Effects of Beetroot Juice Supplementation on Anaerobic Exercise Performance

By

Mark Andrew Pinder

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Food and Human Nutritional Sciences

University of Manitoba

Winnipeg

ABSTRACT

The study objective was to evaluate the dose-dependent effects of dietary nitrate supplement on dynamic, multi-joint exercise performance. Thirteen males (mean age, 25.2 ± 2.7 years; squat 1-repetition-maximum 146 ± 33 kg) completed a randomized, double-blind, crossover study. Participants consumed beetroot juice with 8 mmol (BRH) or 1.2 mmol nitrate (BRL), or a placebo (PLA) for seven days, executed a dynamic resistance exercise, and seven-day washout between treatments. Assessment included plasma nitrate/nitrite (NO_x) levels, blood lactate, gross motor unit efficiency; measured by electromyography (EMG), during isometric contractions pre, intra, and post-exercise, and exercise repetition to failure. BRH elevated ($P < 0.0001$) plasma NO_x compared to BRL and PLA. BRH and BRL increased mean peak EMG amplitude compared to PLA (79.1 ± 16.3%, 77.5 ± 9.7% and 70.0 ± 9.5% respectively), but it was not significant ($P > 0.05$). No differences ($P > 0.05$) were observed in any other outcome. Even 8 mmol nitrate may not decrease neuromuscular fatigue nor enhance resistance exercise performance in some recreational athletes.

ACKNOWLEDGEMENTS

I would like to thank the members of my advisory committee Dr. Michel Aliani and Dr. Jason Peeler for their guidance and support. I would also like to thank my advisor, Dr. Semone Myrie for providing the opportunity to work on this project, along with several other studies and publications we have completed together. Looking back, I can truly say I have learned a great deal from her and possess skills and knowledge that I will carry with me for the rest of my life.

I would also like to thank those that granted me access to certain areas or equipment that I required and without whom, this study would not have been possible. Dr. Cheryl Glazebrook for allowing me to utilize her electromyography equipment and always making sure it was available despite high-demand for the unit in her own lab. Dr. Stephen Cornish for permitting me to access his phlebotomy lab and equipment in the Applied Research Centre. Finally, Shawn Preston for permitting me unsupervised access to the High Performance Room and allowing me to monopolize several pieces of equipment for many hours at a time. Additionally, thank you to all the Bison athletes who agreed to turn off their music and otherwise adapt their training to ensure the testing environment was quiet and consistent for all my sessions.

I would like to acknowledge the volunteers that assisted me during my testing sessions as I couldn't have completed a single session without them. Dianna Omer, Michael Arinze, Dominique Daoust, and Kheshinah Ramdoyal. Also acknowledged from my lab group are Farnaz Farshidfar and Brianne Collette for their assistance in treatment randomization and preparation and also helping with testing sessions.

Finally, I would like to thank the participants for all their time and hard work as without their willingness to suffer, there would be no study!

DEDICATION

I dedicate this thesis to my family, particularly my wife, Tamera Pinder, and my son, Kaden Pinder. Over the course of this study, Tam and I have gotten engaged, bought our first home together, gotten married, and had our son. Perhaps not the typical undertakings when completing Graduate studies and while I don't know if I can say I wouldn't have done anything differently, I wouldn't have wanted to do it with anyone else. Tam, thank you for your patience, understanding, and support throughout this entire process, and thank you for giving me the best possible gift to come home to everyday in Kaden.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1RM	one-repetition maximum
µL	microlitre
µmol	micromole
ADP	adenosine di-phosphate
ALC	Active Living Centre
AMRAP	as many repetitions as possible
ANT	adenosine nucleotide translocase
ARC	Applied Research Centre
ATP	adenosine tri-phosphate
BH_4	tetrahydrobiopterin
BIA	bio-electrical impedance analysis
BMI	body mass index
BP	blood pressure
BRH	high-dose beetroot
BRL	low-dose beetroot
Ca^{2+}	calcium
CO_2	carbon dioxide
CV	coefficient of variation
DHEA	dehydroepiandrosterone
EDTA	ethylenediaminetetraacetic acid
EMG	electromyography
FAD	flavin adenine dinucleotide

FMN	flavin mononucleotide
fROM	full range of motion
GET	gas exchange threshold
GH	growth hormone
HMB	β-hdroxy β-methylbutyrate
HNO_2	nitrous acid
HPR	High Performance Room
IGF-1	insulin-like growth factor
kDa	kilodalton
KNO_3	potassium nitrate
MMOL	millimole
MVC	maximum voluntary contraction
NADPH	nicotinamide adenine dinucleotide phosphate
$NaNO_3$	sodium nitrate
nm	nanometre
NO	nitric oxide
NOS	nitric oxide synthase
NO_x	nitrate/nitrite
NO_2^-	nitrite
NO_3^-	nitrate
O_2	oxygen
PCr	phosphocreatine
PLA	placebo

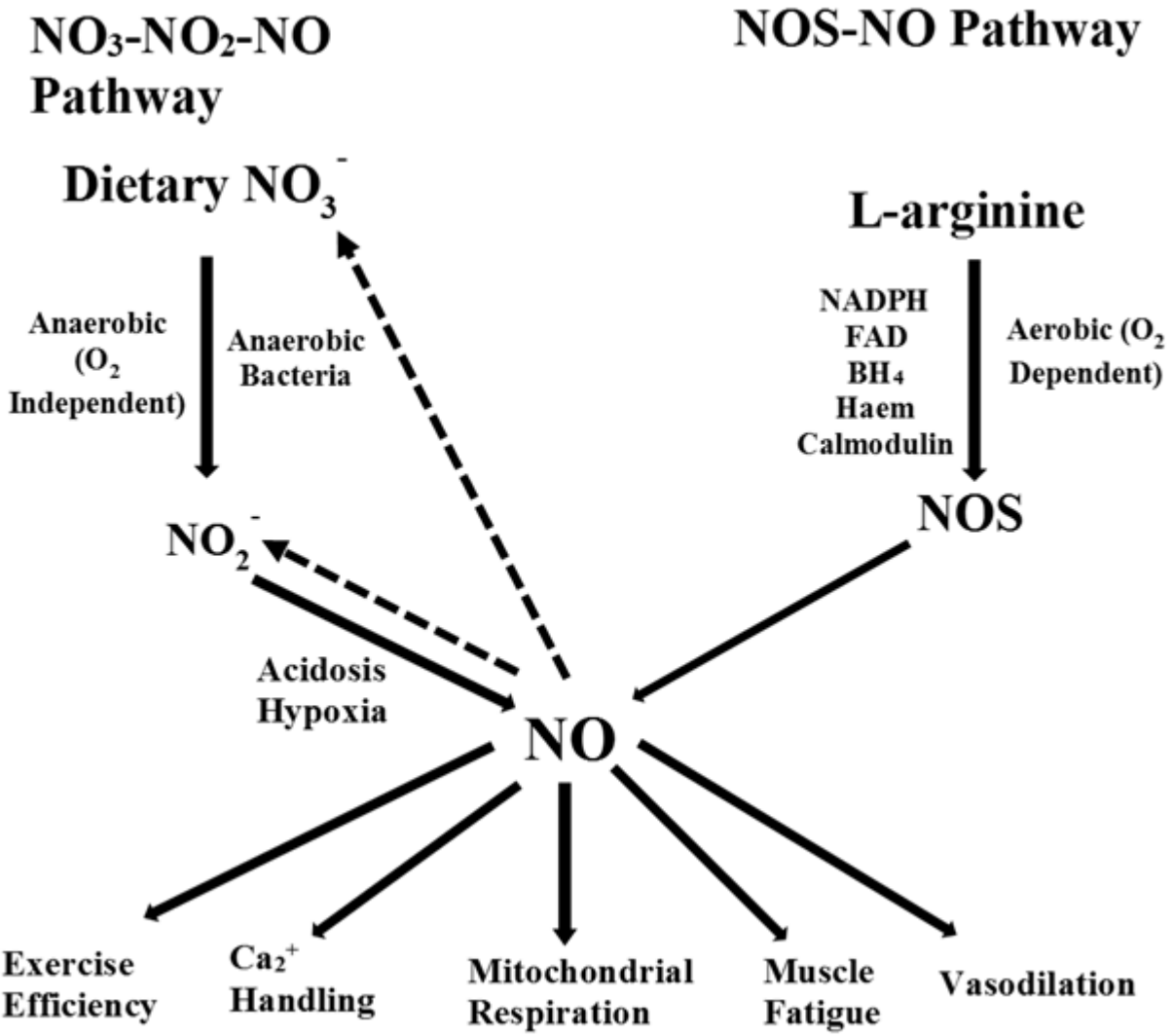
PO	power output
RCFFN	Richardson Centre for Functional Foods and Nutraceuticals
TT	time trial
TTE	time to exhaustion
VO_2	oxygen uptake
VO_{2max}	maximal oxygen uptake
WAnT	Wingate

Chapter I: Literature Review

Introduction

Dietary nitrate (NO_3^-) refers to inorganic NO_3^-, which differs greatly from organic NO_3^-, the latter being commonly found in a variety of compounds known as NO_3^- vasodilators or nitrovasodilators used to treat angina pectoris, myocardial infarction, and congestive heart failure, as well as being used in a variety of explosives such as nitromethane (Torfgard & Ahlner, 1994). Dietary NO_3^- is obtained primarily through the consumption of vegetables, particularly green leafy vegetables such as spinach, lettuce, and beets (McKnight et al., 1997). Once ingested, NO_3^- is converted to nitrite (NO_2^-) and then to nitric oxide (NO) through the nitrate-nitrite-nitric oxide (NO_3^--NO_2^--NO) pathway (Bailey, Vanhatalo, Winyard, & Jones, 2012) (Figure 1). NO is the main bioactive molecule from the pathway that has a variety of functions in the body; exerting physiological effects of interest in both clinical and athletic applications. In summary, some of the main effects of NO include: antimicrobial- It is toxic to some human pathogens such as *E. coli* and *Salmonella* (Dykhuizen et al., 1996). It is also a potent vasodilator capable of lowering blood pressure and improving blood flow (Moncada & Higgs, 2006). Most importantly when discussing athletic applications, it improves exercise tolerance (Bailey et al., 2010; Bailey et al., 2009) and lowers the oxygen (O_2) cost of exercise (Bailey et al., 2009; Lansley, Winyard, Fulford, et al., 2011; Larsen, Weitzberg, Lundberg, & Ekblom, 2007), which essentially allows an individual to perform the same amount of physical work with less O_2. The mechanism responsible for this is still under investigation but it is thought to be related to mitochondrial respiration, either through decreased proton leak through the mitochondrial wall, or NO acting as a supplementary terminal electron acceptor to O_2 (Bailey et al., 2010; Larsen et al., 2011).

Figure 1. (Adapted from Bailey et al. (2012)): Simplified view of pathways responsible for nitric oxide (NO) generation in humans.



Right branch represents a simplified view of conventional L-arginine nitric oxide synthase-nitric oxide (NOS-NO) pathway. Left branch represents the nitrate-nitrite-nitric oxide (NO_3^--NO_2^- NO) pathway. Portions of NO are capable of being recycled back to both NO_3^- and NO_2^- as shown by the dashed arrows. O_2, oxygen; NADPH, nicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; BH_4 tetrahydrobiopterin; Ca_2^+, calcium.

The human body also produces NO through another pathway called the nitric oxide synthase-nitric oxide (NOS-NO) pathway (Figure 1), which is aerobic (O_2 dependent), unlike the NO_3-NO_2-NO pathway which is anaerobic (O_2 independent) (Bailey et al., 2012). The NOS-NO pathway uses the amino acid arginine to produce NO as opposed to the NO_3-NO_2-NO pathway which converts NO_3^- into NO. The two pathways are complimentary to each other (Bailey et al., 2012) with the NO_3-NO_2-NO pathway becoming dominant in acidic (Modin et al., 2001), hypoxic (Stuehr, Santolini, Wang, Wei, & Adak, 2004) tissues such as those found during exercise induced stress (Bailey et al., 2012).

It has been understood for some time that consuming a diet rich in fruits and vegetables has some beneficial health effects in humans, primarily in clinical applications dealing with cardiovascular disease, with green leafy vegetables showing some of the greatest promise for protection against coronary events (Joshipura et al., 2001). This suggested that the effects were not simply attributed to high levels of calcium, potassium and fibre along with low sodium content typically present in fruits and vegetables, but perhaps due to the vasodilatory and blood pressure lowering effects of NO_3^- derived NO (Hord, Tang, & Bryan, 2009). In recent years, building on the knowledge that NO_3^- ingestion provided a viable and substantial pool of physiological NO, research has been conducted into the effects of dietary NO_3^- on athletic performance, specifically on O_2 consumption levels, work capacity and time to completion over a set distance (Dominguez, Cuenca, et al., 2017). Most of these studies have focused on the use of supplements which deliver concentrated doses of NO_3^- in a quick and convenient fashion for use by athletes. This review will explore the structure of the pathway responsible for converting NO_3^- to NO, the physiological effects related to athletic populations and the results of the research conducted thus far through a review and discussion of the literature.

Dietary Nitrate: Overview

Naturally occurring, inorganic NO_3^- that is found in food is referred to as dietary NO_3^-. A misconception is often made that because it is consumed in the diet, that dietary NO_3^- is organic. It is important to remember that the term "organic" in terms of chemistry, generally concerns compounds which contain a carbon atom. Dietary NO_3^- is composed of one nitrogen atom and three O_2 atoms and is therefore classified as inorganic. While there are clinical and therapeutic applications for organic NO_3^-, the focus of this review will be on inorganic NO_3^- consumed orally and any further references to NO_3^- will concern inorganic NO_3^-. NO_3^-, in quantities sufficient to elicit a measurable effect, is primarily (approximately 80%) obtained through ingestion of plant matter (Hord et al., 2009), especially from green leafy vegetables such as spinach, lettuce, arugula and beets (McKnight et al., 1997). NO_3^- itself is not the compound that exerts the beneficial effects of interest on the human body. The bioactive molecule that produces positive physiological influences is NO.

Nitric Oxide: Function, Generation and Physiological Effects in the Body

Nitric oxide: Physiological functions in the body. In mammalian physiology, physiological NO is a signaling molecule that was initially identified as a vasodilator; promoting relaxation of vascular tissue, capable of reducing blood pressure and improving blood flow (Bailey et al., 2012). The ability of dietary NO_3^- to lower blood pressure through its conversion to NO has long been known and utilized in clinical applications, particularly in persons suffering from cardiovascular disease. A single dose of beetroot juice providing 340 mg of NO_3^- has been shown to lower systolic blood pressure by 5.4 mmHg in 6 hours (Coles & Clifton, 2012). A reduction of systolic blood pressure by 5 mmHg is associated with a 10% reduction in the risk of mortality due to cardiovascular disease (Staessen, Wang, & Thijs, 2001). Overall, NO is among

the most investigated molecules in human medicine and physiology with a wide variety of other important and useful functions in the body. Some of the main functions in the body include: antimicrobial- It is toxic to some human pathogens such as *E. coli* and *Salmonella* (Dykhuizen et al., 1996). It is also known to impact processes such as skeletal muscle satellite cell activation following muscle injury (Anderson, 2000), glucose uptake by skeletal muscle (Merry, Lynch, & McConell, 2010), calcium handling (Ca^{2+}) by sarcoplasmic reticulum (Hart & Dulhunty, 2000), neurotransmission (Garthwaite, 2008), skeletal muscle fatigue (Percival, Anderson, Huang, Adams, & Froehner, 2010) and mitochondrial respiration (Brown & Cooper, 1994). Interestingly, and of relevance to athletic populations, NO can increase VO_2 max and reduce the O_2 cost of exercise (Bailey et al., 2009). Generally, while the elevated O_2 cost of exercise above the gas exchange threshold (GET) can be increased with rigorous training (Jones & Poole, 2005), the O_2 cost of submaximal exercise is assumed fixed and is not influenced by factors such as age, gender or fitness level (Poole, Ward, Gardner, & Whipp, 1988), so the fact that increasing NO levels can lower this is of great importance to athletes of all types. This essentially allows an individual to perform the same amount of work with less O_2, which is highly beneficial in hypoxic conditions such as those present under exercise stress. The mechanism through which NO derived from NO_3^-, as opposed to NO synthesized from L-arginine, exerts this effect is not yet well understood but there are several leading theories. First, the mechanisms responsible for reducing NO_2^- to NO are known to be enabled by hypoxia and therefore, it is possible that more NO is produced in areas of muscle tissue that are using more or receiving less O_2. Due to its vasodilatory effects a more consistent O_2 distribution is ensured in skeletal muscle and assists to balance blood flow to meet the O_2 requirement in a given area (Bailey et al., 2009). It is also possible that NO somehow reduces proton leakage through the inner mitochondrial membrane

(Larsen et al., 2011). During mitochondrial respiration, energy is used to pump hydrogen ions through the inner membrane into the inter-membrane space. The inner mitochondrial membrane is slightly permeable and allows some of the hydrogen ions to leak back into the mitochondrial matrix, effectively wasting energy and decreasing the power output of the mitochondria (Larsen et al., 2011). At rest, this leakage phenomenon has been found to be responsible for up to 25% of energy expenditure (Rolfe, Hulbert, & Brand, 1994). Uncoupling proteins can increase the permeability of the wall of the mitochondria allowing more protons to leak through and adenosine nucleotide translocase (ANT) is one of the most potent of these proteins (Brand & Esteves, 2005). NO significantly lowers the expression of ANT resulting in less proton leakage (Brand & Esteves, 2005) and more efficient energy production within the mitochondria, thereby lowering the amount of O_2 required to perform physical work. Another leading theory behind the ability of NO to lower the O_2 cost of exercise is that NO acts as a secondary electron acceptor in place of O_2 at the end of the electron transport chain allowing the same amount of adenosine triphosphate (ATP) to be produced with less O_2 or the continued production of ATP in hypoxic conditions (Basu et al., 2008). Working synergistically with this enhanced ATP production is the potential reduced O_2 and ATP cost of force production in skeletal muscle (Bailey et al., 2010; Fulford et al., 2013) and modification of intracellular Ca^{2+} handling in fast twitch muscle fibers leading to increased muscle force production (Hernandez et al., 2012). It is proposed that NO acts to reduce the O_2 and ATP cost of force production through inhibition of the enzymes actomyosin-ATPase (Walsh, Howlett, Stary, Kindig, & Hogan, 2006) and Ca^{2+}-ATPase (Takaki et al., 1998) which are O_2 dependant and are responsible for a small amount of ATP turnover in muscle (Barclay, Woledge, & Curtin, 2007). The ATP cost of force production is further modulated by NO through reduction of Ca^{2+} cycling (Barclay et al., 2007), a ATP driven

process, and enhanced expression of Ca^{2+} handling proteins allowing for activation of fast-twitch muscle fibers at lower level of stimuli to elicit equal force production (Hernandez et al., 2012). Lowered ATP turnover and enhanced contractile efficiency could result in decreased levels of metabolic waste markers such as adenosine di-phosphate (ADP) and inorganic phosphate accumulation (Bailey et al., 2010). This could lead to delayed muscle fatigue and maintenance of neuromuscular efficiency. These theories are not entirely separate from each other and likely work together to produce the observed results.

In recent years, with a greater understanding of the other beneficial capabilities of physiological NO, research has started to be conducted on the potential of NO to increase athletic performance. Rather than obtaining the high levels of NO_3^- required to see an ergogenic effect from food alone, which is possible but may prove somewhat impractical due to avoidance of fibrous or bulky foods, particularly for athletes near or during competitions, supplements have begun to enter the marketplace that provide concentrated doses of NO_3^- in convenient, easy to consume formats. Most, of these supplements are derived from beets, which are one of the richest sources of dietary NO_3^- available containing >250mg NO_3^-/100g of fresh weight (Hord et al., 2009). Nearly every study conducted to date on the relationship between athletic performance and NO_3^- consumption has used a form of beetroot supplement with the majority of these relying on beetroot juice (Hoon, Johnson, Chapman, & Burke, 2013).

Nitric oxide synthase-Nitric oxide pathway. The NOS-NO pathway (Figure 1) is a well-established pathway that produces NO from the amino acid L-arginine through a series of enzymatic reactions relying on NOS enzymes (Bailey et al., 2012). Three isoforms of NOS enzymes have been identified taking their names from the body systems or cells in which they were first isolated and in order of discovery are known as neuronal (n) NOS, macrophage

(immune) (i) NOS, and endothelial (e) NOS (Stamler & Meissner, 2001). NO, along with L-citrulline, are produced by NOS enzymes through a five electron oxidation reaction of L-arginine in a complex process necessitating the substrates/cofactors; O_2, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH_4), haem and calmodulin (Alderton, Cooper, & Knowles, 2001). The production of NO through the NOS-NO pathway can be impacted by a low bioavailability of any of these compounds including O_2. This is of particular importance when considering the roles NO plays in governing the function of skeletal muscle performing work, which under intense stress develops an acidic, hypoxic environment and impairs the aerobically enzymatic dependant mechanisms involved. Indeed it has been shown that persons with NO synthesis impairments exhibit reduced tolerance to exercise (Lauer et al., 2008).

Nitrate - Nitrite - Nitric oxide conversion pathway. In addition to the NOS-NO pathway, NO is also produced following consumption of dietary NO_3^- through a specialized pathway called the NO_3^--NO_2^--NO pathway (Figure 1). In contrast to the NOS-NO pathway, the NO_3^--NO_2^--NO pathway is anaerobic and therefore is not influenced by O_2 concentration levels. Figure 1 represents a general overview and comparison of the two pathways. The two pathways are complimentary to each other with the NO_3-NO_2-NO pathway becoming dominant in hypoxic, acidic conditions such as those produced during strenuous exercise (Bailey et al., 2012). For this reason, it is considered the primary pathway of interest for athletic populations seeking performance enhancement. The first step in the NO_3-NO_2-NO conversion pathway is consumption of dietary NO_3^- which is rapidly absorbed from the gut and passes into the circulatory system, elevating plasma NO_3^- levels with peak plasma NO_3^- seen at around 60 minutes post-ingestion (Lundberg & Weitzberg, 2009, 2010). Approximately 60% of the

circulating plasma NO_3^- is filtered out by the kidneys and excreted in the urine (Lundberg & Weitzberg, 2009, 2010). Around 25% of the plasma NO_3^- is taken up by the salivary glands providing a continuous stream of NO_3^- which is excreted in the saliva (Lundberg & Govoni, 2004). The salivary glands concentrate the NO_3^- before releasing it into the oral saliva, with NO_3^- levels in saliva ranging anywhere from 10-20 times the concentrations seen in the circulating plasma (Lundberg & Weitzberg, 2010). Once the NO_3^- enters the oral cavity in the saliva, facultative anaerobic bacteria in the mouth convert NO_3^- to NO_2^- through the use of NO_3^- reductase enzymes (Lundberg & Weitzberg, 2009, 2010). The NO_2^- is then swallowed with the saliva and mixes with gastric juices. The acidic conditions of the stomach pronate NO_2^- to nitrous acid (HNO_2), which then breaks down to form various nitrogen oxides, the most important of which is NO (Benjamin et al., 1994). A small amount of swallowed NO_2^- is also absorbed into plasma circulation, elevating circulating NO_2^- levels (Lundberg & Govoni, 2004). The NO_3^--NO_2^--NO pathway is cyclical, which serves to regenerate the highly reactive and short lived NO molecule by converting it back to NO_3^- or NO_2^- so that the process can begin again and produce another molecule of NO (Bailey et al., 2012). The NO_3^--NO_2^--NO can also recycle NO produced from L-arginine in the NOS-NO pathway under normal physiological conditions.

