dot{V}CO_2$ STPD/ $\dot{V}O_2$ STPD

Note that in the above equations, fractions were entered as percentages, hence the frequent division by 100.

Appendix D

Food and Training Log

Today's Date: (Day of testing)
Food Consumption (please report the time of consumption as well): Morning (Breakfast/Snacks): Noon (Lunch/Snacks) Evening/Night (Dinner/Snacks)
Caffeine: YES/NO If yes, when:
Physical Activity: (activity, duration and intensity)
Hours of Sleep:

One day prior to testing:
Food Consumption (please report the time of consumption as well): Morning (Breakfast/Snacks): Noon (Lunch/Snacks) Evening/Night (Dinner/Snacks)
Physical Activity: (type, duration and intensity)

Physical Activity Readiness
Questionnaire - PAR-Q
(revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO	
☐	☐	**1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	**2. Do you feel pain in your chest when you do physical activity?**
☐	☐	**3. In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	**4. Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	**5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	**6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	**7. Do you know of any other reason why you should not do physical activity?**

If you answered YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT _____ WITNESS _____
or GUARDIAN (for participants under the age of majority)

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

CSEP | SCPE

Physical Activity History

1. Type of physical activity (please list all sports/exercises you participate on a regular basis)

Type of sport	Volume (a). how many minutes/session and (b) how many times/week?)
	a) b).
	a) b).
	a). b).
	a). b).
	a). b).

2. How long have you been a competitive cyclist/triathlete? _____

3. Volume of cycling per week: _____hours/week

_____km/week (solely for cycling)

4. Are you in-season or off-season currently? IN-SEASON/ OFF-SEASON

5. Highest level of competition:_____

6. VO_{2max} (if known): _____; Date:_____; Modality (bike, treadmill)_____

7. Last cycling race: _____; Distance:_____; Date:_____

8. Cycling category (if applicable, please list how many years you've competed in each category)

_____

Appendix E

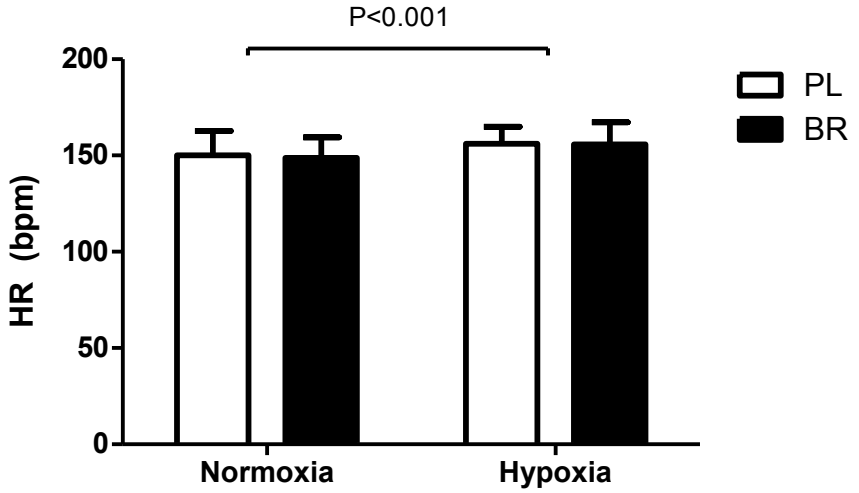

Fig 8: Heart rate (HR) during steady state cycling at 50% maximum power output in hypoxia and normoxia when supplemented with placebo (PL) or beetroot juice (BR). Normoxic and hypoxic trials were significantly different ($P < 0.001$). Error bars represent standard deviation.