L-Arginine Derived Nitric Oxide: Effects on Athletic Performance

L-arginine has been studied as a potential ergogenic aid for both aerobic and anaerobic exercise due to its role in producing NO, as well as its function as a hormone secretagogue. Consumption of L-arginine increases plasma insulin and growth hormone (GH) levels (McConell, 2007), which could increase performance by sparing glycogen stores through increased fatty acid oxidation (Sonksen, 2001). Additionally, GH promotes the release of insulin-like growth factor (IGF)-1 which upregulates protein synthesis and amino acid uptake

(Gibney, Healy, & Sonksen, 2007). Therefore it has been hypothesized that arginine supplementation could increase skeletal muscle mass and strength, leading to better performance (Gibney et al., 2007). However, long term supplementation would likely be required for any appreciable benefit to be realized due to the time required for accumulation of muscle mass caused through hormonal upregulation of muscle protein synthesis and the results would not likely reach practical significance (Gibney et al., 2007). Therefore, studies focusing on L-arginine for performance enhancement have typically centered on its role in NO production. Unlike studies focusing on NO_3^- supplementation as an ergogenic aid for athletic performance where the treatments were studied in isolation from other potentially helpful metabolites, many of the studies on the ergogenic effects of oral L-arginine supplementation have been done in conjunction with other various compounds (Campbell et al., 2006; Paddon-Jones, Borsheim, & Wolfe, 2004; Willoughby, Boucher, Reid, Skelton, & Clark, 2011). Indeed, this makes it difficult to determine whether any observed positive effects came from L-arginine and the NO produced from it, or if the enhancements in performance were in whole, or in large part due to the added components consumed (McConell, 2007). When an acute administration of L-arginine alone has been studied it has often been supplied by direct intravenous infusion with nearly all studies done by oral route containing supplementary compounds. For obvious reasons, when seeking to use L-arginine outside of clinical applications and instead seeking to augment exercise performance through supplementation, infusion techniques prove impractical and oral administration is greatly preferred. When infused prior to exercise and compared with a placebo, Schaefer et al. (2002) observed reduced plasma ammonia and lactate levels following maximal exercise in recreationally active men. However, plasma lactate levels were not altered with L-arginine infusion in trained cyclists following a bout of exercise (McConell, Huynh, Lee-Young,

Canny, & Wadley, 2006). Overall there have been mixed results reported in the studies conducted to test the performance enhancing capabilities of oral consumption of L-arginine alone in athletic populations. Studies focusing on markers of aerobic athletic performance, such as power output or maximal O_2 uptake (VO_{2max}) on either a cycle ergometer (Liu et al., 2009; Sunderland, Greer, & Morales, 2011) or treadmill (Bescos et al., 2009) found no significant benefit of L-arginine supplementation. Other studies have been conducted focusing on response to anaerobic exercise in the form of resistance training (Alvares et al., 2012; Fahs, Heffernan, & Fernhall, 2009). Following supplementation with arginine, an increase in muscle blood volume (Alvares et al., 2012), forearm blood flow and arm circumference with a decrease in brachial artery stiffness (Fahs et al., 2009) were noted, although strength performance measured as peak torque, total work and set total work failed to increase. Anaerobic performance has also been assessed through a study conducted by Olek et al. (2010). Six, healthy, recreationally active volunteers performed three Wingate tests (WAnTs) with 4 minutes of rest between WAnTs following ingestion of 2 g of arginine or placebo 60 minutes prior to exercise in a crossover design. The WAnT is a supramaximal test that involves pedaling "all-out" against a constant load based on participant bodyweight for 30 seconds and measures peak power, mean power and the rate of fatigue (Bar-Or, 1987; Vandewalle, Peres, & Monod, 1987). No significant improvements were seen in mean power, peak power, fatigue index, O_2 uptake, lactate and ammonia concentrations, or plasma NO_3^-/NO_2^- (NOx) levels (Olek et al., 2010).

Nitrate Derived Nitric Oxide: Effects on Athletic Performance

46 studies relating to the effect of dietary NO_3^- consumption on aerobic exercise performance including the type of participants used, supplementation protocols, mode of performance assessment and performance results are summarized in Table 1. Sixteen of the

studies used time trials to gauge the effects of NO_3^- use. Six of the sixteen studies in this category used cyclists with three of these six studies using distance based time trials. Jo et al. (2017) also used a distance-based time trial, but differed from previous works in that the participants were recreationally active as opposed to trained cyclists. Both Cermak, Res, et al. (2012) and Christensen, Nyberg, and Bangsbo (2013) relied solely on energy expenditure based time trials. Hoon, Hopkins, et al. (2014) examined the effect of NO_3^- supplementation on high-intensity performance on cyclists by having participants aim to achieve maximum power output in two 4-minute cycle ergometer bouts. Lowings, Shannon, Deighton, Matu, and Barlow (2017) conducted the only study to date using a swimming time trial in which male and female swimmers completed 168 m (8 x 21 m) backstroke swim in a pool. Bond, Morton, and Braakhuis (2012) used rowers who performed repeated short distance rowing tests on an ergometer, while Hoon, Jones, et al. (2014) had rowers complete a single 2000 meter ergometer time trial. The studies performed by Boorsma, Whitfield, and Spriet (2014), de Castro, Manoel, Figueiredo, Figueiredo, and Machado (2018), Murphy, Eliot, Heuertz, and Weiss (2012), Oskarsson and McGawley (2018), Peacock et al. (2012) and Shannon et al. (2017) tested the effects of NO_3^- supplementation through running time trials. Boorsma et al. (2014) utilized elite distance runners and measured time to completion of a 1500-meter track run, while Shannon et al. (2017) used trained, but non-elite runners and examined time to completion over both 1500 and 10,000-meter treadmill runs. The investigation carried out by de Castro et al. (2018) also recruited trained, but non-elite runners and had them complete a 10,000-meter track run. Oskarsson and McGawley (2018) again used trained, but non-elite distance runners and observed completion of two, five-minute submaximal treadmill runs and a single 1-km treadmill time trial while supplemented with beetroot juice and caffeine both in isolation and together. The studies

by Murphy et al. (2012) and Peacock et al. (2012) both measured performance as time to completion of a 5 km running time trial, but differed in population and means of NO_3^- delivery. Whereas Peacock et al. (2012) used elite cross-country skiers and the pre-beetroot mode of NO_3^- supplementation of sodium NO_3^- ($NaNO_3$), Murphy et al. (2012) recruited recreationally fit men and women and provided them with whole, cooked beetroot to deliver the NO_3^-. Interestingly, while nearly all work to date on the effects of NO_3^- supplementation on athletic performance uses some form of beetroot juice, the study by Murphy et al. (2012) is the only one to exclusively use whole beetroot. Seven of the fourteen studies showed a measurable increase in performance through either a decrease in time to completion for the distance tests, increased power output, or an increase in time to reach the set energy expenditure. The studies that failed to cause measurable improvements in performance typically used elite or highly trained athletes, single bolus doses, or a combination of these factors.

Three studies utilized steady state time to exhaustion tests as their measure of performance outcome. All participants were healthy and participated in recreational sporting activities. Bailey et al. (2010) had participants perform a leg extension time to exhaustion (TTE) test at 30% of their determined maximum voluntary contraction, while the studies conducted by Bailey et al. (2009) and Lansley, Winyard, Fulford, et al. (2011) used cycling and treadmill TTE tests respectively as their assessments for performance outcomes. All three experiments observed a significant increase in time to exhaustion with NO_3^- supplementation compared to placebo.

Three studies had participants perform a variety of different testing modalities at simulated altitudes to help induces hypoxia and promote conversion of NO_2^- to NO. Vanhatalo et al. (2011) used a leg extension TTE at 14.5% O_2 concentration, while Masschelein et al.

(2012) had participants complete a cycle TTE at a simulated 5000 m altitude. Finally, Arnold, Oliver, Lewis-Jones, Wylie, and Macdonald (2015) used two separate treadmill tests to assess performance. The first was an incremental TTE at a simulated 4000m, while the second test was a 10 km TT completed at a simulated 2500 m altitude. The TTE trials performed by Vanhatalo et al. (2011) and Masschelein et al. (2012) both demonstrated increased TTE with NO_3^- supplementation ($P < 0.05$). The TTE and TT test conducted by Arnold et al. (2015) returned results at $p = 0.05$ and $p = 0.6$ respectively.

Twelve investigations employed graded exercise tests in which the participants performed treadmill, cycle and/or arm crank time to exhaustion tests in which the resistance increased over time, with the exception of the studies done by Coggan et al. (2014) who utilised a knee extension test with a variety of angular velocities followed by a 50 contraction fatigue test, and Pinna et al. (2014) who performed a tethered, incremental resistance swimming test to assess performance. Six studies in this category used participants that were classified as healthy and recreationally fit while the other six studies used elite middle and long-distance runners (Balsalobre-Fernandez et al., 2018), trained cyclists and/or triathletes (Bescos et al., 2011; Christensen, Petersen, Friis, Weitzberg, & Nybo, 2017; Larsen et al., 2007), moderately trained swimmers (Pinna et al., 2014), and Thompson et al. (2018) who utilized non-elite team sport athletes. An improvement was seen with NO_3^- supplementation in eleven of the twelve studies in either O_2 cost, time to exhaustion, total distance covered, energy cost or peak power output when compared to placebo, with the study by Coggan et al. (2014) being the exception, seeing an improvement only in maximum velocity in the leg extension test.

Six studies examined how NO_3^- supplementation impacted performance in intermittent exercise through the use of cycling tests or sprinting. While all six studies fall into the same

category of testing the effects on intermittent exercise, they varied greatly in the protocol used for assessment. Wylie, Mohr, et al. (2013) used recreationally fit males and provided them with 16.4 mmol NO_3^- the day before exercise with a further 8.2 mmol 2.5 hours and 4.1 mmol NO_3^- 1.5 hours prior to exercise. The Yo-Yo intermittent recovery test was utilized as the means of assessment which consists of repeated 2 x 20 m back and forth sprints with pre-recorded audio signals increasing speed in a progressive manner with 10 seconds of active rest between sprints until a participant fails to reach the finish line in time on two occasions (Krustrup et al., 2003). Performance was measured by the total distance completed with beetroot producing a 4.2% greater outcome compared to placebo. The study that was undertaken by Byrne, Wardrop, and Storey (2014) is currently only available in an abstract form and as such, limited information is available. Although the authors state that active participants received either a low or high dose of a commercially available beetroot juice in a randomized crossover design, the actual NO_3^- content is not stated. Treatments were provided two hours prior to performing three, 30 second WAnT cycling tests on an ergometer with three minutes of low intensity active cycling between tests. While no significant differences were reported in mean power, peak power, or fatigue index, the values are not provided. Combined with the unreported NO_3^- content of the treatments it is difficult to make any substantial inferences about the results of this particular study at this time. Another study used a modified WAnT protocol, this time with 20 second intervals instead of the traditional 30, and additionally examined isometric, short-duration force production, but differed from all other work due to using adolescents instead adults ages 18 years and older (Bender et al., 2018). A single bolus dose of 12.9 mmol of NO_3^- was given to participants 2.5 hours prior to completing repeated bouts of a modified WAnT protocol along with isometric mid-thigh pulls on a fixed barbell. While no significant effects were seen in the cycling test,

force production in the isometric pull was increased ($P = 0.004$). Aucouturier, Boissiere, Pawlak-Chaouch, Cuvelier, and Gamelin (2015) recruited team sports athletes and had them consume 5.5 mmol of NO_3^- at breakfast for two days preceding testing and also two hours prior to the test. The participants performed an intermittent cycling test consisting of 15 second intervals at 170% of their maximum aerobic power interspersed with 30 second passive recovery periods until volitional exhaustion. Total work performed, the number of repetitions, and time to volitional failure were significantly increased. Thompson et al. (2015) also utilized team sports athletes and provided them with 6.4 mmol of NO_3^- in both the morning and evening for 6 days, with an additional 12.8 mmol on the 7^{th} day 2.5 hours before performance testing. This study differed from all previous work in that it assessed not only physical performance, but also cognitive functioning. Testing consisted of intermittent sprint tests on a cycle ergometer with 100 seconds and 16 seconds of active and passive rest respectively, in between performing 6 second maximal sprints. This was repeated in two-minute blocks over two 40-minute halves with a 15 minute 'half time'. Additionally, twice during each of the 40-minute halves, participants performed 5 x 4 second sprints with 16 seconds of active rest. Participants were asked to complete cognitive tasks lasting 90 seconds at 15 minutes prior to testing, 7.5 minutes during the 'half time', 15 minutes after completion of testing, and during all 100 second recovery periods. The cognitive tasks alternated between Stroop tests, which evaluate information processing speed and selective attention, and Decision-Reaction tests which are used to measure the association between information processing speed and nutrient consumption. Total work performed measured in kJ was found to be significantly different ($P < 0.05$) with NO_3^- compared to placebo, as was reaction time in milliseconds in the second half of testing only ($P < 0.05$). Response accuracy on the cognitive test, however, was not significantly different between the

two treatments ($P > 0.05$). Again using team sports athletes, Wylie et al. (2016) had participants consume 8.2 mmol of NO_3^- from beetroot juice split into two doses for two days, followed by 8.2 mmol in a single dose over the next three days 2.5 hours prior to testing. Participants completed 24 x 6-s all-out sprints, 7 x 30-s all-out sprints, and 6 x 60-s self-paced maximal efforts on days 3, 4, and 5 respectively. Mean power output was significantly increased for the 24 x 6 session, but not in the following sessions with longer sprint times.

Two studies utilized a WAnT protocol similar to those mentioned previously but did not employ repeated bouts such as those used in the studies by Bender et al. (2018) and Byrne et al. (2014). Dominguez, Garnacho-Castano, et al. (2017) had participants consume 5.6 mmol of NO_3^- in the form of beetroot juice 3 hours before completing a standard WAnT protocol and observed a 6% improvement in peak power compared to placebo ($P = 0.034$) along with average power being 6.7% greater at 0-15 seconds of the 30 second test ($P = 0.048$). Kramer, Baur, Spicer, Vukovich, and Ormsbee (2016) also used a single WAnT session to measure performance along with having participants return the following day to complete a Grace protocol. These tests were performed both before and after six days of supplementation. The Grace protocol is commonly seen in CrossFit competitions and involves completing 30 clean-and-jerk repetitions of a 61.37 kg barbell in the shortest amount of time possible. Due to the sport specificity of the Grace protocol, the authors recruited 12 male CrossFit athletes to participate. Unlike the vast majority of previous studies that delivered NO_3^- from beetroot juice alone, Kramer et al. (2016) supplied 8 mmol of NO_3^- to participants daily for 6 days solely from KNO_3^-. Also, in contrast to most other studies, the final dose was consumed ≥ 24 hours prior to the first testing session. They observed an increase in peak power over time in the WAnT with

KNO_3^- ($P = 0.01$) while no such increase was seen with placebo ($P = 0.75$). No improvement was noted in the Grace protocol.

One study examined the effect of blood occlusion and isometric force production (Papadopoulos et al., 2018). Physically active males performed an occlusion-reperfusion maneuver where blood flow to the forearm was stopped for 5 minutes and then quickly allowed to return. Participants then performed a three-minute isometric handgrip test at 30 percent of their maximum voluntary contraction (MVC) strength, measured previously, followed by 15 minutes of rest. A second isometric handgrip test was then performed at 30 percent of their MVC this time with arterial occlusion until the MVC value dropped to 20 percent.

Two studies employed healthy, recreationally active males and utilized electrical stimulation of skeletal muscles to assess the effects of NO_3^- supplementation on performance (Tillin, Moudy, Nourse, & Tyler, 2018; Whitfield et al., 2017). The study by Whitfield et al. (2017) delivered the highest dosage of NO_3^- seen in the literature having participants consume approximately 26 mmol of NO_3^- from beetroot juice per day for seven days. Another uncommon feature of the study design was the splitting of the participants into two groups, with one group undergoing the electrical stimulation tests, while the other had skeletal muscle biopsies for determination of proteins associated with Ca^{2+} handling in the muscle. Participants in the electrical stimulation group underwent two electrically stimulated twitches of the quadriceps muscles with 150V at 0.5 Hz followed by 50 μs pulses at 40% of their MVC values at 10, 20, 30, 50 and 100 Hz, followed a repeat of the two 150V, 0.5 Hz twitches. While no change in maximum voluntary force production or electrically induced tetanic contractions were noted, force production increased at the 10 Hz pulse ($P < 0.05$), as did peak twitch tension ($P < 0.01$). Despite these improvements in force, the muscle biopsy group showed no alterations to the

measured proteins associated with Ca^{2+} handling. Still using a high, but more commonly administered dose of NO_3^- (12.9 mmol) from concentrated beetroot juice, Tillin et al. (2018) used electrical muscle stimulation to compare differences in fatigued and unfatigued leg muscle tissue. Unfatigued performance was assessed by having participants perform ten explosive, three-second MVCs followed by two sets of four-second involuntary tetanic contractions with one contraction each at 10, 20, 50, and 100 Hz per set. After ten minutes of rest, muscular fatigue was induced by having participants perform 60, three second MVCs followed by a repeat of the previous involuntary tetanic contractions. The unfatigued muscle tissue showed no effect with NO_3^- supplementation. In contrast, the fatigued muscle showed a lowered percent decline in explosive, voluntary impulse from the first to the last six MVCs ($P = 0.039$) along with a smaller reduction in the rate of tetanic peak force development in the 20/50 Hz range from the pre to post-fatigue protocol ($P = 0.011$) with NO_3^- compared to placebo.

The remaining study conducted by Flanagan et al. (2016) assessed the effect of NO_3^- on neuromuscular fatigue through electromyography (EMG) measurements and reps to failure performed in a dynamic exercise testing protocol. Participants were asked to complete fatiguing sets at various percentages of their 1 repetition maximum (1RM) in a squat exercise, in an ascending and descending pyramid fashion while having EMG measurements collected during maximum voluntary contractions performed between each of the fatiguing sets, with the final fatiguing set being taken to volitional failure.

The study performed by Bailey et al. (2009) was one of the first published papers to examine how supplemental consumption of NO_3^- derived from beetroot could enhance athletic performance. Eight recreationally active males were used to assess the effectiveness of consuming a beetroot juice supplement delivering 5.5 mmol of NO_3^- per day over a 6-day period.

They found that at low intensity, NO_3^- supplementation served to lower the O_2 cost of exercise and also extended the time it took to reach failure when subjected to intense exercise. Blood pressure was also lowered when the supplements were consumed and plasma NO_3^- levels were significantly elevated as expected. Similar to the study by Bailey et al. (2009), the work of Vanhatalo et al. (2010) also tested the effects of NO_3^- supplementation against a low NO_3^- placebo by using the same beetroot juice supplement and delivering the same level of NO_3^- per day. The study protocols were very similar with both using randomized, crossover designs and measured the effects on performance, blood pressure and plasma NO_3^- levels. The participant group was again comprised of eight healthy individuals, but in contrast to the 2009 study by Bailey et al. which used all males, the experiment performed by Vanhatalo et al. (2010) also included three females. Whereas the Bailey et al. (2009) study looked at the effects of acute supplementation up to six days, the study by Vanhatalo et al. (2010) extended the supplementation period to 15 days to capture the impact of prolonged use of the NO_3^- supplements to determine if there was any adaptation to the supplements resulting in either a positive or negative impact on the outcomes of interest. As in previous studies, blood pressure and the O_2 cost of exercise were both lowered and plasma NO_3^- levels were elevated with acute supplementation and these effects were maintained at the 15-day point. While the lowering of blood pressure through acute supplementation was similar to what was seen in the 2009 study from Bailey et al., the chronic effects of NO_3^- supplementation produced a more pronounced lowering of both systolic and diastolic blood pressure beginning at the 12-day point. VO_{2max}, peak power output and work rate were measured at the GET, that is, the point at which the test participant had attained the maximum rate at which they could intake and utilize O_2 and expel

carbon dioxide (CO_2). No effect was observed in these areas at 2.5 hours post consumption or at the five-day point, but improvements were seen in all areas at the 15-day point.

In 2013 Christensen et al. published a paper detailing the results of their work examining the impact of NO_3^- supplementation on similar factors measured in previous works including those by Bailey et al. (2009) and Vanhatalo et al. (2010) such as plasma NO_3^- levels, VO_2 kinetics, peak power output and time to completion over a set distance. Whereas most research on the subject of the ergogenic effects of NO_3^- supplementation on athletic performance has been conducted in healthy, yet moderately trained participants, the study by Christensen et al. (2013) used elite athletes, specifically cyclists, as their test subjects. In order to produce comparable results, a protocol similar to that of previous studies was implemented consisting of a blinded, randomized crossover design with two, six-day supplemented periods separated by a 14-day washout. The same concentrated beetroot juice supplement was used at the same dose as in the studies by Bailey et al. (2009) and Vanhatalo et al. (2010). Blood pressure was not captured in the study by Christensen et al. (2013) but plasma NO_3^- levels were elevated to comparable levels seen in the prior studies. In contrast to the work performed on sub-elite populations, no improvement was seen in exercise economy when the elite level athletes performed their tests after consuming the beetroot supplements for six days. The authors theorized that athletes of the highest caliber and fitness level, such as those used in their study, may already possess optimal means of NO production and that their performance is limited by other physiological factors not influenced by consumption of dietary NO_3^-. Balsalobre-Fernandez et al. (2018) also conducted a study on NO_3^- from beetroot juice with elite-level athletes in the form of middle and long-distance runners and while no improvements to running economy were observed, likely to very likely improvements were noted for time to exhaustion in and incremental treadmill running test.

Although, while Christensen et al. (2013) used a six day supplementation period, Balsalobre-Fernandez et al. (2018) delivered the NO_3^- supplement for 15 days. Wilkerson et al. (2012) also utilized well trained, though not elite, cyclists in their study prior to the work of Christensen et al. (2013). NO_3^- from beetroot juice was provided in a single bolus dose prior to exercising testing which consisted of a laboratory based 50-mile time trial which is the longest distance studied in relation to NO_3^- supplementation and athletic performance. While time to completion and power output (PO) did not differ significantly between NO_3^- and placebo, O_2 uptake (VO_2) showed a trend towards being lower with NO_3^- ($P = 0.06$). This resulted in a significant increase in the PO/VO_2 ratio indicating a lowered O_2 cost of exercise.

Wylie, Mohr, et al. (2013) conducted the first study on NO_3^- supplementation and athletic performance using intermittent sprinting in increments to assess the influence of NO_3^- on athletic performance. The vast majority of the research on NO_3^- supplementation in athletes has been centered around steady state or incremental exercise which makes the results difficult to extrapolate to athletes requiring intermittent bursts of energy and relying on a mix of aerobic and anaerobic power systems such as soccer players. Wylie, Mohr, et al. (2013) utilized fourteen recreational team sports players. A double-blind, randomized crossover design with a minimum of a 72-hour washout period between treatments was used in which participants were assigned to receive either beetroot juice with high NO_3^- content similar to what has been used in the majority of NO_3^- supplementation studies, or a NO_3^--depleted placebo. Participants consumed two large doses of NO_3^- (approximately 8 mmol each) on the day before their performance test and two more doses of about 8 mmol and 4 mmol of NO_3^- 2.5 hours and 1.5 hours respectively prior to their test. Participants performed the Yo-Yo intermittent recovery test as described previously. When consuming beetroot, participants completed an average distance of 1704 ± 304 m

compared to 1636 ± 288 m on placebo, representing a 4.2% increase in total distance before reaching failure.

A dose response relationship has been proposed in early work and was demonstrated by Wylie, Kelly, et al. (2013). Their work confirmed the dose-dependent effects of beetroot ingestion on plasma NO_3^- and NO_2^- levels with increases in plasma NO_3^- of 334%, 778%, and 1556% and plasma NO_2^- of 121%, 218%, and 338% 2.5 hours following consumption of 4.2, 8.4, and 16.8 mmol NO_3^- respectively (Wylie, Kelly, et al., 2013). This increase was especially noteworthy for plasma NO_2^- levels post ingestion of 8.4 and 16.8 mmol NO_3^-, as previous studies reported 15-150% rises in plasma NO_2^- after acute consumption of 4-6 mmol (Bescos et al., 2011; Lansley, Winyard, Bailey, et al., 2011; Vanhatalo et al., 2010) and chronic consumption of 5-6 mmol per day (Bailey et al., 2010; Bailey et al., 2009; Lansley, Winyard, Fulford, et al., 2011; Vanhatalo et al., 2010) of dietary NO_3^- supplementation from beetroot. Interestingly, the study conducted by Flanagan et al. (2016) elected to deliver NO_3^- from beetroot to their participants through a bar as opposed to beetroot juice and with a much lower dosage of NO_3^- than what had been previously used in the literature with participants receiving only 1.1 mmol NO_3^-. The lowest dose reported in previous works was 4.2 mmol by Hoon, Jones, et al. (2014) though no significant improvement in performance was noted. In contrast, Flanagan et al. (2016) reported significant effects on improving neuromuscular efficiency demonstrated by increasing mean peak EMG amplitude with NO_3^- (83.3 ± 2.6%) compared to placebo (78.8 ± 3.8%; p = 0.04). In the protocol used, EMG amplitude is measured during an MVC performed prior to any exercise or muscular fatigue where the participant isometrically contracts the target muscle as hard as possible while sensors measure the electrical activity in the muscle. The highest value establishes the maximum EMG amplitude and is representative of the highest amount of motor

units able to be detected firing at their highest rates (Hakkinen et al., 1998). MVCs are then performed during or after exercise to determine the peak EMG value, which is the largest EMG amplitude observed during the trial and this is then compared to the maximum value. The inability to continue to reproduce the maximum value suggests the development of neuromuscular fatigue translating to reduced ability to produce forceful contractions of the muscle fibers. The development of neuromuscular fatigue in the study by Flanagan et al. (2016) is suggested by the fact that both the NO_3^- and the placebo treatment failed to reproduce the maximum EMG amplitudes and could be attributed to interference of neuromuscular transmission (Boyas & Guevel, 2011) or the detrimental build-up of metabolic waste (Westerblad, Allen, Bruton, Andrade, & Lannergren, 1998). Bailey et al. (2010) had participants perform 3 MVCs and a test of incremental leg extensions on an ergometer and demonstrated reduced buildup of metabolic wastes including decreased inorganic phosphate and ADP as well as reduced phosphocreatine (PCr) decomposition, suggesting increased ATP production and decreased ATP turnover in the muscle under exercising conditions with NO_3^- supplementation. In the study by Flanagan et al. (2016), the ability of the NO_3^- treatment to elicit a higher mean peak EMG amplitude compared to placebo could suggest resistance to fatigue from one or both of these factors, particularly metabolic waste. Although the testing modalities and outcomes of interest used by Flanagan et al. (2016) differ greatly from previous work and represents the first study to examine the potential of NO_3^- to improve neuromuscular efficiency during dynamic, compound resistance exercise, it is still surprising that such an improvement could be attained with a comparatively low dose of NO_3^- even when considering that, in theory, the NO_3^--NO_2^--NO pathway should perform even better under anaerobic exercise conditions as opposed to the better documented aerobic exercise environment.

Table 1. Summary of Studies Detailing the Effect of Nitrate Supplementation on Athletic Performance.

Reference	Participants, (n)	Supplementation Protocol	Performance assessment	Trial result ± SD
Time Trials				
Lansley, Winyard, Bailey, et al. (2011)[i]	competitive cyclists (9 males)	500 ml beetroot juice (6.2 mmol NO_3^-), 150 min before exercise	4 km cycle time trial	N: 6.27 ± 0.35 min vs. P: 6.45 ± 0.42 min ($p < 0.05$) N: 292 ± 44 vs. P: 279 ± 51 W ($p < 0.05$)
Lansley, Winyard, Bailey, et al. (2011)[ii]	as above	as above	16.1 km cycle time trial	N: 26.9 ± 1.8 vs. P: 27.7 ± 2.1 min ($p < 0.01$) N: 247 ± 44 vs. P: 233 ± 43 W ($p < 0.05$)
Bond et al. (2012)	well-trained junior rowers (14 males)	500 ml beetroot juice (5.5 mmol NO_3^-) per day for 6 days	6 x 500 m rowing ergometer trials	N: 0.4% ± 1.0% (95% CI) improvement vs. P
Cermak, Gibala, and van Loon (2012)	trained cyclists (12 males)	140 ml beetroot juice concentrate/day (8 mmol NO_3^-) for 6 days	10 km cycle time trial	N: 953 ± 18 vs. P: 965 ± 18 s ($p <0.05$) N: 294 ± 12 vs. P: 288 ± 12 W ($p < 0.05$)
Cermak, Res, et al. (2012)	trained cyclists (20 males)	140 ml beetroot juice concentrate (8.7 mmol NO_3^-), 150 min before exercise	energy expenditure based time trial	N: 65.5 ± 1.1 vs. P: 65.0 ± 1.1 s ($p > 0.05$) N: 275 ± 7 vs. P: 278 ± 7 W ($p > 0.05$)
Murphy et al. (2012)	11 recreationally fit (5 men; 6 women)	200 g whole cooked beetroot (≥500 mg (8.1 mmol) NO_3^-) 75 min prior to exercise test	5 km treadmill running time trial	N: 12.3 ± 2.7 vs. P: 11.9 ± 2.6 km/h ($p = 0.06$) mean of 41 sec faster with NO_3^-

Peacock et al. (2012)	well-trained cross-country skiers (10 males)	1 g KNO_3^- (614 mg (9.9 mmol) NO_3^-), 150 min before exercise	5 km running time trial	N: 1005 ± 53 vs. P: 996 ± 49s (p = 0.12)
Wilkerson et al. (2012)	well-trained cyclists (8 males)	500 ml beetroot juice (6.2 mmol NO_3^-), 150 min before exercise	50-mile cycle time trial	N: 136.7 ± 5.6 vs. P: 137.9 ± 6.4 min (p > 0.05) N: 67.4 ± 5.5 vs. P: 65.3 ± 4.8 W L min^{-1} (p < 0.05)
Christensen et al. (2013)	highly trained cyclists (10 males)	500 ml beetroot juice (8.1 mmol NO_3^-) per day for 6 days	energy expenditure based time trial	N: 18:20 vs. P: 18:37 min:s (p > 0.05)
Boorsma et al. (2014)	elite distance runners (8 males)	140 ml beetroot juice concentrate (13.0 mmol NO_3^-) on days 2-7 with lunch; 210 ml (19.5 mmol NO_3^-) on days 1 and 8 2.5 hr prior to performance test	treadmill warm-up run: 7 min @ 50% VO_{2peak}, 7 min @ 65%, 5 min @ 80% 1.5 hr post ingestion. Followed by 1500m time trial	(acute) N: 250.7 ± 4.3 vs. P: 250.4 ± 7.0 s (p > 0.05); (chronic) N: 250.5 ± 6.2 vs. 251.4 ± 7.6 s (p > 0.05) 2 "responders" improved TT by 5.8 and 5.0 s (acute) (p < 0.05) and 7.0 and 0.5 s (chronic) (p < 0.05)
Hoon, Hopkins, et al. (2014)	trained cyclists (26 males)	70 ml beetroot juice concentrate (4.1 mmol NO_3^-) at 150 and 75 min prior to TT1 and an additional 35 ml dose 75 min prior to TT2	4 min cycling time trials separated by 75 min recovery period	Both TT difference in mean vs. placebo: 150-Pre: 0.4 ± 2.0s (p > 0.05) 75-Pre: 0.6 ± 2.0s (p > 0.05) TopUp:0.5 ± 2.0s (p > 0.05) All: 0.5 ± 1.6s (p > 0.05)

Hoon, Jones, et al. (2014)	highly trained rowers (10 males)	70 ml (4.2 mmol NO_3^-) or 140 ml (8.4 mmol NO_3^-) 2 hours prior to exercise	2000 m rowing ergometer time trial	(90% CI) 0.2 ± 2.5s difference between single dose and placebo (likely trivial); (90% CI) 1.6 ± 1.6 s difference between double dose and placebo (possibly beneficial)
Jo et al. (2017)	healthy, recreationally active (15 males; 14 females)	NO_3^- supplement containing beet powder and KNO_3^- (8 mmol NO_3^-) or placebo for 14 days. Both groups consumed single serving of NO_3^- supplement 2.5 hours prior to testing	8.04 km cycling time trial	N: 5.6% reduction for TTC (p = 0.01); 4.2% increase average power (p = 0.04); 2.2% increase average speed (p = 0.02) vs. baseline P: no significant changes
Lowings et al. (2017)	trained swimmers (5 males; 5 females)	140 ml concentrated beetroot juice (12.5 mmol NO_3^-) 3 hours prior to testing	168 m (8 x 21 m lengths) backstroke swimming time trial	N: 131.59 ± 9.09 vs. P: 130.37 ± 8.10 s (p = 0.144)
Shannon et al. (2017)	trained runners (8 males)	140 ml concentrated beetroot juice (12.5 mmol NO_3^-) 3 hr prior to testing	1500 m treadmill running time trial	N: 319.6 ± 36.2 vs. P: 325.7 ± 38.8 s (p < 0.05)
as above	as above	as above	10,000 m treadmill running time trial	N: 2643.1 ± 324.1 vs. P: 2649.9 ± 319.8 s (p > 0.05)

de Castro et al. (2018)	recreational runners (14 males)	420 ml beetroot juice (8.4 mmol NO_3^-) for 3 days with final dose 2 hours prior to testing	10 km track running time trial	TT: N: 50.1 ± 5.3 vs. P: 51.0 ± 5.1 min (p = 0.391) MV: N: 12.1 ± 1.3 vs. P: 11.9 ± 1.2 km/h (p = 0.321)
Oskarsson and McGawley (2018)	healthy endurance runners (9 males; 2 females)	70 ml concentrated beetroot juice (7.3 mmol NO_3^-) both with and without caffeine (4.8 ± 0.4 (4.3-5.6) mg/kg bodyweight) 2.5 hours prior to testing	2 constant load (70% and 80% of VO_{2max}) 5 min treadmill runs and a single 1 km self-paced treadmill TT	No significant (p > 0.05) differences among any treatment or combination of treatments vs. placebo for both submaximal runs or for 1 km TT
Time to Exhaustion Tests				
Bailey et al. (2009)	healthy, recreationally fit (8 males)	500 ml beetroot juice (5.5 mmol NO_3^-) a day for 6 days	cycling TTE @ 70% between GET and VO_{2max}	N: 675 ± 203 vs. P: 583 ± 145 s (p < 0.05)
Bailey et al. (2010)	healthy, recreationally fit (7 males)	500 ml beetroot juice (5.1 mmol NO_3^-) a day for 6 days	leg extension TTE @ 30% MVC	N: 734 ± 109 vs. P: 586 ± 80 s (p < 0.01)
Lansley, Winyard, Fulford, et al. (2011)	healthy, recreationally fit (9 males)	500 ml beetroot juice (6.2 mmol NO_3^-) a day for 6 days	treadmill TTE @ 75% between GET and VO_{2max}	N: 8.7 ± 1.8 vs. P: 7.6 ± 1.5 min (p < 0.01) N: 3.50 ± 0.62 vs. P: 3.77 ± 0.57 l/min (p < 0.01)
Simulated Altitude Tests				
Vanhatalo et al. (2011)	Healthy, recreationally fit (7 males; 2 females)	750 ml beetroot juice (9.3 mmol NO_3^-), 24 hr prior, with last 250 ml 2.5 hr pre-exercise	leg extension TTE @ 14.5% O_2	N: 477 ± 200 vs. P: 393 ± 169 s (p < 0.05) (in hypoxia)

Masschelein et al. (2012)	healthy, recreationally fit (15 males)	0.07 mmol NO_3^- per kg body mass per day (as beetroot juice) for 6 days)	cycle incremental TTE @ simulated 5000 m altitude	N: 597 ± 22 vs. P: 568 ± 23 s ($p < 0.05$)
Arnold et al. (2015)	well-trained competitive runners (10 males)	70 ml beetroot juice concentrate (7 mmol NO_3^-) 2.5 hr prior to exercise tests	1st visit: treadmill incremental TTE @ simulated 4000 m altitude 2nd visit: 10 km TT @ simulated 2500 m altitude	TTE: N: 402 ± 80 vs. P: 393 ± 62 s ($p = 0.05$) TT: N: 2862 ± 233 vs. P: 2874 ± 265 s ($p = 0.6$)
Graded Exercise Tests				
Larsen et al. (2007)	well trained cyclists (9 males)	0.1 mmol NO_3^- per kg body mass per day (as $NaNO_3$) for 3 days, last dose 60 min before exercise	cycle incremental TTE	N: 2.82 ± 0.58 vs P: 2.98 ± 0.57 L/min ($p < 0.02$)
Larsen, Weitzberg, Lundberg, and Ekblom (2010)	healthy (7 males; 2 females)	0.1 mmol NO_3^- per kg body mass per day (as $NaNO_3$) for 2 days	arm crank and cycle incremental TTE	TTE N: 563 ± 30 vs P: 524 ± 31 s ($p = 0.13$) N: 3.62 ± 0.31 vs. P: 3.72 ± 0.33 L/min ($p < 0.05$)
Vanhatalo et al. (2010)	healthy (5 males; 3 females)	500 ml beetroot juice (5.2 mmol NO_3^-) 2.5 hr before exercise	cycle incremental TTE	N: 325 ± 71 vs. P: 322 ± 68 W_{max} ($p > 0.05$)
as above	as above	500 ml beetroot juice (5.2 mmol NO_3^-) per day for 5 days	as above	N: 328 ± 68 vs. P: 323 ± 67 W_{max} ($p > 0.05$)
as above	as above	500 ml beetroot juice (5.2 mmol NO_3^-) per day for 15 days	as above	N: 331 ± 68 vs. P: 323 ± 68 W_{max} ($p < 0.05$)

Bescos et al. (2011)	competitive cyclists or triathletes (11 males)	10 mg NO_3^- per kg body mass (as $NaNO_3$) 3 hr before exercise	cycle incremental TTE	N: 11.2 ± 1.1 vs. P: 11.8 ± 1.1 $mL \cdot min^{-1} \cdot W^{-1}$ ($p = 0.031$) N: 416 ± 32 vs. P: 409 ± 27 s ($p > 0.05$)
Breese et al. (2013)	healthy, recreationally fit (4 males; 5 females)	140 ml beetroot juice concentrate/day (8 mmol NO_3^-) for 6 days	cycle incremental TTE	N: 635 ± 238 vs. P: 521 ± 158 s ($p < 0.05$)
Wylie, Kelly, et al. (2013)	Two phases (s1 and s2) healthy recreationally active men (10 in each phase)	70, 140, or 280 ml beetroot juice (4.2, 8.4, 16.8 mmol NO_3^- respectively) 1, 2, 4, 8, 12, and 24 hr prior to venous blood samples (s1) Same dosage protocol 2.5 hr prior to exercise test (s2)	venous blood samples (s1). 2 x cycle incremental TTE moderate-intensity (93 ± 11W) and 1 x severe-intensity (258 ± 23W) all separated by 5 min (s2)	(s1) NO_3^- peaked at 1 hr post administration of 4.2mmol (160 ± 43μM), 8.4 mmol (269 ± 92μM) and 2 hr 16.8 mmol (581 ± 209μM) (s2) O_2 uptake decreased by 1.7% (8.4 mmol) ($p = 0.06$) and 3.0% (16.8 mmol) ($p < 0.05$) TTE increased for 8.4 and 16.8 mmol by 14% and 12% respectively ($p < 0.05$)

Coggan et al. (2014)	healthy, normally active, non-exercising (7 males, 5 females)	140 ml beetroot juice concentrate 120 min prior to testing	knee extension at various angular velocities; 50 contraction fatigue test	No significant difference ($p > 0.05$) in max isometric torque or peak isokinetic torque or power. Max velocity increased significantly ($p < 0.05$) with NO_3^-. No sig differences ($p > 0.05$) in torque, power, work, or rate of fatigue during 50 contraction fatigue test
Pinna et al. (2014)	moderately trained swimmers (14 males)	500 ml beetroot juice (5.5 mmol NO_3^-) per day for 6 days	swimming incremental TTE	N: 6.7 ± 1.1 vs. C: 6.3 ± 1.0 kg ($p < 0.05$); N: 1.7 ± 0.3 vs. C: 1.9 ± 0.5 $kcal \cdot kg^{-1} \cdot h^{-1}$ ($p < 0.05$)
Bailey et al. (2015)	healthy, recreationally fit (7 males)	unspecified volume concentrated beetroot juice (8.4 mmol NO_3^-) per day for 9 days additional dose (NO_3^- content not specified) provided 2 hours following each exercise test	cycling TTE ramp incremental cadence-specific (35 rpm and 115 rpm)	N: @ 35 rpm: 344 ± 74 vs. P: 341 ± 99 s ($p > 0.05$); @ 115 rpm: N:362 ± 137 vs. P: 297 ± 79 s ($p < 0.05$)

Christensen et al. (2017)	trained cyclists (9 males) and recreationally active (8 males)	150 ml concentrated beetroot juice (9 mmol NO_3^-) 2.5 hours prior to testing on 2 consecutive test days	4 sessions of 3, 6-minute moderate intensity submaximal exercise bouts alternating between arm and leg cycling separated by 15 minutes rest; followed by a final bout of incremental arm (15 W/min) or leg (25 W/min) cycling. Order was alternated so both supplement and placebo treatments experienced arm and leg cycling as incremental test over 4 sessions	Incremental test peak power higher in leg cycling for cyclists N: 418 ± 47 vs. P: 407 ± 46 W ($p <$ 0.05) No other improvements for either group
Balsalobre-Fernandez et al. (2018)	elite middle and long-distance runners (12 males)	70 ml concentrated beetroot juice (6.5 mmol NO_3^-) for 15 days with breakfast	treadmill incremental TTE	TTE: SMD 1.18, 90% CI = -0.14, 2.5) vs. P RPE: SMD = -2.17, 90% CI = --3.23, -1.1) vs. P

Thompson et al. (2018)	non-elite team sport athletes (18 males; 12 females)	70 ml concentrated beetroot juice (6.4 mmol NO_3^-) or 70 ml KNO_3^- water (6.4 mmol NO_3^-) in morning and again in evening over 4-week training period. 140 ml (12.8 mmol NO_3^-) of assigned treatment 2.5 hr prior to testing	cycle incremental TTE	VO_{2peak}: N +11% increase vs. P +6% increase vs. KNO_3 +4% increase ($p < 0.05$)
as above	as above	as above	severe-intensity cycle step TTE	TTE: N +71% increase vs. P +47% increase vs. KNO_3 +42% increase ($p < 0.05$)
Intermittent Exercise Tests				
Wylie, Mohr, et al. (2013)	healthy, recreationally fit (14 males)	280 ml beetroot juice concentrate on day before exercise (16.4 mmol NO_3^-), 140 ml beetroot juice concentrate (8.2 mmol NO_3^-) 2.5 hr prior to exercise and 70 ml beetroot juice concentrate (4.1 mmol NO_3^-) 1.5 hr prior to exercise	intermittent, incremental sprints for distance	N: 1704 ± 304 vs. P: 1636 ± 288 m ($p < 0.05$)
Byrne et al. (2014) Abstract only	healthy, active (8 males)	250 ml or 500 ml beetroot juice 2 hr prior to exercise (NO_3^- content not stated)	supramaximal intermittent cycling test (Wingate protocol)	No significant differences reported in mean power, peak power, or fatigue index. Values not provided

Aucouturier et al. (2015)	healthy, team sport athletes (12 males)	500 ml beetroot juice (5.5 mmol NO_3^-) at breakfast for 2 days preceding exercise tests and 2 hr prior to performance test.	supramaximal intermittent cycling test with 15 sec intervals @ 170% max aerobic power with 30 sec passive recovery periods until volitional exhaustion	N: 472.5 ± 42.5 vs. P: 468.8 ± 43.4 W (p > 0.05); N: 168.1 ± 60.2 vs. P: 142.0 ± 46.8 kJ (p < 0.05); N: 26.1 ± 10.7 vs. P: 21.8 ± 8.0 reps (p < 0.05); N: 19.6 ± 8.1 vs. P: 16.4 ± 6.0 min (p < 0.05)
Thompson et al. (2015)	recreational team sport athletes (16 males)	70 ml concentrated beetroot juice (6.4 mmol NO_3^-) each morning and evening for 6 days. 140 ml (12.8 mmol NO_3^-) on day 7, 2.5 h prior to performance test	two 40 min halves broken into 2 min blocks consisting of intermittent sprint tests on cycle ergometer with 6 s 'all out' sprint, 100 s active rest and 14 s passive rest. Twice in each half performed 5 x 4 s sprints with 16 s active rest. 15 min 'half time'. 90 s cognitive tasks at 15 min before testing, 7.5 min during 'half time', 15 min following testing, and during each 100 s recovery period	Total work: N 123 ± 19 kJ vs. P 119 ± 17 kJ (p < 0.05) N Reaction time: N 817 ± 86 ms vs P 847 ± 114 ms (p < 0.05) in second half Response accuracy: N first half: 6.9 ± 6.1 vs. second half: 7.7 ± 7.3; P first half: 7.3 ± 5.8 vs. second half: 7.1 ± 5.9 (p > 0.05)

Wylie et al. (2016)	team sport players (10 males)	70 ml concentrated beetroot juice (4.1 mmol NO_3^-) in morning and repeat in evening on non-experimental days (1 and 2); 140 ml (8.2 mmol NO_3^-) 2.5 hr prior to testing on experimental days (3, 4, and 5)	24 x 6-s all-out sprints, 7 x 30-s all-out sprints, and 6 x 60-s self-paced maximal efforts on days 3, 4, and 5 respectively	Mean power output: (24 x 6-s) N 568 ± 136 vs. P 539 ± 136 W ($p < 0.05$); (7 x 30-s) N 558 ± 95 vs. P 562 ± 94 W ($p > 0.05$); (6 x 60-s) N 374 ± 57 vs P 375 ± 59 W ($p > 0.05$)
Bender et al. (2018)	active adolescents (16 males)	140 ml concentrated beetroot juice (12.9 mmol NO_3^-) 2.5 hr prior to testing	supramaximal intermittent cycling test (modified Wingate protocol); isometric mid-thigh pull	No significant effects on supramaximal intermittent cycling test. Isometric mid-thigh pull: N 2361.7 ± 641.6 vs. P 2158.3 ± 602.3 newtons ($p = 0.004$)
Supramaximal Tests				
Kramer et al. (2016)	CrossFit athletes (12 males)	1 x 4 mmol KNO_3^- capsule in both morning and evening for 6 days; final dose consumed ≥ 24 hr prior to testing	supramaximal cycling test (Wingate protocol) on day 1; time to completion of 30 clean-and-jerk repetitions with 61.37 kg (Grace protocol)	Peak power increase over time in Wingate: N 889.17 ± 179.69 to 948.08 ± 186.80 W ($p = 0.01$) vs. P 898.08 ± 183.24 to 905.00 ± 157.23 W ($p = 0.75$) No other improvements

Dominguez, Garnacho-Castano, et al. (2017)	trained, non-elite (15 males)	70 ml concentrated beetroot juice (5.6 mmol NO_3^-) 3 hr prior to testing	supramaximal cycling test (Wingate protocol)	6% improvement in peak power for N vs. P (p = 0.034); average power 0-15s 6.7% higher in N vs. P (p = 0.048)
Occlusion Tests				
Papadopoulos et al. (2018)	physically active (16 males)	unspecified volume of beetroot juice (8.1 mmol NO_3^-) 2.5 hours prior to testing	occlusion-reperfusion maneuver followed by IHG @ 30% MVC for 3 minutes followed by IHG @ 30% MVC with arterial occlusion until exhaustion	No differences between N and P during occlusion-reperfusion. IHG: O_2Hb and tHb higher in N vs. P in 2nd and 3rd minute (p < 0.05) and force output higher during 3rd minute N: 10.8 ± 0.7 vs. 9.5 ± 1.2 kg (p < 0.05) IHG with occlusion: Prolonged time to fatigue N: 94.1 ± 5.8 vs. P: 80.1 ± 3.3 s (p < 0.01). No effect on O_2Hb decline and mVO_2.
Electrical Stimulation Tests				

Whitfield et al. (2017)	healthy, recreationally active (16 males)	280 ml concentrated beetroot juice (26 mmol NO_3^-) split into 2 daily doses for 7 days with final dose 90 min prior to testing	2 x electrically stimulated twitches @ 150V, 0.5 Hz followed by 50 µs pulses @ 40% MVC @ 10, 20, 30, 50, and 100 Hz, followed by repeat of 2 twitches. Separate group followed same supplementation protocol but underwent SkM biopsies pre and post-supplementation for determination of proteins associated with calcium handling	No change in maximum voluntary force production N: 602 ± 50 vs. P: 596 ± 56 N or in electrically induced tetanic contractions. Force production increased @ 10 Hz N: 41.1 ± 2.3 vs. P: 37.6 ± 2.4% of peak force ($p < 0.05$) Peak twitch tension: N: 164.0 ± 12.5 vs. P: 136.5 ± 7.2 N ($p < 0.01$) Proteins associated with calcium handling unaltered

Tillin et al. (2018)	healthy, recreationally active (17 males)	140 ml concentrated beetroot juice (12.9 mmol NO_3^-) with 70 ml in morning and 70 ml in evening for 6 days; on 7th day, entire dose 2.5 hours prior to testing	Unfatigued: 10 explosive 3 second MVCs; 2 sets of 4 second involuntary tetanic contractions with one contraction each at 10, 20, 50 and 100 Hz per set. Fatigued: Following 10-minute rest, fatigue protocol of 60, 3 second MVCs followed by repeat of involuntary tetanic contractions	Unfatigued: No effect of N on any measured variables. Fatigued: % decline in explosive, voluntary impulse from first to last 6 MVCs: N: 51.1 ± 13.9 vs. P: 57.3 ± 12.4% (p = 0.039) Low frequency decline in 20/50 Hz for tetanic peak force: N: 12.3 ± 12.0 vs. P: 17.0 ± 10.1% (p = 0.110) Tetanic peak force rate development: N: 12.3 ± 10.4 vs. P: 20.3 ± 9.5% (p = 0.011)
Dynamic Exercise Tests				
Flanagan et al. (2016)	resistance-trained (14 males)	2 bars containing 3 g of concentrated beetroot extract each (1.1 mmol NO_3^- total) with first meal for 3 days. Testing protocol performed on empty stomach on day 4	pyramid style dynamic box squats; MVCs between each dynamic exercise set with EMG data collection	Mean peak EMG (percent maximum): N: 83.3 ± 2.6% vs. P: 78.8 ± 3.8% (p = 0.04) Total repetitions: N: 599 ± 5 vs. P: 608 ± 5 (p > 0.05) Lactate: N: 1.48 ± 0.25 to 7.98 ± 3.27 mmol·L^{-1} vs. P: 1.52 ± 0.27 to 8.02 ± 3.45 mmol·L^{-1} (p > 0.05)

Note. SD = standard deviation; NO_3^- = nitrate; N = nitrate trial; P = placebo trial; C = control trial; W = watts; KNO_3 = potassium nitrate; TT = time trial; CI = confidence interval; TTC = time to completion; MV = mean velocity; TTE = time to exhaustion; GET = gas exchange threshold; MVC = maximum voluntary contraction; $NaNO_3$ = sodium nitrate; W_{max} = peak power; SMD = standardized mean difference; kJ = kilojoules; IHG = isometric hand grip; O_2Hb = oxygenated hemoglobin; tHb = microvascular red blood cell count; mVO_2 = skeletal muscle oxygen consumption; SkM = skeletal muscle; EMG = electromyography

Chapter II: Effects of Beetroot Juice Supplementation on Anaerobic Exercise Performance

Rationale

NO has long been known to have vasodilatory capabilities and to elicit a decrease in blood pressure. More recently, researchers working in the athletic community discovered the potential for NO to improve exercise capacity and tolerance, translating into increased performance. The widely studied NOS-NO pathway was first turned to as a means of producing NO, through its conversion of the amino acid L-arginine, to improve exercise performance. Studies focusing on aerobic exercise found no significant results when participants consumed supplemental L-arginine, regardless of whether consumption was acute or chronic (Bescos et al., 2009; Liu et al., 2009; Sunderland et al., 2011). When anaerobic exercise in the form of resistance training was examined with supplemental L-arginine consumption, some positive effects were observed in muscle blood volume (Alvares et al., 2012), forearm blood flow, arm circumference and brachial artery stiffness (Fahs et al., 2009), though no effects on actual performance occurred. L-arginine also failed to positively influence any performance parameters in a WAnT test (Olek et al., 2010).