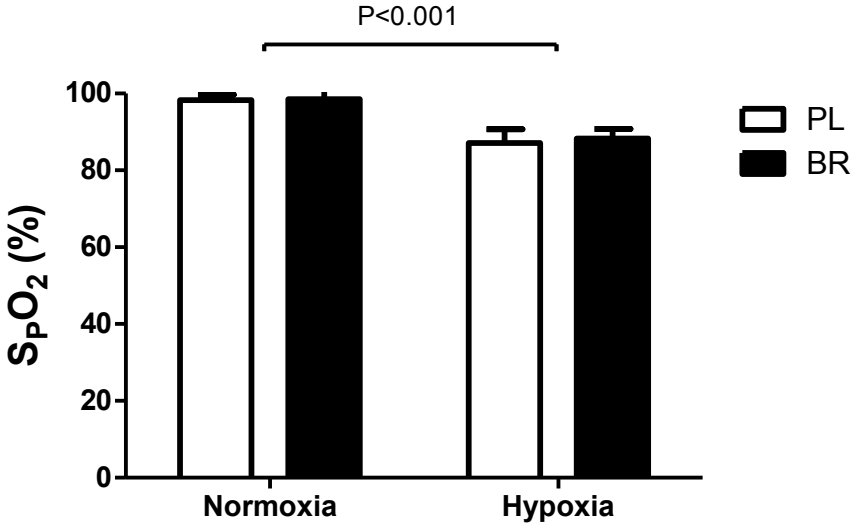

Fig 9: Pulse oxygen saturation (SpO_2) during steady state cycling at 50% maximum power output in hypoxia and normoxia when supplemented with placebo (PL) or beetroot juice (BR). Normoxic and hypoxic steady state were significantly different ($P < 0.001$). Error bars represent standard deviation.

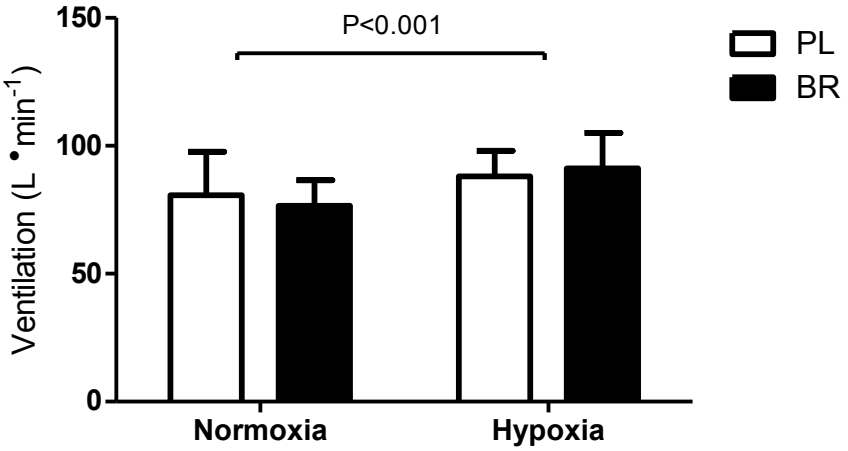

Fig 10: Pulmonary ventilation (V_E) during steady state cycling at 50% of maximum power output in hypoxia and normoxia when supplemented with placebo (PL) or beetroot juice (BR). Hypoxic and normoxic steady state were significantly different ($P < 0.001$). Error bars represent standard deviation.

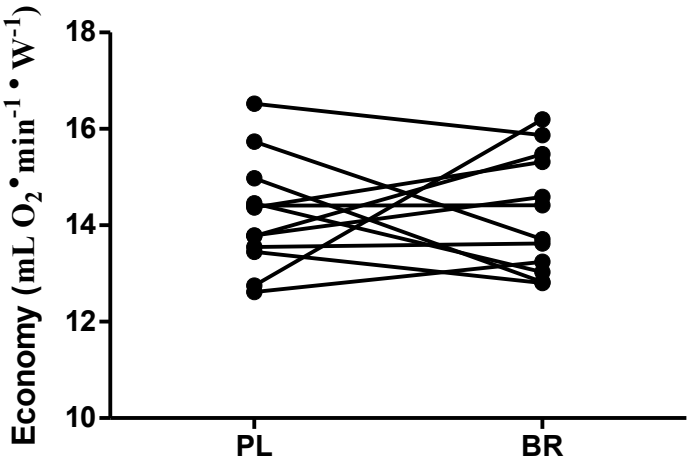

Fig 11: Individual subject economy (mL $O_2 \cdot min^{-1} \cdot W^{-1}$) data during steady state cycling at 50% of maximum power output in normoxia when supplemented with placebo (PL) or beetroot juice (BR).