With L-arginine failing to show any ergogenic effects, focus shifted to the less understood NO_3^--NO_2^--NO pathway and its production of NO from dietary NO_3^-. Numerous studies have been conducted demonstrating the ergogenic effects of dietary NO_3^- supplementation (Dominguez, Cuenca, et al., 2017; Jones, 2014). While early work provided NO_3^- in the form of $NaNO_3^-$ (Bescos et al., 2011; Larsen et al., 2007, 2010) or potassium NO_3^- (KNO_3^-) (Peacock et al., 2012), the overwhelming majority of studies have used beetroot juice to deliver dietary NO_3^- to testing participants. Interestingly, some of the L-arginine studies measured plasma NOx levels as a marker of NO production (Alvares et al., 2012; Bescos et al.,

2009; Liu et al., 2009; Olek et al., 2010) due to the fact that NO is short-lived and is recycled back to both NO_3^- and NO_2^-, but all failed to show an increase in plasma NOx levels. While the NOS-NO pathway functions in only one direction, with NO being the end product, the NO_3^--NO_2^--NO pathway is cyclical allowing for the recycling of NO back into NO_3^- and NO_2^- to again be converted back to NO. This failure to raise plasma NOx levels with L-arginine supplementation could indicate that L-arginine supplementation cannot actually increase NO levels sufficiently to produce any benefits beyond simple vasodilation and increased blood flow. This inability to raise NO levels with L-arginine could be due to the activity of liver enzymes known as arginases that are active in the urea cycle and compete with the NOS-NO pathway for L-arginine (Mori & Gotoh, 2000). Arginase activity is elevated in athletes when performing intense exercise and may limit the availability of L-arginine for NO production through the NOS-NO pathway (Sureda et al., 2006). NO generation from the NOS-NO pathway is further restricted during exercise due to the hypoxic and acidic conditions found in exercising tissue undergoing oxidative stress (Huang, Xiao, Samii, Vita, & Keaney, 2001; Stuehr et al., 2004), particularly contracting skeletal muscles. In contrast, the single electron reduction of NO_2^- to NO is enhanced under these types of conditions (Castello, David, McClure, Crook, & Poyton, 2006). With these factors in mind, supplementation with dietary NO_3^- may elicit performance enhancing benefits where L-arginine has failed. This appears to often be the case in terms of studies conducted on aerobic athletic performance under a wide variety of testing parameters and dosage strategies (Aucouturier et al., 2015; Bailey et al., 2009; Bond et al., 2012; Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011; Vanhatalo et al., 2010; Wylie, Kelly, et al., 2013; Wylie, Mohr, et al., 2013). Yet to date, little work has focused on the potential for dietary NO_3^- supplementation to enhance anaerobic performance outcomes.

Early studies have shown positive effects on increasing endurance or delaying fatigue in intermittent bouts of short-term high-intensity exercise (Aucouturier et al., 2015; Thompson et al., 2015; Wylie et al., 2016) with mixed results for improvement of mean or peak power output (Aucouturier et al., 2015; Bender et al., 2018; Byrne et al., 2014; Wylie et al., 2016). While Bender et al. (2018) failed to see improvements in any parameter during repeated bouts of a modified WAnT protocol, a significant (P =0.004) increase in force production was observed during an isometric mid-thigh pull. In single-bout high-intensity exercise using a WAnT protocol both Kramer et al. (2016) and Dominguez, Garnacho-Castano, et al. (2017) reported increases in peak power following NO_3^- supplementation from KNO_3 capsules and beetroot juice respectively, with the latter also noting higher average power in the first 15 seconds of the test. Both Whitfield et al. (2017) and Tillin et al. (2018) applied electrical stimulation to induce involuntary tetanic muscle contractions following NO_3^- supplementation. Whitfield et al. (2017) observed no change in maximum voluntary or electrically induced involuntary force production overall ($P > 0.05$) but did note an increase in force production at low electrical frequency (10 Hz) ($P < 0.05$) as well as an increase in peak twitch tension during supramaximal (150 V) electrically induced muscle twitches ($P < 0.01$). The study by Tillin et al. (2018) specifically examined differences between unfatigued and fatigued muscle tissue. Participants in the unfatigued group performed ten explosive, three second MVCs followed by two sets of four second involuntary tetanic contractions at various frequencies. Following a ten-minute rest period, fatigue was induced by performing 60, three second MVCs followed again by two sets of four second involuntary tetanic contractions at various frequencies. While no effects from NO_3^- were seen in the unfatigued muscles, fatigued tissues demonstrated a lowered percent decline in explosive, voluntary impulses from the first to the last six MVCs ($P = 0.039$) and an increased rate of peak

force development in the involuntary tetanic contractions (P = 0.011). Flanagan et al. (2016) was one of the first studies that examined the impact of NO_3^- supplementation on anaerobic exercise performed with a dynamic, multi-joint resistance protocol and measured a variety of factors including neuromuscular fatigue by means of muscle activity measured through EMG (Flanagan et al., 2016). While a significant (P =0.04) improvement to reproducibility of peak EMG was reported, the type of NO_3^- supplement given to participants differed from all previous work on NO_3^- and delivered a fraction of the lowest dose ever shown to have an ergogenic effect. Almost all studies on NO_3^- supplementation regardless of participant demographics, testing modalities, type of exercise, or outcomes of interest have used beetroot juice in various concentrations to provide NO_3^- to participants (Dominguez, Cuenca, et al., 2017; Jones, 2014), with dosages ranging from 4.2 mmol (Wylie, Kelly, et al., 2013) all the way to 26 mmol (Whitfield et al., 2017). In contrast, the study by Flanagan et al. (2016), while still deriving their NO_3^- from beetroot, used bars containing three grams of concentrated beetroot extract per bar and had participants consume two bars per day for three days, equalling slightly more than 1.1 mmol of NO_3^- per day. A far lower dosage of NO_3^- than anything previously studied and a relatively short period of supplementation. Additionally, the testing protocol was performed on an empty stomach with the last NO_3^- treatment consumed approximately 24 hours prior to testing. No plasma levels were reported for either NO_3^- or NO_2^- further compounding difficulties in comparing the study by Flanagan et al. (2016) to any other works on NO_3^- supplementation literature. While the protocol used for inducing and measuring neuromuscular fatigue was well designed with great potential to transfer to real-world training and performance scenarios in anaerobic or explosive type multi-joint movements, the fact that significant improvements were made with such a small dose of NO_3^- from an unstudied delivery method make the results

questionable and highlights the need for comparison to a better established, ergogenic dose of NO_3^- from beetroot juice along with verification of plasma NO_x levels.

Research Objectives

The overall objective of this study is to assess the effects of dietary NO_3^- supplementation in the form of beetroot juice on exercise performance and neuromuscular fatigue in a dynamic exercise test protocol with EMG data collected from MVCs performed before and during the dynamic exercise testing and having participants complete as many repetitions as possible (AMRAP) on the final set of the protocol. The specific objectives are to determine if NO_3^- supplementation in a dose-dependent manner, when compared to placebo will:

1) elevate plasma NOx levels, and affect blood lactate levels
2) decrease neuromuscular fatigue as seen through greater percent maintenance of maximum EMG amplitude
3) increase the number of repetitions to failure during the final set of a testing session at a given percentage of 1 RM
4) have a more profound effect on all measured anaerobic outcomes with an established effective dose of 8 mmol compared to an unsubstantiated dose of 1.2 mmol

Hypotheses

1) Supplementation with dietary NO_3^- for seven days prior to performing the dynamic exercise testing protocol will elevate plasma NOx levels.
2) Supplementation with dietary NO_3^- seven days prior to performing the dynamic exercise testing protocol will affect blood lactate levels.

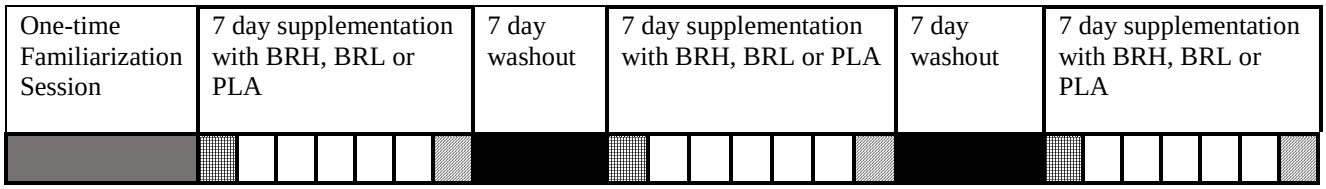
3) Supplementation with dietary NO_3^- for seven days prior to performing the dynamic exercise testing protocol will increase the ability to maintain peak EMG amplitude as a percent of maximum.
4) Supplementation with dietary NO_3^- for seven days prior to performing the dynamic exercise testing protocol will increase the number of repetitions to failure during the final set (60% 1RM) of the testing protocol.
5) A dose response effect will be seen with 8 mmol of NO_3^- producing more pronounced effects in each participant compared to both 1.2 mmol of NO_3^- and placebo. A dose of 1.2 mmol of NO3- is expected to elicit minimal improvements in all parameters compared to placebo with the exception of plasma NOx values which are anticipated to be elevated above placebo but far lower than with 8 mmol.

Methodology

Study design. Participants were randomly assigned in a double blind, randomized, crossover design to receive 7 days of supplementation of the high dose of beetroot juice (8 mmol NO3-/day) treatment (BRH), low dose of beetroot juice (1.2 mmol NO3-/day) treatment (BRL), or placebo (negligible NO3-) treatment (PLA). BRH and BRL were administered as 87 ml and 13 ml respectively of a commercially available, concentrated beetroot juice supplement (Beet It Sport Shot, James White Drinks, UK). The placebo treatment consisted of 83 ml of black cherry juice (Just Black Cherry, Smucker Quality Beverages, Inc., USA). A sufficient volume of water was added to all treatments to bring the total volume of each daily treatment to 500 ml and were provided to the participants in 7 sealed, opaque containers for each phase of supplementation. All persons involved in the study were unaware of which treatment each individual was on at any point until all testing and data analysis had been completed. Overall, all participants received

BRH, BRL, and PLA during the 7-day supplementation periods with each period separated by a 7-day washout (Figure 2). Treatments were consumed over 2-3 doses throughout the day on days 1-6 of each supplementation period. On day 7, participants were instructed to consume the entire treatment 2.5 hours prior to their testing session appointment. Participants visited the performance laboratory located in the High Performance Room (HPR) in the Active Living Centre (ALC) located at the University of Manitoba Ft. Garry Campus, a total of four times; a familiarization followed by 3 testing sessions (Figure 2).

Figure 2. Graphic representation of the crossover design and testing protocol.



= Familiarization session; = Blood pressure and anthropometrics including height in m, weight in kg, body fat percentage and lean mass in kg; = Blood draw, blood pressure, anthropometrics as previously mentioned and dynamic exercise/physical performance testing including electromyography; = Washout period

Participants. Participants were recruited through in person recruitment drives and by study flyers posted at authorized locations frequented by the target population. To be eligible to participate in this study, volunteers were required to meet the following criteria:

1) Must not have used any anabolic or ergogenic supplement (creatine, HMB, thermogenics, ribose, pro-hormones ((e.g., dehydroepiandrosterone (DHEA)), 7-keto-DHEA, etc.) in the last 1-3 months.

2) Must be currently engaging in a recreational resistance training program a minimum of 2 times per week for at least the last 3 months.

3) Must have at least 6 months experience in recreational exercise.

4) Must be between 18 to 40 years of age.

5) Must be in good physical health, determined by the physical activity readiness questionnaire (PAR-Q) (Warburton et al., 2011).

Fourteen resistance-trained males were recruited, with 13 completing the study. One participant after completion of the exercise familiarization session, but prior to any treatments and study measurements dropped out of the study. The participant did not attend the next scheduled study appointment and attempts at communication were not returned, therefore, no reason for withdrawal was recorded. Each participant was required to demonstrate proper squatting technique prior to proceeding with the study. At each blood collection, participants were asked if they had any change in supplementation or medication since the last time they had been questioned. Participants were instructed to refrain from chewing gum or using any antibacterial mouthwash throughout the duration of the study as this can impair NO_3^- conversion (Govoni, Jansson, Weitzberg, & Lundberg, 2008). All participants were engaged in regular resistance training throughout the duration of their participation in the study, although they were instructed not to perform any strenuous lower body exercise for at least 48 hours prior to testing sessions. Participants reported no major changes in training frequency, volume, or intensity during their participation in the study. All participants received written and oral information about the study procedures, potential benefits, risks and/or side effects related to participating in the study and were given time to review. Written informed consent was obtained from all

participants prior to participation. This study was approved by the University of Manitoba Joint-Faculty Research Ethics Board.

Study measurement procedures.

Exercise familiarization session. During the familiarization session, participants were exposed to the experimental conditions and equipment. This was done to ensure that participants were comfortable using the testing apparatus, to establish and record proper foot and bar placement, to verify acceptable squat depth for each individual, and to determine 1RM and target repetitions at all percentage loads of 1RM for the dynamic exercise testing protocol. For familiarization and all testing sessions, participants were instructed to avoid strenuous exercise for 24 hours (48 hours for lower body) and to abstain from caffeine for at least 12 hours prior to their appointment time. Participants were directed to wear comfortable, loose fitting clothing that would allow for ease of movement as well as supportive, flat soled shoes. Participants were required to wear the same shoes for all testing sessions. No squat shoes, weightlifting belts, compression shorts, knee or wrist wraps, chalk or lifting aids of any kind were permitted at any point during the study.

Prior to being randomized to a treatment group for the first phase and before any other study interventions, participants reported to the HPR to complete a familiarization session. Upon arrival at the HPR participants were asked to warmup on an exercise bike (Pro/Trainer, WattBike, UK) at a self-selected, but light to moderate pace and intensity for 10 minutes. Participants were then familiarized with the smith machine testing apparatus that would be used in all subsequent sessions. Full range of motion (fROM), defined as achieving at least a 90° angle of knee flexion with a nearly upright trunk (Figure 3), along with foot position and bar placement were determined and recorded for all participants. Repetitions performed in the

succeeding testing sessions were not counted if fROM was not achieved. Following determination of fROM, participants were tested for 1RM and maximum number of repetitions at 60%, 70%, 80% and 90% of 1RM on the smith machine squat.

Figure 3. Acceptable squat form for bottom portion of repetition in dynamic exercise testing protocol and position for all MVCs.

Photo by M. Pinder.

Anthropometric and blood collection sessions. On day 1 of each supplementation period (the day following familiarization testing in phase 1 and the day following the washout period for phases 2 and 3) after at least 10 hours overnight fast, the participants reported to the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), located at the University of Manitoba Ft. Garry Campus, between 7 and 8 am for anthropometric and blood pressure (BP) testing as well as venous blood sampling to determine the levels of plasma NO_x (see Assessments below) (Figure 2). All AM fasted blood draws were performed at the same time of day (± 1 h).

On day 7 of each supplementation period, participants reported to the Applied Research Centre (ARC) located in the ALC for anthropometrics, BP measurement, blood sampling, delivery of urine sample, and dynamic exercise testing (See Assessments below) (Figure 4).

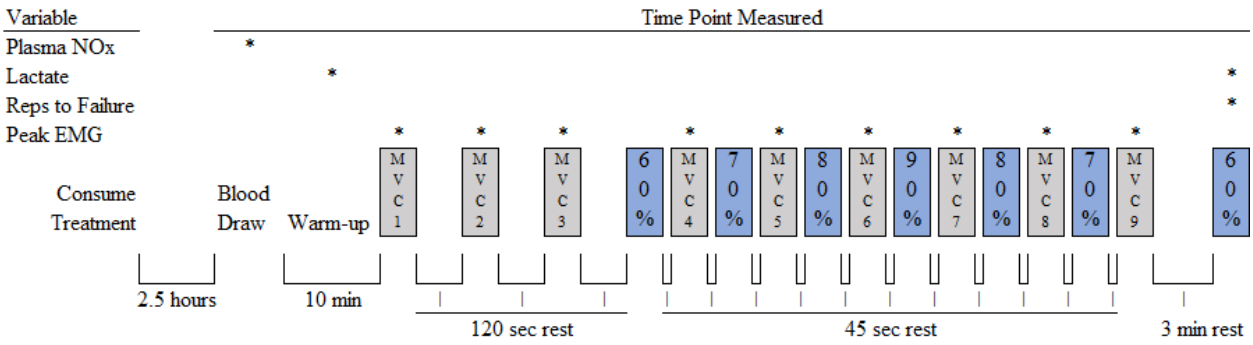
Throughout the study, blood samples were collected a total of 6 times from each participant (morning of day 1 prior to consuming any treatment; day 7 immediately before the testing procedure). The exact same procedures described here for day 1 were utilized for the blood draw and anthropometric testing on day 7 prior to exercise testing, although the day 7 blood draw was non-fasted and was typically performed in the late morning or in the afternoon depending on the appointment time of the participant. Upon arrival at the centre(s) and prior to the blood draw, participants were allowed to rest while answering questions to ensure compliance with any study requests and to confirm that there had been no change in supplementation or medication since participants were last asked.

Dynamic exercise testing protocol day 7. Following the day 7 blood draw, participants were then taken to the HPR for exercise testing and electromyography measurements (Figure 4). Participants completed the same bike warmup as described for the familiarization session.

Following the warmup, the electromyography (EMG) sensors were attached (see Assessments below). Following the electrode placement, a blood lactate sample was obtained from a fingertip of each participant (see Assessments below). Participants then began the dynamic exercise testing procedure (Figure 4). During the procedure, participants received no verbal feedback or encouragement aside from verbal cues mentioned below. Participants began by performing a series of 3, 3-second isometric squat MVCs (MVC 1-3) (positioning shown in Fig. 3) separated by 2 minutes of passive rest. The peak EMG amplitude from these 3 MVCs served as the maximum EMG amplitude and was used to normalize the data from the subsequent MVCs (MVC 4-9) completed during the session. MVCs were performed by placing the participant under the bar at a minimum of 90° angle of knee flexion with the bar racked high on the shoulders. Prior to commencing the session, the bar was loaded with each participants' 1RM weight plus an additional 25% to ensure that maximum force could be exerted without moving the bar. Two Smith machines were utilized during the testing sessions with one machine being loaded and positioned for MVCs and the other loaded and positioned for fatiguing sets. Once participants were in position, they received a "3, 2, 1, lift" cue following which they were to fully exert themselves in an attempt to lift the bar for a period of 3 seconds during which muscle activity was recorded from the EMG sensors attached to the quadricep. Following the third MVC (MVC3), participants were given another 2 minutes of passive rest before beginning the fatiguing portion of the protocol. Participants performed dynamic sets at 60%, 70%, 80%, 90%, 80% and 70% of 1RM using the target repetition ranges determined during the familiarization session. Each participant was positioned with the same foot and bar placement used in the familiarization session and given their target rep range for that set before receiving a "lift" command. Each dynamic set was followed by 45 seconds of rest during which participants

walked approximately 10 meters to the Smith machine set up for MVCs. Participants were again positioned to perform an MVC and received the lifting countdown and cue mentioned previously, at the end of the 45 second rest period. Participants were given another 45 second rest period while walking back to the Smith machine loaded for the next prescribed percentage of 1RM load. This procedure was repeated until completing the final MVC (MVC9) following the second dynamic set at 70% of 1RM. Participants were then given a 3-minute rest period followed by a final dynamic set at 60% 1RM in which participants completed an AMRAP set until volitional or actual failure. Upon completion of the dynamic exercise testing procedure, participants again had a fingertip cleaned, lanced, and a blood sample was taken for measurement of blood lactate.

Figure 4. Dynamic exercise testing protocol.



NO_x, Nitrate/Nitrite; EMG, electromyography; MVC, maximum voluntary contraction; %, the percentage of 1 repetition maximum for a given set.

Assessments.

EMG sensor placement and signal collection. Upon completing the bike warm up, the EMG sensors (Trigno, Delsys Incorporated, USA) were affixed to the right vastus lateralis muscle with the length of the rectangular sensors running parallel to the femur and the locations were marked in the first testing session and recorded for future sessions. Prior to affixing the

electrodes, the skin was shaved to remove any hair in the selected attachment zone, and cleaned with 70% ethanol wipes. The electrodes were then placed against the skin and pressure was applied to ensure proper bonding of the adhesive. Adhesive tape was then wrapped around the electrodes and thigh to further secure the sensors. A test contraction of the target muscle was then performed by having the participant perform an isometric contraction of the quadricep to ensure the sensors were collecting and transmitting the electromyography signal to the wireless receiver unit. EMG sensor signals were collected during all MVCs using unit acquisition software (EMGworks Acquisition version 4.3.2, Delsys Incorporated, USA) and saved for later analysis. Motion and noise artifact filters were applied to the signals during collection.

AMRAP. Following the final MVC measurement (MVC9), participants were allowed to rest for three minutes before completing a final set at 60% of their respective 1 RMs attempting to perform as many fROM repetitions as possible in a controlled manner before reaching volitional or absolute failure. Number of repetitions in this set were recorded for each treatment**.**

Blood lactate. Following placement of the EMG electrodes, a 70% ethanol wipe was used to clean a fingertip of each participant on the right hand and a lancet (Autolet Impression, Owen Mumford, USA) was used to obtain a fingertip blood sample for blood lactate testing with a portable lactate meter (Lactate Plus, Nova Biomedical, USA). This measurement was repeated at the end of each testing session immediately following the final dynamic squat set at 60% 1RM.

Blood and urine collection. Following anthropometric measurements, the blood draw was then performed by a trained phlebotomist during which approximately 20 ml of blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. EDTA tubes were immediately placed in a refrigerator at 4° C for a period not exceeding 90 minutes. The tubes were then

transferred to a refrigerated centrifuge set to 4° C and centrifuged at 3,000 rpm for 20 minutes. Plasma was divided into 0.5 and 1 ml aliquots, placed in 1.5 ml Eppendorf tubes and stored at -80° C until analysis.

Participants were provided with sealed containers to collect a urine sample to be turned in the day of their day 7 performance test. Participants were instructed to collect a midstream sample in the first urination upon waking and place the samples in the refrigerator until transporting them to the testing centre. Once received by study personnel, the urine samples were stored in a refrigerator at 4° C for a period of no more than 5 hours and were then aliquoted into 1.5 ml Eppendorf tubes at volumes of 1 ml and placed in a -80° C freezer to be tested for levels of NO_x.

Blood pressure. Following a minimum of five minutes rest at the beginning of each blood draw session, peripheral blood pressure was recorded in triplicate from the brachial artery on the right arm while participants were in a supine position using an automated sphygmomanometer (Series 10, Omron, USA). BP was recorded as the average of the 3 measurements.

Anthropometrics. Anthropometric measurements were also taken following BP measurement and immediately prior to the blood draw and included height, weight, body mass index (BMI), and body composition, including body fat percentage, by bio-electrical impedance analysis (BIA). All anthropometric measurements were collected with minimal clothing without shoes or socks and with all metal removed from the body. Height in cm was recorded using a stadiometer, while weight in kg was obtained on a digital scale (7562EF, Taylor Precision Products, USA). BMI calculations were performed by dividing weight in kg by height in meters squared (kg/m^2). BIA measures total body resistance through electrodes placed on the wrist,

hand, ankle and foot on the right side of the body and was collected with a four-electrode portable unit (Quantum II, RJL Systems, Michigan, USA).

Dietary intake records. Detailed dietary data was collected from the participants on at least 3 occasions; that is, dietary intake data was collected during each treatment phase, using a 3-day food record method to determine participants' intake of all food and drink. In the 72-hour period preceding the first exercise test, participants recorded their food, and were asked to replicate this diet as closely as possible in the 72 hours preceding subsequent tests. Additionally, participants were provided with a list of nitrate-rich foods to abstain from, apart from the study treatments, for 48 hours prior to all performance tests.

Data Analysis.

EMG analysis. Stored EMG files were uploaded into the analysis software (EMGworks Analysis version 4.3.2, Delsys Incorporated, USA). Peak EMG values were obtained by identifying the highest EMG value recorded during the 3 MVCs (MVC 1-3) at the beginning of each dynamic exercise testing session. Peak EMG values then functioned as the maximum EMG amplitude and were used to normalize values from the subsequent EMG tests for their respective sessions. The ability to reproduce maximum EMG values was determined by comparing average peak MVC EMG levels for each session and all time points among the 3 treatments.

Plasma NO_x analysis. Plasma analysis for NO_x content was performed using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, USA). The kit uses 96-well plates with an enzymatic reduction of NO_3^- followed by addition of Griess reagents to form a deep purple azo compound which then undergoes photometric measurement. The first step of the procedure involved the pre-assay preparation of the six reagents used.

1. The contents of the NO_3^-/NO_2^- Assay Buffer vial was diluted to 100 ml with Ultrapure water (Milli-Q Reference, MilliporeSigma, USA). The buffer was stored at 4° C when not in use.
2. The contents of the NO_3^- Reductase Enzyme Preparation vial was reconstituted with 1.2 ml of Assay Buffer. The vial was kept on ice during use and stored at -20° C when not in use. Freezing and thawing of the solution was restricted to one time.
3. The contents of the NO_3^- Reductase Cofactors Preparation vial was reconstituted with 1.2 ml of Assay Buffer. The vial was kept on ice during use and stored at -20° C when not in use. Freezing and thawing of the solution was restricted to one time.
4. The contents of the NO_3^- Standard vial were reconstituted with 1.0 ml of Assay Buffer. Reconstituted solution was vortexed (Vortex-Genie, Scientific Industries, Inc., USA) to ensure all lyophilized powder, including any on vial stopper, was mixed into solution. The vial was stored at 4° C when not in use.
5. The contents of the NO_2^- Standard vial were reconstituted with 1.0 ml of Assay Buffer. Reconstituted solution was vortexed to ensure all lyophilized powder, including any on vial stopper, was mixed into solution. The vial was stored at 4° C when not in use.
6. The contents of the Griess Reagents R1 and R2 vials were ready for use and required no preparation. The vials were stored at 4° C when not in use.

Once pre-assay preparation of the reagents was complete, 30 kilodalton (kDa) molecular weight cut-off filters (VWR, USA) were pre-rinsed with Ultrapure water to reduce background

absorbance due to the presence of hemoglobin and improve colour formation by the Griess Reagents. Participant plasma samples were then thawed and two aliquots of 0.5 ml of sample were placed into two molecular weight cut-off filters (1.0 ml of sample per participant per time point) and ultrafiltered in a high-speed centrifuge (Avanti J-30I, Beckman Coulter, USA) at 100,000 g for 20 minutes. While plasma samples were being centrifuged, set up of the 96-well plate began (Figure 5.). Adjustable pipettors (Research Plus, Eppendorf, Germany) were used to deliver all samples and reagents to the wells. Prior to pipetting each sample or reagent, the pipette tip was equilibrated by slowly filling the tip and gently expelling the contents several times. Care was taken to not expose the pipette tip to any reagent(s) already in the well. First, the nitrate standard curve was prepared. All curve and sample wells were plated in duplicate. 0.9 ml of Assay Buffer was placed in a clean test tube to which 0.1 ml of reconstituted NO_3^- Standard was added and vortexed. This stock standard had a NO_3^- concentration of 200 µM. The standard curve preparation with reagent volumes and final NO_3^- concentration are displayed in Figure 6.

Figure 5. Standard curve and duplicate sample layout on 96-well plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards; S1-S40 = Sample wells.

Figure 6. Nitrate standard curve preparation on 96-well plate.

Well	Nitrate Standard (µL)	Assay Buffer (µL)	Final Nitrate Concentration* (µL)
A1, A2	0	200	0
B1, B2	5	75	5
C1, C2	10	70	10
D1, D2	15	65	15
E1, E2	20	60	20
F1, F2	25	55	25
G1, G2	30	50	30
H1, H2	35	45	35

*Concentration calculated for final 200 µL assay volume following the addition of Griess Reagents.

Once the standard curve wells were prepared, the microfiltered plasma samples were added to the assay unknown wells in volumes of 40 µL per well. 40 µL is the maximum amount of plasma filtrate allowed according to the assay kit instructions. 40µL of Assay Buffer was then added to all unknown wells to bring their volume to 80 µL to match the NO_3^- standard wells. All reagents mentioned after this point were added to all standard and unknown wells with the exception of A1 and A2 which served as blanks. 10 µL of the Enzyme Cofactor Mixture was then added to each of the wells followed immediately by 10 µL of the NO_3^- Reductase Mixture. The plate was then covered with the provided plate cover and incubated at room temperature for three hours. The plate was not agitated in anyway during the incubation time.

Following the three-hour incubation, the plate cover was removed and 50 µL of Griess Reagent R1 was added to each of the wells followed immediately by 50 µL of Griess Reagent R2 bringing the volume of all wells to 200 µL. The colour was then allowed to develop for a minimum of 10 minutes, uncovered, at room temperature. The absorbance of the deep purple

azo developed was then read at 540 nm using a microplate reader (Synergy H4, Biotek Instruments Inc., USA). Plasma analysis procedure was repeated for all samples whose percent coefficient of variation (%CV) between the duplicate wells was over 10%.

Absorbance values of the blank wells were subtracted from all other wells and a standard curve was plotted by creating a plot of absorbance at 540 nm as a function of NO_3^-. Sample NO_x concentrations were then determined using the following formula shown in Figure 7.

Figure 7. Formula for determination of plasma Nitrate and Nitrite concentrations from colorimetric analysis.

$$[NO_3^- + NO_2^-]\ (\mu M) = \left(\frac{A_{540} - \text{y-intercept}}{\text{slope}} \right)\left(\frac{200\ \mu L}{\text{volume of sample used } (\mu L)} \right) \text{ x dilution}$$

NO_3^-, Nitrate; NO_2^-, Nitrite; µM, micromole; A_{540}, Mean absorbance value of duplicate samples at 540 nanometres (nm) from the colorimetric analysis; µL, microlitre.

Dietary analysis. Dietary analysis was completed by inputting all food record data and analysing with Food Processor software (ESHA Research, USA). Three-day food records were collected from participants during each treatment phase representing the 72-hour period prior to each testing session. Records were analysed for differences in energy intake, protein, carbohydrate and fat among treatments.

Statistical analysis. Data analysis was performed using IBM SPSS 25 (IBM, USA). One-way repeated-measures ANOVAs were used to assess the effects of the treatments on anthropometrics, systolic and diastolic blood pressure, plasma NO_x values, plasma lactate, AMRAP, and MVC mean peak EMG amplitude. All data are presented as means ± SD, unless

otherwise stated. Results were accepted as statistically significant when $P < 0.05$. 95% confidence intervals were reported where relevant.

The required sample size of 14 was determined using a standard sample size equation (Figure 8) based on variability using the study by Flanagan et al. (2016). The required number of participants was determined to be 6.4 per arm, however, it is hypothesized that in the current study, 1.2 mmol of NO_3^- will have minimal effect on any measured parameters compared to placebo and therefore, the study was treated as a two-arm investigation requiring 13 individuals. An extra participant was added to allow for possible dropout.

Figure 8. Formula used for sample size determination.

$$n = \frac{2(Z_a + Z_{1-\beta})^2 \sigma^2}{\Delta^2}$$

$$n = \frac{2(1.96 + 0.8416)^2 (0.032)^2}{(0.05)^2}$$

$$n = 6.4$$

Z_a, constant set according to accepted error (α) set at 0.05 for a two-tailed test; $Z_{1-\beta}$, constant set according to power (β) at 0.80 (80%); σ, estimated standard deviation; Δ, effect size

Results

Thirteen males completed the present study (mean age, 25.2 ± 2.7 years; height, 176.1 ± 8.1 cm; body mass, 82.6 ± 15.3 kg; BMI, 26.7 ± 5.0; squat 1 repetition maximum [1RM] 146 ± 33 kg) (Table 2). The supplementation protocol used was well tolerated by the participants and

no adverse side effects were reported. However, some participants did report beeturia (pink urine), and red stool, both of which are common with beet consumption.

Table 2. Participant baseline descriptives.

Variable	Baseline	Min	Max
Age (years)	25.2 ± 2.7	20	30
Height (cm)	176.1 ± 8.1	167	192
Weight (kg)	82.6 ± 15.3	69.8	125.5
BMI (kg/m^2)	26.7 ± 5.0	20.8	41.0
BF (%)	20.9 ± 6.6	13.6	35.0
1 RM (kg)	146 ± 33	95	200

BMI, body mass index; BF, body fat; 1 RM, 1-repetition maximum.

Anthropometrics. In general, body mass, BMI and body fat percentage did not differ significantly ($P > 0.05$) from baseline at any point, nor was there a significant difference ($P > 0.05$) between BRH, BRL or PLA (Table 3).

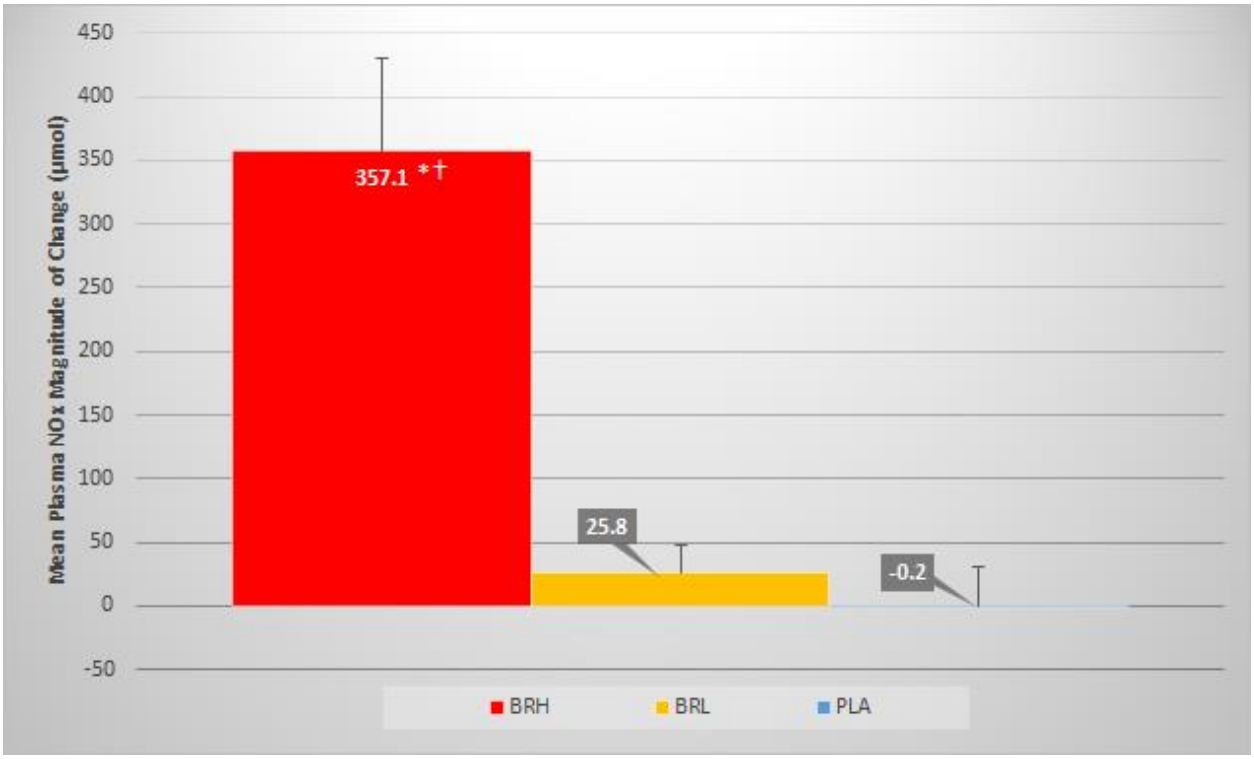
Table 3. Anthropometric measurements of study participants.

	Treatment							
Variable	BRH		BRL		PLA		*P*-values	
	Day 1	Day 7	Day 1	Day7	Day1	Day7	Time	Treatment
Weight kg	82.6 ± 15.2	82.3 ± 14.9	82.5 ± 15.1	82.6 ± 15.2	82.5 ± 15.2	82.6 ± 15.1	0.571	
BMI (kg/m^2)	26.7 ± 5.0	26.5 ± 4.9	26.7 ± 5.0	26.7 ± 5.0	26.7 ± 5.0	26.7 ± 5.0	0.211	
BF%	20.9 ± 6.5	20.8 ± 6.5	20.8 ± 6.6	20.9 ± 6.5	20.9 ± 6.6	20.9 ± 6.6	0.940	
Weight (kg) Change	-0.2 ± 0.8		-0.1 ± 0.7		0.1 ± 0.4			0.460
BMI (kg/m^2) Change	-0.1 ± 0.3		0.0 ± 0.2		0.0 ± 0.1			0.297
BF% Change	-0.1 ± 0.2		0.0 ± 0.5		0.0 ± 0.3			0.526

BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment; BMI, body mass index; BF%, body fat percentage.

Plasma NOx values. The BRH treatment significantly elevated levels of plasma NO_x (357.1 ± 73.6 µmol) compared to both BRL (25.8 ± 22.6 µmol) and PLA (-0.2 ± 31.7 µmol) ($P < 0.0001$). BRL did not elicit a significant change compared to PLA ($P = 0.170$) (Figure 9).

Figure 9. Mean plasma NO_x (µmol) magnitude of change from pre to post-supplementation.

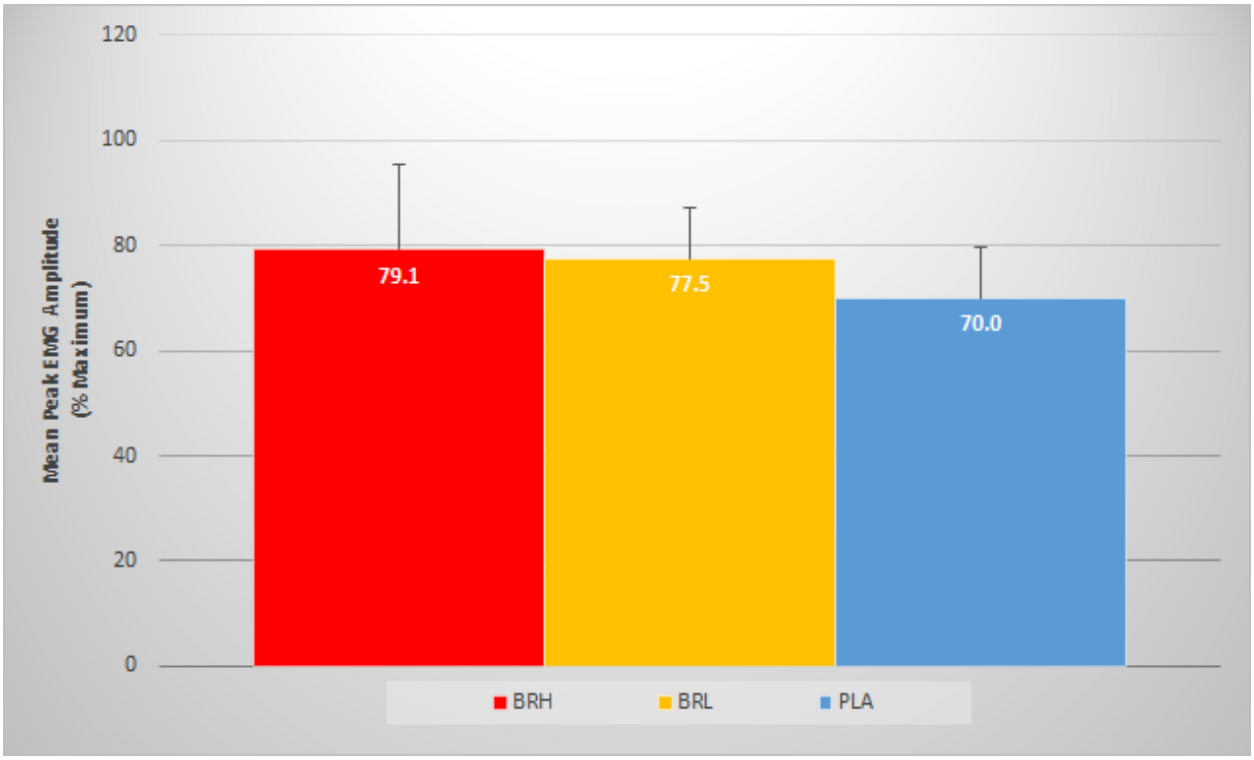


NO_x, Nitrate/Nitrite; BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment; *, significant difference compared to BRL; †, significant difference compared to PLA

Maximum voluntary contraction mean peak EMG amplitude. One participant's mean peak EMG amplitude was found to be much lower than the group mean and was determined to be an outlier and was therefore excluded from the analysis (n=12). Participants demonstrated greater total mean peak EMG amplitude on both BRH and BRL when compared to PLA (79.1% (95% CI: 68.8-89.5) and 77.5% (95% CI: 71.4-83.6) respectively vs. 70.0% (95% CI: 64.0-76.1)) although none of the results were significant ($P > 0.05$) (Figure 10). During the time point comparisons of peak EMG amplitude across treatments, three outliers were

discovered in the MVC post 60% (MVC4), two outliers were discovered in the MVC post 70% (MVC5) and one outlier was discovered in each of the MVCs post 80% (MVC6) and post the second set at 70% (MVC9). They were excluded from their respective time point analysis. While both BRH and BRL produced greater peak EMG amplitude at all time points compared to PLA, none of the results were significant ($P > 0.05$).

Figure 10. Mean peak EMG amplitude displayed as a percent of maximum EMG amplitude.



EMG, electromyography; BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment

Mean peak EMG across treatments as well as peak EMG values at all time points across treatments were also analyzed by splitting participants into high ($\geq$ 25.8 kg/m^2; n = 7) and low (<25.8 kg/m^2; n = 6) BMI groups to ensure BMI did not have an effect on reproducibility of

maximum EMG amplitude. Previously mentioned outliers were removed where applicable. Both high and low BMI groups did not show significance ($P > 0.05$) for mean peak EMG among all treatments. Two consecutive time points in the low BMI group showed a significant ($P < 0.05$) difference between BRH and PLA (Table 4). The MVC following the first 70% set (MVC5) returned values of 86.9% (95% CI: 64.3-109.6) for BRH compared to 64.7% (95% CI: 51.4-77.9) for PLA ($P = 0.033$). In the MVC following the first 80% set (MVC6), values were 80.9% (95% CI: 63.2-98.6) for BRH compared to 61.4% (95% CI: 51.1-71.7) for PLA ($P = 0.041$). All other peak EMG values across treatments by BMI showed no significant differences ($P > 0.05$).

Table 4. MVC values for low BMI (< 25.8 kg/m^2) group (n = 6).

EMG Timepoint	Treatment		Mean Difference (95% CI)	*P*-values
	BRH	PLA		
MVC 5	86.9% (64.3-109.6)	64.7% (51.4-77.9)	22.3% (2.3-42.2)	0.033
MVC 6	80.9% (63.2-98.6)	61.4% (51.1-71.7)	19.5% (0.96-38.0)	0.041

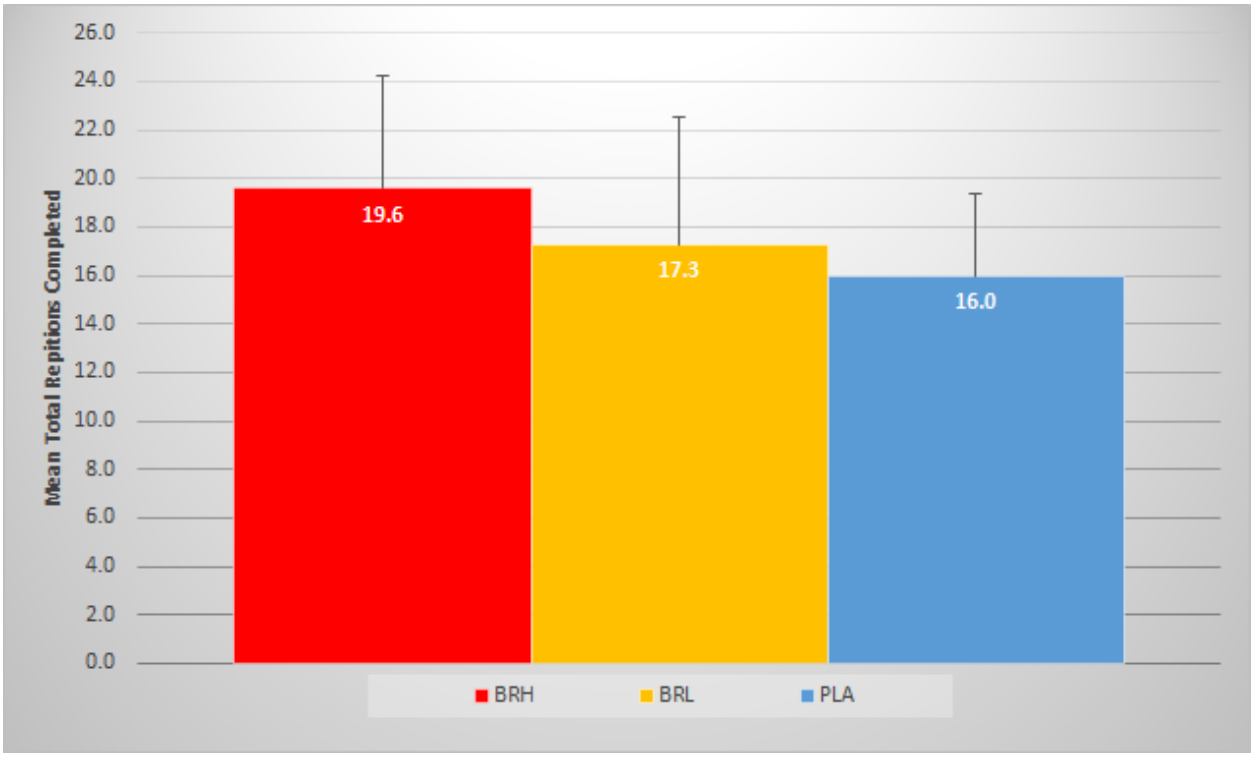
EMG, electromyography; BRH, high-dose nitrate (8 mmol) treatment; PLA, placebo treatment; MVC, maximum voluntary contraction.

AMRAP. Two participants were found to be outliers for the AMRAP set during the PLA treatment and were excluded from analysis (n=11). Total repetitions completed in the final set (60% 1RM) of the dynamic exercise testing were increased with both BRH and BRL compared to PLA (19.6 (95% CI: 16.4-22.7) and 17.3 (95% CI: 13.7-20.8) respectively vs. 16.0 (95% CI: 13.8-18.2)), however none of these results were significant ($P > 0.05$) (Figure 11).

AMRAP results were also examined by splitting the participants into high (≥ 25.8 kg/m^2; n = 7) and low (<25.8 kg/m^2; n = 6) BMI groups to ensure that BMI did not impact the number

of reps completed before reaching failure in the final set. Previously mentioned outliers were excluded where appropriate. Both high and low BMI groups did not differ in the number of reps completed to failure across treatments ($P > 0.05$).

Figure 11. Mean total repetitions completed in the final set of the dynamic exercise protocol by treatment.



BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment

Plasma lactate. The magnitude of change in plasma lactate from start to end of the dynamic exercise testing protocol did not differ significantly among any of the treatments ($P > 0.05$) nor did post exercise lactate levels (Table 5).

Table 5. Pre and post-exercise lactate levels (mmol/L) by treatment.