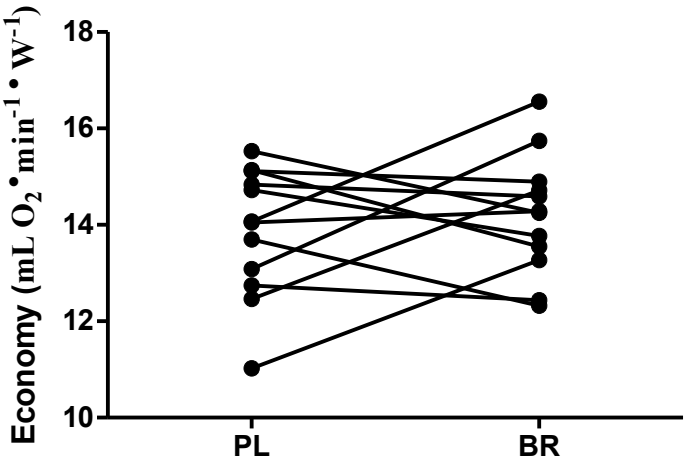

Fig 12: Individual subject economy (mL $O_2 \cdot min^{-1} \cdot W^{-1}$) data during steady state cycling at 50% of maximum power output in hypoxia when supplemented with placebo (PL) or beetroot juice (BR).

Appendix F

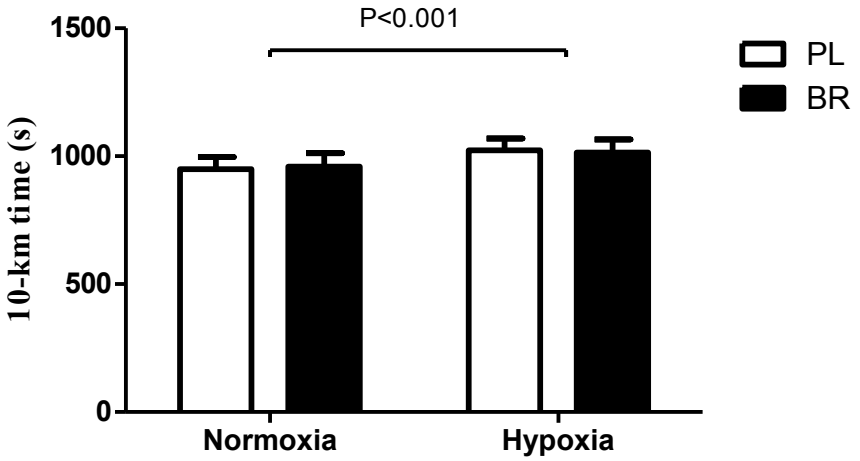

Fig 13: Time (s) to complete a 10-km time trial in normoxia and hypoxia when supplemented with either placebo (PL) or beetroot juice (BR). Hypoxic and normoxic trials were significantly different ($P < 0.001$). Error bars represent standard deviation.

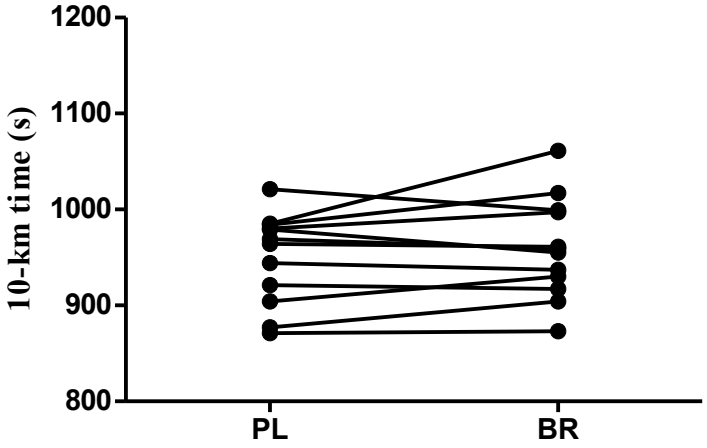

Fig 14: Individual subject data for the 10-km time trial (s) in normoxia when supplemented with either placebo (PL) or beetroot juice (BR).