Treatment	Pre-exercise Lactate	Min	Max	Post-exercise Lactate	Min	Max	*P*-value Post-exercise Lactate	Magnitude of Change	Min	Max	*P*-value Magnitude of Change
BRH	3.0 ± 1.6	1.1	7.1	14.8 ± 3.3	10.1	19.4		11.8 ± 2.6	8.8	16.2	
BRL	3.4 ± 1.7	1.1	6.5	15.1 ± 3.8	9.2	21.2	0.809	11.6 ± 4.2	4.5	19.3	0.145
PLA	3.7 ± 1.5	1.3	6.9	14.6 ± 3.3	10.3	19.8		10.9 ± 3.0	7.8	15.6	

BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment

Blood pressure. No significant differences were observed between treatments for systolic or diastolic blood pressure post-supplementation, and for the magnitude of change on systolic or diastolic blood pressure from pre to post-supplementation ($P > 0.05$) (Table 6).

Table 6. Pre and post-supplementation blood pressure measurements in the study participants.

Treatment	Day 1 Systolic	Day 7 Systolic	*P*-value Day 7 Systolic	Magnitude of Change	*P*-value Magnitude of Change	Day 1 Diastolic	Day 7 Diastolic	*P*-value Day 7 Diastolic	Magnitude of Change	*P*-value Magnitude of Change
BRH	123 ± 11	132 ± 10		8 ± 9		71 ± 8	71 ± 8		0 ± 7	
BRL	119 ± 12	133 ± 11	0.361	15 ± 11	0.145	69 ± 8	71 ± 6	0.333	2± 6	0.522
PLA	122 ± 11	130 ± 9		8 ± 9		68 ± 7	69 ± 8		1 ± 8	

Means of systolic, diastolic, and magnitude of change displayed as mmHg ± standard deviation. BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment; *P*-value indicates the change among treatments.

Dietary records. One participant failed to turn in any dietary records and was excluded from analysis (n=12). No differences were found for mean energy, protein, carbohydrate or fat among treatments ($P > 0.05$) (Table 7).

Table 7. Dietary intake of participants by treatment.

	Treatment			
Variable	**BRH**	**BRL**	**PLA**	***P*-values**
Energy Intake (kcal)	2527 ± 444	2517 ± 444	2524 ± 445	0.944
Protein (g)	160 ± 37	155 ± 39	162 ± 38	0.162
Carbohydrate (g)	284 ± 49	288 ± 49	280 ± 53	0.457
Fat (g)	83 ± 14	83 ± 12	84 ± 13	0.580

BRH, high-dose nitrate (8 mmol) treatment; BRL, low-dose nitrate (1.2 mmol) treatment; PLA, placebo treatment.

Discussion

The present study examined biochemical, neuromuscular, and physical responses to two dosage levels of a NO_3^--rich concentrated beetroot juice supplement and a placebo. Plasma samples were analysed for levels of plasma NO_x and compared among treatments. Gross motor unit activity was assessed through EMG measurements during maximal isometric contractions before and during a fatiguing, dynamic squat exercise protocol using an ascending and descending pyramid scheme at various percentages of 1 RM. We also investigated the number of repetitions that could be performed before reaching failure following the fatiguing, dynamic squat protocol as well as changes in blood lactate, body mass and composition, and blood pressure.

Body weight and body composition did not differ from pre to post-supplementation or between treatments minimizing the possibility that any variability in results could be attributed to changes in anthropometrics. Where appropriate, participants were stratified by the median of BMI among participants (25.8 kg/m^2), as this is very close to the 25 kg/m^2 distinction between healthy and overweight (WHO, 2000). Participants were placed into high (n=7) and low (n=6)

groups ($\geq$ 25.8 kg/m^2 and < 25.8 kg/m^2 respectively) to examine if physical size had an impact on the recorded outcomes. In particular, EMG amplitude is known to have a large amount of variability between individuals (Balogh, Hansson, Ohlsson, Stromberg, & Skerfving, 1999). In our current investigation, in addition to using the first three MVCs of each session (MVC1-3) to determine the maximum EMG amplitude, these MVCs also served to establish a value for each participant to normalize the subsequent recorded values against, to attempt to deal with this limitation (Nordander et al., 2003). The inter-individual variability associated with EMG amplitude is largely due to variations of the tissue layers between the electrodes and muscles both in terms of thickness and electrical conductivity with the size of the muscle also playing a role (De Luca, 1979; Stegeman, Blok, Hermens, & Roeleveld, 2000). In the current study, while body fat percentage as measured by BIA reveals information regarding total body fat, the specific machine used did not provide any data related to fat distribution including subcutaneous thickness. Skin-caliper or preferably ultrasound measurements are the accepted modes for determining subcutaneous tissue thickness and body fat distribution (Nordander et al., 2003) but were not available in the current study. BMI allows for an accessible, yet rough estimation of subcutaneous tissue thickness and was therefore employed in an attempt to manage the inter-individual variability of the EMG measurements related to subcutaneous tissue differences among participants.

The results showed that BRH at an estimated 8 mmol/L nitrate significantly elevated plasma NO_x levels compared to both the BRL at 1.2 mmol/L nitrate and the negligible nitrate PLA. In general, the literature shows that NO_3^- supplementation has a plasma dose response relationship, and the plasma NO_x levels observed in this study for BRH and PLA closely reflect established values (Wylie, Kelly, et al., 2013). To date, only Flanagan et al. (2016) have utilized

a dosage of 1.1 mmol of NO_3^- with the next lowest dose in the literature being 4.2 mmol (Hoon, Jones, et al., 2014; Wylie, Kelly, et al., 2013). The studies that have used a dose of 4.2 mmol of NO_3^- failed to elicit any performance improvements, thus it was surprising that Flanagan et al. (2016) claimed significant improvements in performance outcomes with only 1.1 mmol of NO_3^- . Albeit the mode of delivery of beetroot NO_3^- was in the form of a snack bar in Flanagan et al (2016) compared to the usual beetroot juice. Unfortunately, plasma values were not reported by Flanagan et al. (2016), but the failure to produce any ergogenic benefits in the present study under very similar exercise protocol conditions with dosages of 8 and 1.2 mmol of NO_3^- generating plasma NO_x values of 357.1 and 25.8 µmol respectively, calls into question whether the results of Flanagan et al. (2016) was related to NO_3^- consumption and therefore plasma level. Based on our study results, a 1.2 mmol of NO_3^- was simply too low to produce sufficient levels of the NO end product to elicit physiological benefits in the proposed mechanisms for performance enhancement with NO_3^- supplementation.

Although NO_3^--rich supplementation with BRH and BRL lead to increased mean peak EMG amplitude compared to PLA (79.1 ± 16.3%, 77.5 ± 9.7% and 70.0 ± 9.5% respectively), none of the results were significant between the treatments ($P > 0.05$). Additionally, EMG measurements were compared among treatments for all time points with BRH resulting in higher peak EMG amplitudes for all time points compared to BRL and PLA but these results were not significant ($P > 0.05$). When stratified by BMI into high (n=7) and low (n=6) groups (≥ 25.8 kg/m^2 and < 25.8 kg/m^2 respectively) BRH produced greater peak EMG amplitude compared to PLA in the MVCs following both the first fatiguing set at 70% 1 RM (MVC5) (86.9 ± 21.6% vs. 64.7 ± 12.6% ($P = 0.033$)) and the first fatiguing set at 80% 1 RM (MVC6) (80.9 ± 16.9% vs. 61.4 ± 9.8% ($P = 0.041$)) for the low BMI group. However, we do not believe the improvement

in this small section of the outcome is sufficient to claim a substantial benefit that would be reflected in actual use for training or competition and could be attributed to simple variability in the EMG measurements. Had more of the timepoint analyses or the overall peak EMG measurements achieved significance for the low BMI group, then the previously mentioned variability in subcutaneous tissue could be theorized to be playing a role in this study. However, with only two time points for EMG amplitude achieving significance, we do not feel the evidence is strong enough in the current study to warrant a statement regarding potential ergogenic effects of NO_3^- on the time point analysis of EMG amplitude. None of the results differed for the high BMI group.

In the present study, the occurrence of neuromuscular fatigue during the dynamic squat exercise protocol is revealed by the inability of participants to reproduce maximum EMG amplitudes across all treatments. This could be due to interferences in neuromuscular transmission (Boyas & Guevel, 2011) or metabolic by-product accrual (Westerblad et al., 1998). Previous research has shown that NO_3^- supplementation with beetroot can help attenuate the detrimental accumulation of such by-products. In the study by Bailey et al. (2010), participants consumed 500 ml of beetroot juice (5.1 mmol NO_3^-) and performed a session of three MVCs along with an incremental leg extension ergometer test to exhaustion and showed reduced decomposition of PCr along with decreased buildup of inorganic phosphate and ADP. While a reduction in metabolic by-products was observed by Bailey et al. (2010) with 5.1 mmol of NO_3^-, no ergogenic benefit was observed in the present study despite a higher dosage level of 8 mmol. Although these metabolites were not measured in the present study, it is possible that the reduction of these compounds did not reach a sufficient level to be beneficial when examining neuromuscular fatigue. More recently, Bender et al. (2018) reported increased force production

during a 4-second isometric mid-thigh pull after a single-bolus high dose (12.9 mmol) of NO_3^-. This type of contraction is similar to those observed during full isometric contractions such as MVCs like those performed in the current study. While the reduction of neuromuscular fatigue inducing compounds, along with increased contractile efficiency and force production, should allow for greater maintenance of neuromuscular efficiency as higher peak EMG values expressed as a percentage of maximum EMG amplitude, any enhancement observed in the present study was not great enough to produce a significant ergogenic benefit when compared to placebo, with the exception of MVCs 5 and 6 for the low BMI group performed during the dynamic squat protocol when participants were stratified by BMI.

It is possible that measurable improvements in neuromuscular fatigue under anaerobic conditions, as opposed to an incremental test to exhaustion (Bailey et al., 2010), due to reduced metabolic by-products requires a more substantial dose of NO_3^- comparable to that utilized by Bender et al. (2018). Using a similar testing protocol to the one undertaken in our investigation, Flanagan et al. (2016) reported an approximate four to five percent increase in mean peak EMG amplitude with NO_3^- supplementation compared to placebo ($P = 0.04$). However, this EMG improvement is surprising considering the NO_3^- dosage of 1.1 mmol, which is markedly lower than any level delivered to participants in any previous literature showing ergogenic effects with NO_3^- (Dominguez, Cuenca, et al., 2017; Jones, 2014). Furthermore, in the Flanagan et al. (2016) report, the NO_3^- dosage was delivered using a new type of NO_3^- supplement bar, which differed greatly from the more widely studied beetroot juice used in the present study and the vast majority of other investigations. In our examination of neuromuscular efficiency and physical performance with NO_3^- supplementation we compared both a typical dosage of NO_3^- (8 mmol) from beetroot juice shown to enhance performance in various outcomes and under a variety of

conditions (Aucouturier et al., 2015; Bailey et al., 2010; Breese et al., 2013; Cermak, Gibala, et al., 2012; Dominguez, Garnacho-Castano, et al., 2017; Lansley, Winyard, Bailey, et al., 2011; Wylie et al., 2016) as well as a similar dosage (1.2 mmol) to that used by Flanagan et al. (2016), yet still failed to show improvements in peak EMG amplitude at both dosage levels compared to placebo. This makes the results of Flanagan et al. (2016) all the more unexpected and raises questions regarding the bar-type supplement used in that study.

A post-hoc power analysis revealed that the present study may have been underpowered and could have benefitted from a substantially larger sample size of about 32 (Appendix C), which is about 2.5 times more than numbers in this report. This underpowered in sample size, combined with the large standard deviation seen in the EMG measurements, likely contributed to obscuring any statistical significance that may have been observed with an increased number of participants.

We also assessed the maximum number of repetitions that could be performed to failure at 60% 1 RM in the final set of the dynamic squatting protocol, however, the number of repetitions did not differ significantly ($P > 0.05$) among treatments for all participants or when stratified by BMI. With the previously mentioned synergistic influences on skeletal muscle environment and neuromuscular efficiency seen with NO_3^- supplementation from the published literature, it could be possible that an individual would be able to complete a greater number of repetitions at a given level of resistance before buildup of metabolic by-products and/or impairment of neuromuscular communication leads to decline of contractile efficiency in anaerobic exercise conditions. In the present study the number of repetitions completed at 60% of 1 RM completed in the final set of the dynamic squat protocol did not differ by treatment, nor did a total number of session repetitions. Despite reporting a reduction in neuromuscular fatigue,

Flanagan et al. (2016) also did not find a significant difference in the total number of repetitions among treatments performed across the entire fatiguing protocol, although the number of repetitions completed in the final set alone were not reported. Similar to the effects on EMG measurements in the current investigation, a larger dose of NO_3^- may lead to improvements for this outcome of physical performance. Additionally, a large standard deviation in the present study likely contributed to the confounding of any potential ergogenic effects that could have been observed in this outcome.

In the present study, blood lactate measurements did increase due to the dynamic exercise testing protocol; however, the lactate values did not differ significantly among treatments during pre to post-exercise. This is similar to the results for lactate stated by Flanagan et al. (2016), although based on reports from other studies some alteration of blood lactate could have been expected from the 8 mmol dose of NO_3^- compared to the 1.2 mmol and placebo treatments. The impact of NO_3^- supplementation on blood lactate levels has however shown mixed results in recent studies with some showing decreased elevation compared to placebo (Christensen et al., 2017; Thompson et al., 2017), while others have shown greater elevation of lactate in response to exercise with NO_3^- supplementation (Dominguez, Garnacho-Castano, et al., 2017; Shannon et al., 2017; Wylie et al., 2016). Interestingly, the elevation of lactate in response to exercise is greater with NO_3^- supplementation, as opposed to placebo, has been proposed to be an indicator of physiological benefit (Wylie et al., 2016). It has been suggested that a greater rise in plasma lactate following NO_3^- supplementation could be indicative of elevated perfusion to type II muscle fibers (Ferguson et al., 2013; Ferguson et al., 2014) which are the dominant fiber type active in anaerobic exercise conditions and have higher lactate production (Esbjornsson-Liljedahl, Sundberg, Norman, & Jansson, 1999). Increased muscle perfusion could lead to

greater lactate effluence (Juel, 1997) and this would align with the known ability of NO_3^- supplementation, through conversion to NO, to promote vasodilation and increase blood flow to hypoxic tissues (Bailey et al., 2012; Cosby et al., 2003). While this is an interesting theory, it does not explain the fact that both post-exercise lactate values and magnitude of change from pre to post-exercise lactate values did not differ significantly among treatments. With the mixed results both in the literature and in the present study, it is clear that the relationship between NO_3^- supplementation and lactate under exercising conditions warrants further investigation.

Both systolic and diastolic blood pressure did not differ among treatments ($P > 0.05$) on day seven of supplementation, nor did the systolic or diastolic magnitude of change observed between the pre-supplementation measurements on day one and the day seven measurements. It is well established that dietary NO_3^- has the ability to reduce blood pressure through vasodilation (Coles & Clifton, 2012; Hord et al., 2009; Moncada & Higgs, 2006) which can translate to improved blood flow to exercising tissues and is indeed one of the mechanisms thought to be responsible for the observed ergogenic effects of NO_3^- supplementation (Bailey et al., 2009). The failure to notice alterations in blood pressure between treatments in the present study could be largely attributed to the variations in the time between the day one and day seven measurements. While the day one measurements were recorded early in the morning after an overnight fast, the day seven measurements were recorded an average of 6-7 hours later after participants had followed their normal eating habits up until that point during the day. Due to the need to collect a fasted blood sample pre-supplementation on day one, and the logistics of collecting a blood sample prior to the dynamic exercise testing sessions on day seven 2.5 hours after consuming the final treatment dose in each phase, it was not possible to measure blood pressure under the same physiological conditions or at the same time of day for participants.

Variations in blood pressure are known to be impacted by a wide variety of factors including sleep patterns, stress, hydration, food intake and humoral aspects such as balance of certain hormones including angiotensin, bradykinins, and insulin (Giuseppe Mancia et al., 1983; G. Mancia & Grassi, 2000). These influences combine to cause variations in blood pressure of more than 50-60 mmHg over a 24-hour period and 15-20 mmHg when day-night differences are observed (Giuseppe Mancia et al., 1983). This variability in blood pressure could mask any effect that could otherwise be noted following NO_3^- supplementation and could explain the lack of decrease in blood pressure with NO_3^- ingestion even at the 8 mmol dose.

Analysis of participant food records in the present study revealed no differences in protein, carbohydrate, fat, or total caloric intake among treatments. This helps to ensure that any variation in measured parameters across treatments cannot be attributed to differences in energy status or macronutrient variation.

Generalizability

When considering the results from the present study and the implications therein, it is important to keep several factors in mind. There is much yet to be learned about the effects of NO_3^- supplementation on muscular fatigue and performance in short-duration, high-intensity multi-joint exercise scenarios. The current investigation utilized young, recreationally active adult males, similar to the majority of research conducted to date on NO_3^- supplementation (Dominguez, Cuenca, et al., 2017; Van De Walle & Vukovich, 2018). It has been shown that highly trained or elite athletes have a lowered response to the ergogenic benefits of NO_3^- supplements in aerobic testing (Van De Walle & Vukovich, 2018), and this may very well be the case with anaerobic or muscular-resistance testing. It is also important to remember that the present study had participants ranging in age from 20 to 30 years old, which reflects the current

literature in terms of the research population demographic pertaining to NO_3^- supplementation effect on exercise performance in which nearly all participants have been between the ages of 18 to 40 years old (Van De Walle & Vukovich, 2018). Thus, caution should be used when attempting to generalize the results of this or other studies on NO_3^- supplementation and exercise performance to older populations. A wide variety of metabolic and functional changes occur with age, including alteration of endocrine function, development of chronic diseases, insulin resistance, increased inflammation and higher risk of nutritional deficiencies, all of which can contribute to the age-related loss of skeletal mass and function known as sarcopenia (Fielding et al., 2011). These shifts in body composition and functionality make results of studies performed on younger individuals difficult to apply to older populations and any recommendations should be approached with caution. Investigations concerning the efficacy of NO_3^- supplementation on improving exercise performance in adults over the age of 40 years old must be performed on those age groups to develop specific recommendations.

Limitations

Several limitations were identified in the present study. A high amount of variability was noted among participants for the EMG measurement resulting in a large standard deviation among the observed values. Taking this large variability in EMG measurement into consideration, a post hoc power analysis indicated that a considerably larger sample size of about n = 32 participants would be needed to observe a significant difference in results between treatments compared to the n = 13 used in the current study. A greater homogeneity relating to body size and body composition may assist in reducing the variability in the EMG measurements due to the effects of subcutaneous tissue thickness and muscle size on EMG variability discussed previously. A combination of a larger sample size with greater homogeneity in body size and

composition may have helped to increase the power of the present study by reducing variability and drawing from a greater number of observations. The addition of a more substantial dose of NO_3^-, in the range of 12-16 mmol compared to the standard dose of 8 mmol that served as the high-dose in the current investigation, reflecting what has been used previously in the literature on aerobic exercise performance may have also been beneficial in determining the ergogenic threshold, if any, for NO_3^- supplementation and neuromuscular efficiency and exercise performance in a muscular fatiguing, multi-joint exercise scenario.

Conclusion

Overall, the present study did not show any significant ergogenic effects with 8 mmol of NO_3^- supplementation from beetroot juice compared to 1.2 mmol or placebo on neuromuscular fatigue, physical performance, or biochemical markers. This is in stark contrast to the research conducted by Flanagan et al. (2016) that reported a four to five percent increase in the ability to maintain peak EMG amplitude as a percent of maximum EMG amplitude, possibly indicating a reduction in neuromuscular fatigue or attenuation of metabolic by-product accumulation with 1.1 mmol of NO_3^-. While the NO_3^- was administered through a food bar as opposed to the more common beetroot juice in Flanagan et al. (2016), it is difficult to accept that a dose several times lower than anything ever previously shown to be effective could produce such a result due to NO_3^- content alone, regardless of mode of delivery, despite the author claims that any macro and/or micronutrient variations between the supplement and placebo would not impact performance (Flanagan et al., 2016). Although plasma NO_x values were not reported by (Flanagan et al., 2016), the difference in plasma NO_x levels between 8 and 1.2 mmol observed in the present study, combined with no significant improvements to the measured performance

outcomes, further contribute to the unlikelihood of the significant results attributed solely to NO_3^- by (Flanagan et al., 2016).

Future directions

While NO_3^- supplementation is generally established as an effective ergogenic aid in endurance or aerobic-type activities for most people, there remains a great deal of work to be done to elucidate potential benefits and applications for NO_3^- and anaerobic exercise. More research is also needed to firmly establish the theories surrounding how NO_3^- supplementation through its conversion to NO_2^- and NO can enhance exercise performance and the level of synergy between these theories. Finally, many questions exist surrounding the relationship of dietary NO_3^- and lactate with the current literature providing a number of conflicting results and theories. Were the current study to be repeated, a more substantial dose of NO_3^- in the range of 12-16 mmol could be compared to the "high-dose" of 8 mmol utilized in our investigation to further explore the dose-dependency of NO_3^- supplementation, as these dosage ranges have now been well documented in the literature on aerobic exercise yet remain to be explored under the conditions utilized in the present study. It would be of interest to determine whether or not a larger dose of NO_3^- would have a greater impact on the measured parameters, particularly the peak EMG amplitudes and the number of repetitions performed before reaching failure in the final set of the dynamic exercise protocol. While the results in these areas were not significant in the present study, there did appear to be a small impact with higher NO_3^- consumption and therefore, it could be theorized that a larger dose may elicit the improvements that were originally hypothesized. Going beyond BMI stratification, it would also be useful to determine subcutaneous tissue thickness by means of ultrasound or at least skin fold measurements, for

future work with EMG amplitudes to help further minimize the variability associated with EMG measurements between individuals.

Future studies, particularly on neuromuscular fatigue and anaerobic exercise, should focus on consistent dosing and delivery of NO_3^-; exploration of higher NO_3^- dosages similar to the high-end seen in the literature on aerobic performance; use of proven testing modalities, especially those that transfer well to real-world athletic performance or training; and ensuring homogeneity within test subjects which could be argued was a limitation of the current study in terms of participant size.

References

Alderton, W. K., Cooper, C. E., & Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochem J, 357*(Pt 3), 593-615.

Alvares, T. S., Conte, C. A., Paschoalin, V. M., Silva, J. T., Meirelles Cde, M., Bhambhani, Y. N., & Gomes, P. S. (2012). Acute l-arginine supplementation increases muscle blood volume but not strength performance. *Appl Physiol Nutr Metab, 37*(1), 115-126. doi:10.1139/h11-144

Anderson, J. E. (2000). A role for nitric oxide in muscle repair: nitric oxide-mediated activation of muscle satellite cells. *Mol Biol Cell, 11*(5), 1859-1874.

Arnold, J. T., Oliver, S. J., Lewis-Jones, T. M., Wylie, L. J., & Macdonald, J. H. (2015). Beetroot juice does not enhance altitude running performance in well-trained athletes. *Appl Physiol Nutr Metab, 40*(6), 590-595. doi:10.1139/apnm-2014-0470

Aucouturier, J., Boissiere, J., Pawlak-Chaouch, M., Cuvelier, G., & Gamelin, F. X. (2015). Effect of dietary nitrate supplementation on tolerance to supramaximal intensity intermittent exercise. *Nitric Oxide, 49,* 16-25. doi:10.1016/j.niox.2015.05.004

Bailey, S. J., Fulford, J., Vanhatalo, A., Winyard, P. G., Blackwell, J. R., DiMenna, F. J., . . . Jones, A. M. (2010). Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol (1985), 109*(1), 135-148. doi:10.1152/japplphysiol.00046.2010

Bailey, S. J., Vanhatalo, A., Winyard, P. G., & Jones, A. M. (2012). The nitrate-nitrite-nitric oxide pathway: Its role in human exercise physiology. *European Journal of Sport Science, 12*(4), 309-320.

Bailey, S. J., Varnham, R. L., DiMenna, F. J., Breese, B. C., Wylie, L. J., & Jones, A. M. (2015). Inorganic nitrate supplementation improves muscle oxygenation, O2 uptake kinetics and exercise tolerance at high but not low pedal rates. *J Appl Physiol (1985)*, jap 01141 02014. doi:10.1152/japplphysiol.01141.2014

Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., Dimenna, F. J., Wilkerson, D. P., . . . Jones, A. M. (2009). Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol (1985), 107*(4), 1144-1155. doi:10.1152/japplphysiol.00722.2009

Balogh, I., Hansson, G. A., Ohlsson, K., Stromberg, U., & Skerfving, S. (1999). Interindividual variation of physical load in a work task. *Scand J Work Environ Health, 25*(1), 57-66.

Balsalobre-Fernandez, C., Romero-Moraleda, B., Cupeiro, R., Peinado, A. B., Butragueno, J., & Benito, P. J. (2018). The effects of beetroot juice supplementation on exercise economy, rating of perceived exertion and running mechanics in elite distance runners: A double-blinded, randomized study. *PLoS One, 13*(7), e0200517. doi:10.1371/journal.pone.0200517

Bar-Or, O. (1987). The Wingate anaerobic test. An update on methodology, reliability and validity. *Sports Med, 4*(6), 381-394.

Barclay, C. J., Woledge, R. C., & Curtin, N. A. (2007). Energy turnover for Ca2+ cycling in skeletal muscle. *J Muscle Res Cell Motil, 28*(4-5), 259-274. doi:10.1007/s10974-007-9116-7

Basu, S., Azarova, N. A., Font, M. D., King, S. B., Hogg, N., Gladwin, M. T., . . . Kim-Shapiro, D. B. (2008). Nitrite reductase activity of cytochrome c. *J Biol Chem, 283*(47), 32590-32597. doi:10.1074/jbc.M806934200

Bender, D., Townsend, J. R., Vantrease, W. C., Marshall, A. C., Henry, R. N., Heffington, S. H., & Johnson, K. D. (2018). Acute beetroot juice administration improves peak isometric force production in adolescent males. *Appl Physiol Nutr Metab, 43*(8), 816-821. doi:10.1139/apnm-2018-0050

Benjamin, N., O'Driscoll, F., Dougall, H., Duncan, C., Smith, L., Golden, M., & McKenzie, H. (1994). Stomach NO synthesis. *Nature, 368*(6471), 502. doi:10.1038/368502a0

Bescos, R., Gonzalez-Haro, C., Pujol, P., Drobnic, F., Alonso, E., Santolaria, M. L., . . . Galilea, P. (2009). Effects of dietary L-arginine intake on cardiorespiratory and metabolic adaptation in athletes. *Int J Sport Nutr Exerc Metab, 19*(4), 355-365.

Bescos, R., Rodriguez, F. A., Iglesias, X., Ferrer, M. D., Iborra, E., & Pons, A. (2011). Acute administration of inorganic nitrate reduces VO(2peak) in endurance athletes. *Med Sci Sports Exerc, 43*(10), 1979-1986. doi:10.1249/MSS.0b013e318217d439

Bond, H., Morton, L., & Braakhuis, A. J. (2012). Dietary nitrate supplementation improves rowing performance in well-trained rowers. *Int J Sport Nutr Exerc Metab, 22*(4), 251-256.

Boorsma, R. K., Whitfield, J., & Spriet, L. L. (2014). Beetroot juice supplementation does not improve performance of elite 1500-m runners. *Med Sci Sports Exerc, 46*(12), 2326-2334. doi:10.1249/mss.0000000000000364

Boyas, S., & Guevel, A. (2011). Neuromuscular fatigue in healthy muscle: underlying factors and adaptation mechanisms. *Ann Phys Rehabil Med, 54*(2), 88-108. doi:10.1016/j.rehab.2011.01.001

Brand, M. D., & Esteves, T. C. (2005). Physiological functions of the mitochondrial uncoupling proteins UCP2 and UCP3. *Cell Metab, 2*(2), 85-93. doi:10.1016/j.cmet.2005.06.002

Breese, B. C., McNarry, M. A., Marwood, S., Blackwell, J. R., Bailey, S. J., & Jones, A. M. (2013). Beetroot juice supplementation speeds O2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. *Am J Physiol Regul Integr Comp Physiol, 305*(12), R1441-1450. doi:10.1152/ajpregu.00295.2013

Brown, G. C., & Cooper, C. E. (1994). Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett, 356*(2-3), 295-298.

Byrne, G., Wardrop, B., & Storey, A. (2014). The effect of beetroot juice dosage on high intensity intermittent cycling performance. *Journal of Science and Cycling, 3*(2), 56.

Campbell, B., Roberts, M., Kerksick, C., Wilborn, C., Marcello, B., Taylor, L., . . . Kreider, R. (2006). Pharmacokinetics, safety, and effects on exercise performance of L-arginine alpha-ketoglutarate in trained adult men. *Nutrition, 22*(9), 872-881. doi:10.1016/j.nut.2006.06.003

Castello, P. R., David, P. S., McClure, T., Crook, Z., & Poyton, R. O. (2006). Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab, 3*(4), 277-287. doi:10.1016/j.cmet.2006.02.011

Cermak, N. M., Gibala, M. J., & van Loon, L. J. (2012). Nitrate supplementation's improvement of 10-km time-trial performance in trained cyclists. *Int J Sport Nutr Exerc Metab, 22*(1), 64-71.

Cermak, N. M., Res, P., Stinkens, R., Lundberg, J. O., Gibala, M. J., & van Loon, L. J. (2012). No improvement in endurance performance after a single dose of beetroot juice. *Int J Sport Nutr Exerc Metab, 22*(6), 470-478.

Christensen, P. M., Nyberg, M., & Bangsbo, J. (2013). Influence of nitrate supplementation on VO(2) kinetics and endurance of elite cyclists. *Scand J Med Sci Sports, 23*(1), e21-31. doi:10.1111/sms.12005

Christensen, P. M., Petersen, N. K., Friis, S. N., Weitzberg, E., & Nybo, L. (2017). Effects of nitrate supplementation in trained and untrained muscle are modest with initial high plasma nitrite levels. *Scand J Med Sci Sports, 27*(12), 1616-1626. doi:10.1111/sms.12848

Coggan, A. R., Leibowitz, J. L., Kadkhodayan, A., Thomas, D. P., Ramamurthy, S., Spearie, C. A., . . . Peterson, L. R. (2014). Effect of acute dietary nitrate intake on maximal knee extensor speed and power in healthy men and women. *Nitric Oxide*. doi:10.1016/j.niox.2014.08.014

Coles, L. T., & Clifton, P. M. (2012). Effect of beetroot juice on lowering blood pressure in free-living, disease-free adults: a randomized, placebo-controlled trial. *Nutr J, 11*, 106. doi:10.1186/1475-2891-11-106

Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., Martyr, S., . . . Gladwin, M. T. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat Med, 9*(12), 1498-1505. doi:10.1038/nm954

de Castro, T. F., Manoel, F. A., Figueiredo, D. H., Figueiredo, D. H., & Machado, F. A. (2018). Effect of beetroot juice supplementation on 10-km performance in recreational runners. *Appl Physiol Nutr Metab*. doi:10.1139/apnm-2018-0277

De Luca, C. J. (1979). Physiology and mathematics of myoelectric signals. *IEEE Trans Biomed Eng, 26*(6), 313-325.

Dominguez, R., Cuenca, E., Mate-Munoz, J. L., Garcia-Fernandez, P., Serra-Paya, N., Estevan, M. C., . . . Garnacho-Castano, M. V. (2017). Effects of Beetroot Juice Supplementation on Cardiorespiratory Endurance in Athletes. A Systematic Review. *Nutrients, 9*(1). doi:10.3390/nu9010043

Dominguez, R., Garnacho-Castano, M. V., Cuenca, E., Garcia-Fernandez, P., Munoz-Gonzalez, A., de Jesus, F., . . . Mate-Munoz, J. L. (2017). Effects of Beetroot Juice Supplementation on a 30-s High-Intensity Inertial Cycle Ergometer Test. *Nutrients, 9*(12). doi:10.3390/nu9121360

Dykhuizen, R. S., Frazer, R., Duncan, C., Smith, C. C., Golden, M., Benjamin, N., & Leifert, C. (1996). Antimicrobial effect of acidified nitrite on gut pathogens: importance of dietary nitrate in host defense. *Antimicrob Agents Chemother, 40*(6), 1422-1425.

Esbjornsson-Liljedahl, M., Sundberg, C. J., Norman, B., & Jansson, E. (1999). Metabolic response in type I and type II muscle fibers during a 30-s cycle sprint in men and women. *J Appl Physiol (1985), 87*(4), 1326-1332. doi:10.1152/jappl.1999.87.4.1326

Fahs, C. A., Heffernan, K. S., & Fernhall, B. (2009). Hemodynamic and vascular response to resistance exercise with L-arginine. *Med Sci Sports Exerc, 41*(4), 773-779. doi:10.1249/MSS.0b013e3181909d9d

Ferguson, S. K., Hirai, D. M., Copp, S. W., Holdsworth, C. T., Allen, J. D., Jones, A. M., . . . Poole, D. C. (2013). Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats. *J Physiol, 591*(Pt 2), 547-557. doi:10.1113/jphysiol.2012.243121

Ferguson, S. K., Holdsworth, C. T., Wright, J. L., Fees, A. J., Allen, J. D., Jones, A. M., . . . Poole, D. C. (2014). Microvascular oxygen pressures in muscles comprised of different fiber types: Impact of dietary nitrate supplementation. *Nitric Oxide*. doi:10.1016/j.niox.2014.09.157

Fielding, R. A., Vellas, B., Evans, W. J., Bhasin, S., Morley, J. E., Newman, A. B., . . . Zamboni, M. (2011). Sarcopenia: an undiagnosed condition in older adults. Current consensus definition: prevalence, etiology, and consequences. International working group on sarcopenia. *J Am Med Dir Assoc, 12*(4), 249-256. doi:10.1016/j.jamda.2011.01.003

Flanagan, S. D., Looney, D. P., Miller, M. J., DuPont, W. H., Pryor, L., Creighton, B. C., . . . Kraemer, W. J. (2016). The Effects of Nitrate-Rich Supplementation on Neuromuscular Efficiency during Heavy Resistance Exercise. *J Am Coll Nutr, 35*(2), 100-107. doi:10.1080/07315724.2015.1081572

Fulford, J., Winyard, P. G., Vanhatalo, A., Bailey, S. J., Blackwell, J. R., & Jones, A. M. (2013). Influence of dietary nitrate supplementation on human skeletal muscle metabolism and force production during maximum voluntary contractions. *Pflugers Arch, 465*(4), 517-528. doi:10.1007/s00424-013-1220-5

Garthwaite, J. (2008). Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci, 27*(11), 2783-2802. doi:10.1111/j.1460-9568.2008.06285.x

Gibney, J., Healy, M. L., & Sonksen, P. H. (2007). The growth hormone/insulin-like growth factor-I axis in exercise and sport. *Endocr Rev, 28*(6), 603-624. doi:10.1210/er.2006-0052

Govoni, M., Jansson, E. A., Weitzberg, E., & Lundberg, J. O. (2008). The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide, 19*(4), 333-337. doi:10.1016/j.niox.2008.08.003

Hakkinen, K., Kallinen, M., Izquierdo, M., Jokelainen, K., Lassila, H., Malkia, E., . . . Alen, M. (1998). Changes in agonist-antagonist EMG, muscle CSA, and force during strength training in middle-aged and older people. *J Appl Physiol (1985), 84*(4), 1341-1349. doi:10.1152/jappl.1998.84.4.1341

Hart, J. D., & Dulhunty, A. F. (2000). Nitric oxide activates or inhibits skeletal muscle ryanodine receptors depending on its concentration, membrane potential and ligand binding. *J Membr Biol, 173*(3), 227-236.

Hernandez, A., Schiffer, T. A., Ivarsson, N., Cheng, A. J., Bruton, J. D., Lundberg, J. O., . . . Westerblad, H. (2012). Dietary nitrate increases tetanic [Ca2+]i and contractile force in mouse fast-twitch muscle. *J Physiol, 590*(Pt 15), 3575-3583. doi:10.1113/jphysiol.2012.232777

Hoon, M. W., Hopkins, W. G., Jones, A. M., Martin, D. T., Halson, S. L., West, N. P., . . . Burke, L. M. (2014). Nitrate supplementation and high-intensity performance in competitive cyclists. *Appl Physiol Nutr Metab, 39*(9), 1043-1049. doi:10.1139/apnm-2013-0574

Hoon, M. W., Johnson, N. A., Chapman, P. G., & Burke, L. M. (2013). The effect of nitrate supplementation on exercise performance in healthy individuals: a systematic review and meta-analysis. *Int J Sport Nutr Exerc Metab, 23*(5), 522-532.

Hoon, M. W., Jones, A. M., Johnson, N. A., Blackwell, J. R., Broad, E. M., Lundy, B., . . . Burke, L. M. (2014). The effect of variable doses of inorganic nitrate-rich beetroot juice on simulated 2,000-m rowing performance in trained athletes. *Int J Sports Physiol Perform, 9*(4), 615-620. doi:10.1123/ijspp.2013-0207

Hord, N. G., Tang, Y., & Bryan, N. S. (2009). Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am J Clin Nutr, 90*(1), 1-10. doi:10.3945/ajcn.2008.27131

Huang, A., Xiao, H., Samii, J. M., Vita, J. A., & Keaney, J. F., Jr. (2001). Contrasting effects of thiol-modulating agents on endothelial NO bioactivity. *Am J Physiol Cell Physiol, 281*(2), C719-725.

Jo, E., Fischer, M., Auslander, A. T., Beigarten, A., Daggy, B., Hansen, K., . . . Wes, R. (2017). The effects of multi-day vs. Single pre-exercise nitrate supplement dosing on simulated cycling time trial performance and skeletal muscle oxygenation. *J Strength Cond Res*. doi:10.1519/jsc.0000000000001958

Jones, A. M. (2014). Influence of dietary nitrate on the physiological determinants of exercise performance: a critical review. *Appl Physiol Nutr Metab, 39*(9), 1019-1028. doi:10.1139/apnm-2014-0036

Jones, A. M., & Poole, D. C. (2005). Oxygen uptake dynamics: from muscle to mouth--an introduction to the symposium. *Med Sci Sports Exerc, 37*(9), 1542-1550.

Joshipura, K. J., Hu, F. B., Manson, J. E., Stampfer, M. J., Rimm, E. B., Speizer, F. E., . . . Willett, W. C. (2001). The effect of fruit and vegetable intake on risk for coronary heart disease. *Ann Intern Med, 134*(12), 1106-1114.

Juel, C. (1997). Lactate-proton cotransport in skeletal muscle. *Physiol Rev, 77*(2), 321-358. doi:10.1152/physrev.1997.77.2.321

Kramer, S. J., Baur, D. A., Spicer, M. T., Vukovich, M. D., & Ormsbee, M. J. (2016). The effect of six days of dietary nitrate supplementation on performance in trained CrossFit athletes. *J Int Soc Sports Nutr, 13*, 39. doi:10.1186/s12970-016-0150-y

Krustrup, P., Mohr, M., Amstrup, T., Rysgaard, T., Johansen, J., Steensberg, A., . . . Bangsbo, J. (2003). The yo-yo intermittent recovery test: physiological response, reliability, and validity. *Med Sci Sports Exerc, 35*(4), 697-705. doi:10.1249/01.mss.0000058441.94520.32

Lansley, K. E., Winyard, P. G., Bailey, S. J., Vanhatalo, A., Wilkerson, D. P., Blackwell, J. R., . . . Jones, A. M. (2011). Acute dietary nitrate supplementation improves cycling time trial performance. *Med Sci Sports Exerc, 43*(6), 1125-1131. doi:10.1249/MSS.0b013e31821597b4

Lansley, K. E., Winyard, P. G., Fulford, J., Vanhatalo, A., Bailey, S. J., Blackwell, J. R., . . . Jones, A. M. (2011). Dietary nitrate supplementation reduces the O2 cost of walking and running: a placebo-controlled study. *J Appl Physiol (1985), 110*(3), 591-600. doi:10.1152/japplphysiol.01070.2010

Larsen, F. J., Schiffer, T. A., Borniquel, S., Sahlin, K., Ekblom, B., Lundberg, J. O., & Weitzberg, E. (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab, 13*(2), 149-159. doi:10.1016/j.cmet.2011.01.004

Larsen, F. J., Weitzberg, E., Lundberg, J. O., & Ekblom, B. (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf), 191*(1), 59-66. doi:10.1111/j.1748-1716.2007.01713.x

Larsen, F. J., Weitzberg, E., Lundberg, J. O., & Ekblom, B. (2010). Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Radic Biol Med, 48*(2), 342-347. doi:10.1016/j.freeradbiomed.2009.11.006

Lauer, T., Heiss, C., Balzer, J., Kehmeier, E., Mangold, S., Leyendecker, T., . . . Rassaf, T. (2008). Age-dependent endothelial dysfunction is associated with failure to increase

plasma nitrite in response to exercise. *Basic Res Cardiol, 103*(3), 291-297. doi:10.1007/s00395-008-0714-3

Liu, T. H., Wu, C. L., Chiang, C. W., Lo, Y. W., Tseng, H. F., & Chang, C. K. (2009). No effect of short-term arginine supplementation on nitric oxide production, metabolism and performance in intermittent exercise in athletes. *J Nutr Biochem, 20*(6), 462-468. doi:10.1016/j.jnutbio.2008.05.005

Lowings, S., Shannon, O. M., Deighton, K., Matu, J., & Barlow, M. J. (2017). Effect of Dietary Nitrate Supplementation on Swimming Performance in Trained Swimmers. *Int J Sport Nutr Exerc Metab, 27*(4), 377-384. doi:10.1123/ijsnem.2016-0251

Lundberg, J. O., & Govoni, M. (2004). Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med, 37*(3), 395-400. doi:10.1016/j.freeradbiomed.2004.04.027

Lundberg, J. O., & Weitzberg, E. (2009). NO generation from inorganic nitrate and nitrite: Role in physiology, nutrition and therapeutics. *Arch Pharm Res, 32*(8), 1119-1126. doi:10.1007/s12272-009-1803-z

Lundberg, J. O., & Weitzberg, E. (2010). NO-synthase independent NO generation in mammals. *Biochem Biophys Res Commun, 396*(1), 39-45. doi:10.1016/j.bbrc.2010.02.136

Mancia, G., Ferrari, A., Gregorini, L., Parati, G., Pomidossi, G., Bertinieri, G., . . . Zanchetti, A. J. C. r. (1983). Blood pressure and heart rate variabilities in normotensive and hypertensive human beings. *53*(1), 96-104.

Mancia, G., & Grassi, G. (2000). Mechanisms and clinical implications of blood pressure variability. *J Cardiovasc Pharmacol, 35*(7 Suppl 4), S15-19.

Masschelein, E., Van Thienen, R., Wang, X., Van Schepdael, A., Thomis, M., & Hespel, P. (2012). Dietary nitrate improves muscle but not cerebral oxygenation status during exercise in hypoxia. *J Appl Physiol (1985), 113*(5), 736-745. doi:10.1152/japplphysiol.01253.2011

McConell, G. K. (2007). Effects of L-arginine supplementation on exercise metabolism. *Curr Opin Clin Nutr Metab Care, 10*(1), 46-51. doi:10.1097/MCO.0b013e32801162fa

McConell, G. K., Huynh, N. N., Lee-Young, R. S., Canny, B. J., & Wadley, G. D. (2006). L-Arginine infusion increases glucose clearance during prolonged exercise in humans. *Am J Physiol Endocrinol Metab, 290*(1), E60-E66. doi:10.1152/ajpendo.00263.2005

McKnight, G. M., Smith, L. M., Drummond, R. S., Duncan, C. W., Golden, M., & Benjamin, N. (1997). Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut, 40*(2), 211-214.

Merry, T. L., Lynch, G. S., & McConell, G. K. (2010). Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction. *Am J Physiol Regul Integr Comp Physiol, 299*(6), R1656-1665. doi:10.1152/ajpregu.00433.2010

Modin, A., Bjorne, H., Herulf, M., Alving, K., Weitzberg, E., & Lundberg, J. O. (2001). Nitrite-derived nitric oxide: a possible mediator of 'acidic-metabolic' vasodilation. *Acta Physiol Scand, 171*(1), 9-16. doi:10.1046/j.1365-201X.2001.00771.x

Moncada, S., & Higgs, E. A. (2006). Nitric oxide and the vascular endothelium. *Handb Exp Pharmacol*(176 Pt 1), 213-254.

Mori, M., & Gotoh, T. (2000). Regulation of nitric oxide production by arginine metabolic enzymes. *Biochem Biophys Res Commun, 275*(3), 715-719. doi:10.1006/bbrc.2000.3169

Murphy, M., Eliot, K., Heuertz, R. M., & Weiss, E. (2012). Whole beetroot consumption acutely improves running performance. *J Acad Nutr Diet, 112*(4), 548-552. doi:10.1016/j.jand.2011.12.002

Nordander, C., Willner, J., Hansson, G. A., Larsson, B., Unge, J., Granquist, L., & Skerfving, S. (2003). Influence of the subcutaneous fat layer, as measured by ultrasound, skinfold calipers and BMI, on the EMG amplitude. *Eur J Appl Physiol, 89*(6), 514-519. doi:10.1007/s00421-003-0819-1

Olek, R. A., Ziemann, E., Grzywacz, T., Kujach, S., Luszczyk, M., Antosiewicz, J., & Laskowski, R. (2010). A single oral intake of arginine does not affect performance during repeated Wingate anaerobic test. *J Sports Med Phys Fitness, 50*(1), 52-56.

Oskarsson, J., & McGawley, K. (2018). No individual or combined effects of caffeine and beetroot-juice supplementation during submaximal or maximal running. *Appl Physiol Nutr Metab, 43*(7), 697-703. doi:10.1139/apnm-2017-0547

Paddon-Jones, D., Borsheim, E., & Wolfe, R. R. (2004). Potential ergogenic effects of arginine and creatine supplementation. *J Nutr, 134*(10 Suppl), 2888S-2894S; discussion 2895S.

Papadopoulos, S., Dipla, K., Triantafyllou, A., Nikolaidis, M. G., Kyparos, A., Touplikioti, P., . . . Zafeiridis, A. (2018). Beetroot Increases Muscle Performance and Oxygenation During Sustained Isometric Exercise, but Does Not Alter Muscle Oxidative Efficiency and Microvascular Reactivity at Rest. *J Am Coll Nutr, 37*(5), 361-372. doi:10.1080/07315724.2017.1401497

Peacock, O., Tjonna, A. E., James, P., Wisloff, U., Welde, B., Bohlke, N., . . . Sandbakk, O. (2012). Dietary nitrate does not enhance running performance in elite cross-country skiers. *Med Sci Sports Exerc, 44*(11), 2213-2219. doi:10.1249/MSS.0b013e3182640f48

Percival, J. M., Anderson, K. N., Huang, P., Adams, M. E., & Froehner, S. C. (2010). Golgi and sarcolemmal neuronal NOS differentially regulate contraction-induced fatigue and vasoconstriction in exercising mouse skeletal muscle. *J Clin Invest, 120*(3), 816-826. doi:10.1172/jci40736

Pinna, M., Roberto, S., Milia, R., Marongiu, E., Olla, S., Loi, A., . . . Crisafulli, A. (2014). Effect of beetroot juice supplementation on aerobic response during swimming. *Nutrients, 6*(2), 605-615. doi:10.3390/nu6020605

Poole, D. C., Ward, S. A., Gardner, G. W., & Whipp, B. J. (1988). Metabolic and respiratory profile of the upper limit for prolonged exercise in man. *Ergonomics, 31*(9), 1265-1279. doi:10.1080/00140138808966766

Rolfe, D. F., Hulbert, A. J., & Brand, M. D. (1994). Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen-consuming tissues of the rat. *Biochim Biophys Acta, 1188*(3), 405-416.

Schaefer, A., Piquard, F., Geny, B., Doutreleau, S., Lampert, E., Mettauer, B., & Lonsdorfer, J. (2002). L-arginine reduces exercise-induced increase in plasma lactate and ammonia. *Int J Sports Med, 23*(6), 403-407. doi:10.1055/s-2002-33743

Shannon, O. M., Barlow, M. J., Duckworth, L., Williams, E., Wort, G., Woods, D., . . . O'Hara, J. P. (2017). Dietary nitrate supplementation enhances short but not longer duration running time-trial performance. *Eur J Appl Physiol, 117*(4), 775-785. doi:10.1007/s00421-017-3580-6

Sonksen, P. H. (2001). Insulin, growth hormone and sport. *J Endocrinol, 170*(1), 13-25.

Staessen, J. A., Wang, J. G., & Thijs, L. (2001). Cardiovascular protection and blood pressure reduction: a meta-analysis. *Lancet, 358*(9290), 1305-1315. doi:10.1016/S0140-6736(01)06411-X

Stamler, J. S., & Meissner, G. (2001). Physiology of nitric oxide in skeletal muscle. *Physiol Rev, 81*(1), 209-237.

Stegeman, D. F., Blok, J. H., Hermens, H. J., & Roeleveld, K. (2000). Surface EMG models: properties and applications. *J Electromyogr Kinesiol, 10*(5), 313-326.