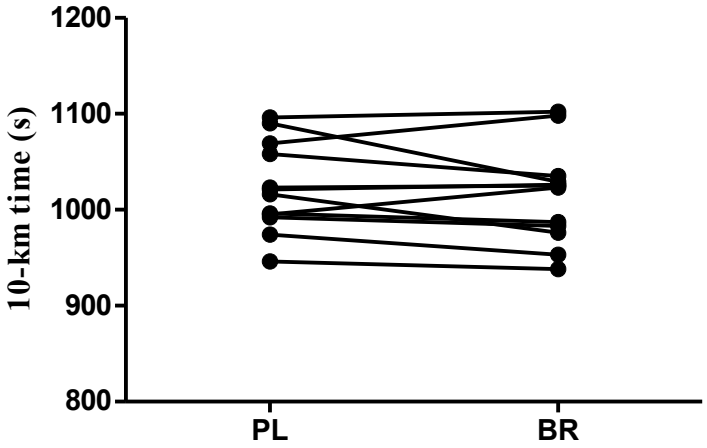

Fig 15: Individual subject data for the 10-km time trial (s) in hypoxia when supplemented with either placebo (PL) or beetroot juice (BR).

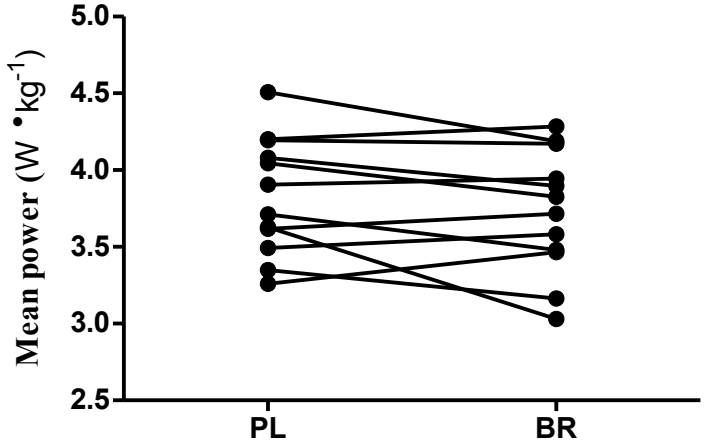

Fig 16: Individual subject data for mean power output ($W \cdot kg^{-1}$) during the 10-km time trial in normoxia when supplemented with either placebo (PL) or beetroot juice (BR).

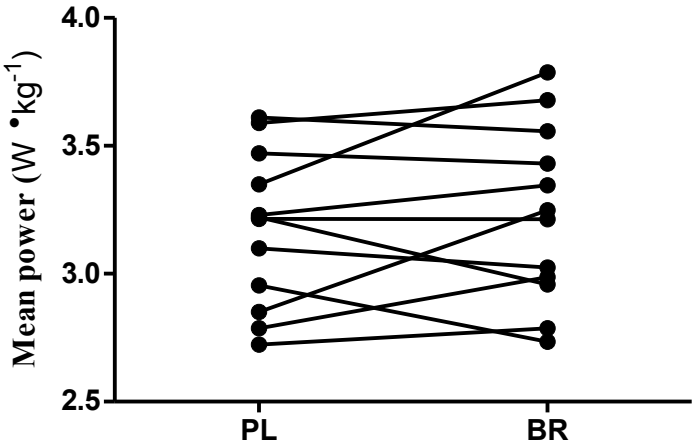

Fig 17: Individual subject data for mean power output ($W \cdot kg^{-1}$) during the 10-km time trial in hypoxia when supplemented with either placebo (PL) or beetroot (BR).