Stuehr, D. J., Santolini, J., Wang, Z. Q., Wei, C. C., & Adak, S. (2004). Update on mechanism and catalytic regulation in the NO synthases. *J Biol Chem, 279*(35), 36167-36170. doi:10.1074/jbc.R400017200

Sunderland, K. L., Greer, F., & Morales, J. (2011). VO2max and ventilatory threshold of trained cyclists are not affected by 28-day L-arginine supplementation. *J Strength Cond Res, 25*(3), 833-837. doi:10.1519/JSC.0b013e3181c6a14d

Sureda, A., Batle, J. M., Tauler, P., Ferrer, M. D., Tur, J. A., & Pons, A. (2006). Vitamin C supplementation influences the antioxidant response and nitric oxide handling of erythrocytes and lymphocytes to diving apnea. *Eur J Clin Nutr, 60*(7), 838-846. doi:10.1038/sj.ejcn.1602388

Takaki, M., Kohzuki, H., Sakata, S., Ohga, Y., Shimizu, S., Ishidate, H., . . . Suga, H. (1998). Oxygen consumption and motility of mechanically unloaded myocardial slices. *Adv Exp Med Biol, 453*, 499-506.

Thompson, C., Vanhatalo, A., Kadach, S., Wylie, L. J., Fulford, J., Ferguson, S. K., . . . Jones, A. M. (2018). Discrete physiological effects of beetroot juice and potassium nitrate

supplementation following 4-wk sprint interval training. *J Appl Physiol (1985), 124*(6), 1519-1528. doi:10.1152/japplphysiol.00047.2018

Thompson, C., Wylie, L. J., Blackwell, J. R., Fulford, J., Black, M. I., Kelly, J., . . . Jones, A. M. (2017). Influence of dietary nitrate supplementation on physiological and muscle metabolic adaptations to sprint interval training. *J Appl Physiol (1985), 122*(3), 642-652. doi:10.1152/japplphysiol.00909.2016

Thompson, C., Wylie, L. J., Fulford, J., Kelly, J., Black, M. I., McDonagh, S. T., . . . Jones, A. M. (2015). Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *Eur J Appl Physiol*. doi:10.1007/s00421-015-3166-0

Tillin, N. A., Moudy, S., Nourse, K. M., & Tyler, C. J. (2018). Nitrate Supplement Benefits Contractile Forces in Fatigued but Not Unfatigued Muscle. *Med Sci Sports Exerc, 50*(10), 2122-2131. doi:10.1249/mss.0000000000001655

Torfgard, K. E., & Ahlner, J. (1994). Mechanisms of action of nitrates. *Cardiovasc Drugs Ther, 8*(5), 701-717.

Van De Walle, G. P., & Vukovich, M. D. (2018). The Effect of Nitrate Supplementation on Exercise Tolerance and Performance: A Systematic Review and Meta-Analysis. *J Strength Cond Res, 32*(6), 1796-1808. doi:10.1519/jsc.0000000000002046

Vandewalle, H., Peres, G., & Monod, H. (1987). Standard anaerobic exercise tests. *Sports Med, 4*(4), 268-289.

Vanhatalo, A., Bailey, S. J., Blackwell, J. R., DiMenna, F. J., Pavey, T. G., Wilkerson, D. P., . . . Jones, A. M. (2010). Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental

exercise. *Am J Physiol Regul Integr Comp Physiol, 299*(4), R1121-1131. doi:10.1152/ajpregu.00206.2010

Vanhatalo, A., Fulford, J., Bailey, S. J., Blackwell, J. R., Winyard, P. G., & Jones, A. M. (2011). Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia. *J Physiol, 589*(Pt 22), 5517-5528. doi:10.1113/jphysiol.2011.216341

Walsh, B., Howlett, R. A., Stary, C. M., Kindig, C. A., & Hogan, M. C. (2006). Measurement of activation energy and oxidative phosphorylation onset kinetics in isolated muscle fibers in the absence of cross-bridge cycling. *Am J Physiol Regul Integr Comp Physiol, 290*(6), R1707-1713. doi:10.1152/ajpregu.00687.2005

Warburton, D. E., Gledhill, N., Jamnik, V. K., Bredin, S. S., McKenzie, D. C., Stone, J., . . . Shephard, R. J. (2011). Evidence-based risk assessment and recommendations for physical activity clearance: Consensus Document 2011. *Appl Physiol Nutr Metab, 36 Suppl 1*, S266-298. doi:10.1139/h11-062

Westerblad, H., Allen, D. G., Bruton, J. D., Andrade, F. H., & Lannergren, J. (1998). Mechanisms underlying the reduction of isometric force in skeletal muscle fatigue. *Acta Physiol Scand, 162*(3), 253-260. doi:10.1046/j.1365-201X.1998.0301f.x

Whitfield, J., Gamu, D., Heigenhauser, G. J. F., LJC, V. A. N. L., Spriet, L. L., Tupling, A. R., & Holloway, G. P. (2017). Beetroot Juice Increases Human Muscle Force without Changing Ca2+-Handling Proteins. *Med Sci Sports Exerc, 49*(10), 2016-2024. doi:10.1249/mss.0000000000001321

WHO. (2000). *Obesity: preventing and managing the global epidemic. Report of a WHO consultation*. (0512-3054 (Print)

0512-3054).

Wilkerson, D. P., Hayward, G. M., Bailey, S. J., Vanhatalo, A., Blackwell, J. R., & Jones, A. M. (2012). Influence of acute dietary nitrate supplementation on 50 mile time trial performance in well-trained cyclists. *Eur J Appl Physiol, 112*(12), 4127-4134. doi:10.1007/s00421-012-2397-6

Willoughby, D. S., Boucher, T., Reid, J., Skelton, G., & Clark, M. (2011). Effects of 7 days of arginine-alpha-ketoglutarate supplementation on blood flow, plasma L-arginine, nitric oxide metabolites, and asymmetric dimethyl arginine after resistance exercise. *Int J Sport Nutr Exerc Metab, 21*(4), 291-299.

Wylie, L. J., Bailey, S. J., Kelly, J., Blackwell, J. R., Vanhatalo, A., & Jones, A. M. (2016). Influence of beetroot juice supplementation on intermittent exercise performance. *Eur J Appl Physiol, 116*(2), 415-425. doi:10.1007/s00421-015-3296-4

Wylie, L. J., Kelly, J., Bailey, S. J., Blackwell, J. R., Skiba, P. F., Winyard, P. G., . . . Jones, A. M. (2013). Beetroot juice and exercise: pharmacodynamic and dose-response relationships. *J Appl Physiol (1985), 115*(3), 325-336. doi:10.1152/japplphysiol.00372.2013

Wylie, L. J., Mohr, M., Krustrup, P., Jackman, S. R., Ermiotadis, G., Kelly, J., . . . Jones, A. M. (2013). Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *Eur J Appl Physiol, 113*(7), 1673-1684. doi:10.1007/s00421-013-2589-8

APPENDICES

Appendix A. Mean dietary intake of participants for energy (kcal), protein (g), carbohydrate (g) and fat (g) from 3-day food records by treatment.

	BRH			
Participant ID	Energy Intake (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
BR001	3135	210	342	103
BR002	3018	197	355	90
BR003	2588	148	301	88
BR004	2672	188	290	80
BR005	2493	172	278	77
BR006	2208	155	244	68
BR007	2137	141	238	69
BR008	2390	138	275	82
BR009	2374	152	248	86
BR010	0	0	0	0
BR011	3331	214	369	111
BR012	2074	107	245	74
BR013	1904	102	221	68
	BRL			
Participant ID	Energy Intake (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
BR001	3106	192	364	98
BR002	2992	214	327	92
BR003	2425	125	299	81
BR004	2608	175	288	84
BR005	2405	161	258	81
BR006	2239	147	253	71
BR007	2311	132	277	75
BR008	2177	127	244	77
BR009	2511	144	279	91
BR010	0	0	0	0
BR011	3416	224	387	108
BR012	2056	114	238	72
BR013	1962	106	236	66
	PLA			
Participant ID	Energy Intake (kcal)	Protein (g)	Carbohydrate (g)	Fat (g)
BR001	3083	220	310	107
BR002	3058	221	341	90
BR003	2626	144	310	90
BR004	2499	175	281	75
BR005	2528	166	277	84
BR006	2217	161	220	77
BR007	2144	121	262	68
BR008	2324	141	251	84
BR009	2385	150	255	85
BR010	0	0	0	0
BR011	3383	208	397	107
BR012	2137	121	240	77
BR013	1908	112	212	68

Appendix B. Electromyography output from maximum voluntary contractions in Volts.

	BRH								
Participant ID	**MVC1**	**MVC2**	**MVC3**	**MVC4**	**MVC5**	**MVC6**	**MVC7**	**MVC8**	**MVC9**
BR001	0.004974	0.005538	0.004429	0.005530	0.004824	0.004881	0.005837	0.003979	0.004342
BR002	0.005668	0.007956	0.007245	0.007006	0.007090	0.006882	0.007256	0.006874	0.006800
BR003	0.003315	0.004588	0.003855	0.004584	0.004511	0.004345	0.004488	0.004021	0.004352
BR004	0.003241	0.003088	0.004586	0.004062	0.003801	0.004822	0.004561	0.004173	0.005579
BR005	0.004479	0.003770	0.003533	0.002784	0.003986	0.003564	0.003603	0.003157	0.003241
BR006	0.002673	0.002852	0.003636	0.003714	0.002746	0.002490	0.002787	0.002762	0.002187
BR007	0.007483	0.006754	0.006890	0.007405	0.010168	0.008204	0.007073	0.007695	0.008280
BR008	0.002901	0.002262	0.002695	0.000699	0.001941	0.001869	0.001619	0.001644	0.001631
BR009	0.001829	0.001991	0.001904	0.007537	0.001704	0.001723	0.002196	0.002039	0.001699
BR010	0.008681	0.008854	0.006514	0.006619	0.005365	0.005900	0.006595	0.005649	0.004797
BR011	0.001110	0.001574	0.000964	0.000961	0.000682	0.000877	0.000944	0.000814	0.001158
BR012	0.003074	0.002957	0.002511	0.001606	0.001600	0.001664	0.001474	0.001483	0.001136
BR013	0.014359	0.006610	0.006421	0.006123	0.006012	0.006283	0.006265	0.006123	0.005552
	BRL								
Participant ID	**MVC1**	**MVC2**	**MVC3**	**MVC4**	**MVC5**	**MVC6**	**MVC7**	**MVC8**	**MVC9**
BR001	0.008023	0.006413	0.007464	0.004817	0.006150	0.005889	0.005068	0.005315	0.005959
BR002	0.004774	0.006887	0.006986	0.006015	0.005877	0.006006	0.006300	0.005478	0.005239
BR003	0.003408	0.004531	0.003491	0.004519	0.004311	0.004430	0.004268	0.003962	0.004180
BR004	0.005924	0.004858	0.003721	0.004211	0.004230	0.004654	0.005919	0.005172	0.004586
BR005	0.004839	0.004435	0.004922	0.004418	0.003991	0.005154	0.005108	0.005187	0.005115
BR006	0.002559	0.002941	0.004156	0.003548	0.003013	0.002746	0.003006	0.002558	0.002448
BR007	0.008783	0.008161	0.008717	0.009676	0.006655	0.005791	0.007491	0.007987	0.006671
BR008	0.004004	0.002412	0.002646	0.002890	0.004291	0.002736	0.003319	0.002487	0.002274
BR009	0.002412	0.001991	0.002227	0.002667	0.002671	0.002447	0.002294	0.002764	0.002585
BR010	0.004571	0.005710	0.005655	0.003991	0.004685	0.004973	0.005107	0.005007	0.005260
BR011	0.001379	0.001636	0.001774	0.000902	0.000535	0.000673	0.000610	0.000577	0.000444
BR012	0.002389	0.002310	0.002965	0.002162	0.001757	0.002069	0.001559	0.001417	0.001379
BR013	0.006366	0.004861	0.004719	0.007281	0.004697	0.005002	0.005005	0.004837	0.004508
	PLA								
Participant ID	**MVC1**	**MVC2**	**MVC3**	**MVC4**	**MVC5**	**MVC6**	**MVC7**	**MVC8**	**MVC9**
BR001	0.005407	0.006828	0.005847	0.004978	0.004982	0.004238	0.004632	0.003694	0.005105
BR002	0.005562	0.008022	0.006493	0.007093	0.005757	0.006307	0.005703	0.005863	0.004473
BR003	0.002916	0.002702	0.002843	0.003163	0.002634	0.002424	0.002650	0.004780	0.003130
BR004	0.003229	0.003179	0.003101	0.002002	0.001790	0.001830	0.002700	0.002172	0.001571
BR005	0.006089	0.004210	0.003590	0.004601	0.003829	0.003918	0.004344	0.003058	0.004252
BR006	0.002874	0.002333	0.004515	0.002592	0.002528	0.002575	0.002193	0.002433	0.002558
BR007	0.015338	0.007648	0.011433	0.007555	0.007720	0.006615	0.006382	0.007716	0.008208
BR008	0.003697	0.002156	0.002918	0.002255	0.003725	0.003035	0.003373	0.002636	0.002554
BR009	0.002823	0.003673	0.004774	0.003640	0.004441	0.002990	0.004245	0.003314	0.003705
BR010	0.005294	0.003673	0.003297	0.003384	0.003306	0.003952	0.004154	0.004435	0.003907
BR011	0.002159	0.001416	0.001782	0.001579	0.001363	0.001457	0.001561	0.001165	0.000901
BR012	0.002487	0.002637	0.002225	0.001416	0.001422	0.001306	0.001638	0.001213	0.001626
BR013	0.012197	0.007402	0.013034	0.010873	0.009029	0.008956	0.008186	0.015611	0.009448

Appendix C. Formula and calculation for post-hoc power analysis and sample size determination.

$$n = \frac{2(Z_{\alpha} + Z_{1-\beta})^{2}\sigma^{2}}{\Delta^{2}}$$

$$n = \frac{2(1.96 + 0.8416)^{2}\,(0.129)^{2}}{(0.091)^{2}}$$

$$n = 31.5$$

Appendix D. Consent form used to obtain consent from participants.

UNIVERSITY OF MANITOBA

Human Nutritional Sciences

Semone Myrie, RD, PhD
565W Duff Roblin Building
Winnipeg, Manitoba
Canada R3T 2N2

Telephone (204) 474-7290
Fax (204) 474-7592
myrie@cc.umanitoba.ca

Consent Form

Title: Evaluation of Various Doses of Beetroot Juice on Anaerobic Performance

Investigators: Semone Myrie, RD, PhD and Mark Pinder, MSc Candidate (997-4402 umpinder@myumanitoba.ca)

This consent form, a copy of which will be left with you for your records and reference, is only part of the process of informed consent. It should give you the basic idea of what the research is about and what your participation will involve. If you would like more detail about something mentioned here, or information not included here, you should feel free to ask. Please take the time to read this carefully and to understand any accompanying information.

This study is being conducted by Mark Pinder, a Masters student in the Department of Human Nutritional Sciences as part of his thesis, under the supervision of Dr. Semone Myrie, RD, PhD (474-7290).

Purpose of the Study

The purpose of this study is to examine the effect of varying nitrate doses of beetroot juice supplementation on dynamic, multi-joint resistance exercise performance in active, trained individuals. Performance will be assessed based on parameters including neuromuscular efficiency, anaerobic performance capability, oxygen consumption, plasma nitrate/nitrite levels, specific muscle tissue biomarkers including lactate, and anthropometric measurements of select muscle groups.

Study Procedures

Prior to beginning the study, you will be required to complete a screening questionnaire to let us know if you are in good health and fit to participate in the study. The screening questionnaire will ask questions about your general health, medical history, use of supplements and current level of physical activity, to determine whether you are eligible to participate.
Any change in your health status at any point during the study needs to be reported to the study investigators.

During the course of the study you will be asked to refrain from chewing gum or using anti-bacterial mouthwash as these are known to reduce to effectiveness of the supplements. Should you choose to participate, you will be asked to complete 4 testing sessions throughout the study over the course of approximately 5 weeks. Each session will require approximately 1 hour of your time.

The study will consist of 4 testing sessions: pre-trial familiarization session (no supplement), and on day 7 of each of the 3 treatment periods. Following the non-supplemented familiarization session, you will receive one of either 500 ml of beetroot juice supplement with moderate dose of active ingredient (i.e., nitrate), or 500 ml of beetroot juice with higher dose of active ingredient, or a placebo to consume daily for the next 7 days. The treatments will be provided in sealed, opaque containers along with storage instructions. On days 1 through 6 you will consume the 500 ml treatments in 3 equal doses throughout the day. On the day of your supplemented performance tests (i.e. the 7^{th} day in each treatment period) you will consume the beverage in its entirety about 2.5 hours prior to the test. The study coordinator will review this in detail with you. You will be instructed to maintain your normal dietary and training habits over the course of the study with the exception of certain foods rich in the active ingredient of interest (nitrate) from which you will be asked to abstain from consuming for the 48 hours period prior to each performance test. We will ask you to wear a heart rate monitor during your training sessions. This is optional; you do not have to wear the monitor.
This study is a crossover design, which means you are randomly assigned one of the treatment dosage levels or the placebo upon your enrollment in the study. You will receive both treatments and the placebo in random order and you will not be aware of this order until the study is complete. You do not get to choose which group you are in; the treatments are randomly assigned by the principal investigator using a randomization program.
Throughout the study treatment periods (pre-trial, phase1, phase2, and phase 3) you will be asked to provide a urine sample on the morning of each testing day to measure hydration status by means of a urine specific gravity test. We will also store the urine sample for analysis of nitrate/nitrite and other metabolites related to nitrate metabolism. The urine sample (approximately 25 ml) will be collected from your first urination upon waking in a sealed container that will be provided to you.
You will be asked to document your food and fluid, and physical activity throughout the study treatment periods (pre-trial, phase1, phase 2, and phase 3).

Testing sessions will be performed as dynamic box squats on a standard Smith machine conducted at the Active Living Centre, Fort Garry Campus, University of Manitoba. The exercise protocol will use a pyramid sequence with an initial load of 60% 1RM and increasing by 10% per set to 90%, before descending back to 60% in 10% increments. 1RM and maximum repetitions at each %RM load will be determined during familiarization visit. During the final 60% load set, participants will perform repetitions to failure.

Wireless EMG sensors will be placed on the quadriceps muscle to collect signals from the vastus lateralis to record both maximum EMG amplitude, representing maximal motor unit firing, and peak EMG amplitude during maximum voluntary contractions (MVC). Following electrode placement, participants will perform 3 3-second isometric box squats MVCs prior to the dynamic exercise testing as well as between each set of dynamic exercise.

Prior to consuming any treatment on day 1 of each phase and 5 minutes following the familiarization (pre-trial) test and all subsequent performance tests, you will be asked to provide a blood sample. Approximately 25 ml will be taken on each blood draw. Blood will be used to measure nitrate/nitrite levels and other biochemical markers including lactate.

Each blood test will take approximately 5 minutes. The total amount of blood drawn throughout the study will be approximately 175 ml.

Before the performance tests in all phases, we would like to measure your height, weight and resting blood pressure. We would also like to perform a bioelectrical impedance analysis (BIA) to measure your fat free mass, fat mass and total body water. A BIA test consists of attaching a small surface electrode to the hand and foot on one side of the body and passing a small current between them. You will be required to remove all metal from your person prior to this test. The resistance to the current provides the information to calculate the body composition measurements.

Responsibilities of Volunteers

Volunteers will be asked to follow the study procedures including abstain from chewing gum or using anti-bacterial mouthwash throughout the course of the study as these are known to reduce the effectiveness of the supplements being tested. A list of nitrate-rich foods to abstain from for 48 hours prior to performance testing will be provided to all participants.

Blood Tests, BIA, and Performance Testing

Subjects should arrive 10 minutes prior to their scheduled assessment time. Prior to testing the subject should follow the pre-assessment instructions in order to maximize the accuracy of the testing procedures.

- Have filled out all necessary forms (Informed Consent/ Medical Background Questionnaire, Food Record)
- Have NOT trained for a minimum of 24 hours prior to assessment
- Have NOT performed any lower body specific training for 72 hours prior to the assessment
- Remove all metallic objects including zippers (as metal may affect bone density values, thus, affecting body composition values)
- Have consumed the designated supplement treatment as instructed 2.5 hours prior to the performance test.
- Be reasonably hydrated
- Wear comfortable clothing suitable for the box squat test to be performed

Risks and Discomforts

The beetroot supplements and the active ingredients contained within them are all natural and have been shown to be safe for human consumption in numerous clinical trials. A known side-effect is a pink discoloration of urine and feces caused by the pigments found in the beetroot. This is normal and poses no health risk. Beet juice is a known laxative and diuretic, and can sometimes lead to intestinal upset. Beet juice in sufficient quantities has also been shown to lower blood pressure, and as a consequence, may lead to weakness and dizziness.

The performance tests are strenuous but are considered safe for healthy individuals. The risks associated with the dynamic exercise performance test are fatigue, shortness of breath and minor muscle and joint pain. Although designed to be strenuous, the test is safe for individuals in good health. If you should feel fatigue or dizziness at any time you can request to stop the test.
Blood sampling may have some rare risks, like placing a needle into a vein which may contribute to infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. As part of the process for blood sampling, to help minimize risks for infection the phlebotomist will start by performing his/her hand hygiene (wash with soap and water) and wearing clean gloves before collecting blood from any participant. Next, the phlebotomist will disinfect the site on the participant that will be used for blood collection using 70% isopropyl alcohol swab. After blood is drawn and the needle removed from the vein, and upon completion of the finger prick test, the phlebotomist will apply gentle pressure to the puncture site using clean gauge or cotton ball to stop bleeding, and a bandage is applied. Infection risk is also minimized by the use of prepackaged sterilized equipment, and all needles are disposed after a single use.
There are no known risks associated with the blood pressure measurement, the BIA assessment or the heart rate monitor.

Benefits

You may not directly benefit from participation in this research; however, this study should contribute to a better understanding of the effects of nitrate rich beetroot supplements and the optimal dose for enhancing athletic performance. You will also receive free supplements, free performance testing, free body composition testing, and access to your test results when they become available. You may receive your test results at your preference in either electronic format or as a hard copy available from the study coordinator.

Confidentiality

Medical / research records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. All records will be kept in a locked secure area and only those persons identified as requiring access to your records will have opportunity to review or copy your medical / research records. Information gathered in this research study may be published or presented in public forums; however your name and other identifying information will not be used or revealed. Personal information such as your name, address, telephone number and/or any other identifying information will be protected. If the results of the study are published, your identity will remain confidential. No information revealing any personal information such as your name, address or telephone number will be made publicly available. All questionnaires will be kept in a locked cabinet. Study biological samples will be stored in freezers at the Richardson Center for Functional Foods and Nutraceutical (RCFFN), University of Manitoba or Duff Roblin Building, University of Manitoba. Only the study coordinators and the principal investigator will have access to the samples and questionnaires. Your samples will not be used for any additional analyses, nor stored for beyond 01/20, nor shared with any other group, other than is indicated in the protocol, without your specific consent.
Personal information such as your name, address, telephone number and/or any other identifying information will be protected. No information revealing any personal information such as your

name, address, telephone number will be made publicly available. Your participation in the study is confidential.

Remuneration and Feedback

No remuneration will be provided for participation in this study. Feedback about the research results will be provided to participants as an electronic document or a paper copy through regular mail.

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Personal data of participants will be deleted within 3 months of the end of the study. The anonymous data will be kept longer, but not beyond 01/20. Access to information on the questionnaire and blood samples will be limited strictly to the researchers named above. All data will be shredded/destroyed after the time has expired.

Voluntary Participation/Withdrawal from the Study

Your signature on this form indicates that you have understood to your satisfaction the information regarding participation in the research project and agree to serve as a participant. In no way does this waive your legal rights nor release the researchers, sponsors, or involved institutions from their legal and professional responsibilities. You may refuse to participate or you may withdraw from the study at any time. Your decision to take part in this study is voluntary.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff. If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions. We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

The University of Manitoba may look at the research records to see that the research is being done in a safe and proper way.

Feel free to ask for clarification or new information throughout your participation by contacting the principal researcher: Semone Myrie, RD, PhD, myrie@cc.umanitoba.ca or 204-474-7290.

This research has been approved by the Joint-Faculty Research Ethics Board at the University of Manitoba. If you have any concerns or complaints about this project you may contact any of the above-named persons or the Human Ethics Coordinator 474-7122, or e-mail humanethics@umanitoba.ca. A copy of this consent form has been given to you to keep for your records and reference.

Dissemination

The results of the study will be written up in Mark Pinder's master's thesis and may be published in recognized scientific journals and presented to public groups such as at scientific meetings and seminars. Additionally, study participants will receive their individual results along with the mean value obtained from the whole study population and a summary of findings. However, participants will not be able to have access to the individual results of other study participants. Please indicate below how you would like to receive your results and a summary of the study findings:

Email:

Ground mail (provide mailing address): _____

Medical Care for Injury Related to the Study

In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities.

I am aware that there are risks associated with placing a needle into a vein for blood sampling, including risk of bleeding, pain or bruising at the site, and possible infection.

Yes ☐ No ☐

_____

Participant's Signature Date

_____

Researcher and/or Delegate's Signature Date