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Inorganic Nitrate Supplementation and Endurance Exercise Performance

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A thesis submitted for admission to the degree of
Doctor of Philosophy

Discipline of Exercise and Sport Science
Faculty of Health Sciences
The University of Sydney
February 2015
STUDENT DECLARATION

I hereby declare that this thesis is my own work and does not, to the best of my knowledge, contain material from any other source unless due acknowledgement is made. The thesis was completed under the guidelines set out by The University of Sydney’s Faculty of Health Sciences, for the degree of Doctorate of Philosophy and has not been submitted for a degree or diploma at any other academic institution.

Candidate: 

Date: 02.10.2014
ABSTRACT

**Background:** Supplementation with inorganic nitrate (NO$\text{}_3$$^-$) has recently been shown to improve exercise capacity in healthy adults. However it is unclear if these research findings extrapolate to exercise performance in well-trained endurance athletes. Given the physiological effects it is purported to elicit, NO$\text{}_3$$^-$ may be a useful ergogenic aid particularly in high-intensity sporting events, however its efficacy is yet to be established.

**Aims:** The aims of this thesis were to: (1) Undertake a systematic review of the available literature concerning inorganic NO$\text{}_3$$^-$ supplementation in exercise and quantify its effect on exercise performance in healthy adults by meta-analysis; (2) Determine if acute supplementation with NO$\text{}_3$$^-$ improves simulated track cycling performance in well-trained cyclists; (3) Examine the effect of supplementation on plasma [NO$\text{}_3$$^-$] and [nitrite (NO$\text{}_2$$^-$)], respectively, and their association with changes in performance; (4) Investigate the effect of altering the dose and timing of NO$\text{}_3$$^-$ ingestion on exercise performance and plasma [NO$\text{}_3$$^-$] and [NO$\text{}_2$$^-$]; (5) Examine the effect of variable acute NO$\text{}_3$$^-$ doses on simulated competition performance in well-trained rowers; (6) Investigate the effect of NO$\text{}_3$$^-$ supplementation on muscular function and the role of vascular and peripheral adaptations in performance improvement.

**Methods and Results:** Currently available literature (up to and including August 2012) was systematically reviewed to assess the effect of NO$\text{}_3$$^-$ supplementation on exercise in healthy populations. Seventeen studies were found that directly compared exercise outcomes following inorganic NO$\text{}_3$$^-$ supplementation with a control/placebo trial. Meta-analysis of the data found a significant, moderate benefit (ES: effect size, CI: confidence intervals; ES = 0.79, 95% CI: 0.23–1.35) of supplementation on time to exhaustion tests ($p = 0.006$). There was a small but insignificant beneficial effect on performance for time trials (ES = 0.11, 95% CI: −0.16–0.37) and graded exercise tests (ES = 0.26, 95% CI: −0.10–0.62). The review of available literature also identified a lack of consistency between studies with the dosing protocol (i.e. timing and amount of NO$\text{}_3$$^-$) as well as the source of NO$\text{}_3$$^-$). Additionally, very few studies had investigated the effect of NO$\text{}_3$$^-$ supplementation on endurance performance in well-trained individuals.

In the second study of the thesis, the effect of nitrate-rich beetroot juice (BRJ) supplementation on the performance of two bouts of maximal 4 min cycling was assessed.
In a Latin-square crossover design, 26 well trained cyclists completed four separate supplementation trials in which they consumed: CONTROL: BRJ providing <0.1 mmol NO₃⁻; 150-PRE: BRJ containing 4.1 mmol NO₃⁻, 150 min prior to the first bout of cycling; 75-PRE: BRJ containing 4.1 mmol NO₃⁻, 75 min prior to the first bout of cycling; TOPUP: BRJ containing 4.1 mmol NO₃⁻, 150 min prior to the first bout of cycling plus an addition half dose (2 mmol NO₃⁻) 75 min prior to the second bout. Performance was assessed by two 4 min time trials, which were separated by 75 min, with the goal to achieve the highest mean power possible in each time trial. Blood samples were collected at four separate times and analyzed for plasma [NO₂⁻]. Relative to placebo, the mean effect of all three NO₃⁻ treatments (i.e. 150-PRE, 75-PRE, TOPUP) was an unclear, small improvement in the first time trial (+1.3% to mean power, 90% confidence limits ±1.7%) and a possible, slight reduction in performance in the second time trial (-0.3%, ±1.6%). Differences between NO₃⁻ treatments were unclear. Peak plasma [NO₂⁻] was highest when NO₃⁻ was consumed 75 min before the first time trial. The unclear effect of supplementation on performance was likely due to the underestimation of the NO₃⁻ amount required to elicit ergogenic effects in trained individuals, whose physiological adaptations to exercise training may reduce the effectiveness of NO₃⁻.

In the third study, the effect of multiple doses of NO₃⁻ (using BRJ) on 2,000 m rowing performance was examined. Ten well-trained male rowers completed 3 supplementation trials in a randomized crossover fashion. In each trial they consumed: PLACEBO: 140 ml BRJ containing <0.1 mmol NO₃⁻; SINGLE: 140 ml BRJ containing 4.2 mmol NO₃⁻; DOUBLE: 140 ml BRJ containing 8.4 mmol NO₃⁻. All BRJ was consumed 120min before the 2,000m time trial. Blood samples were collected before ingestion of BRJ and immediately before commencement of the time trial for plasma [NO₂⁻] and [NO₃⁻] analysis. A possibly beneficial effect was found for DOUBLE compared with SINGLE (mean difference: -1.8 ± 2.1 s; 0.5 ± 1.0% improvement) and PLACEBO (-1.6 ± 1.6 s; 0.4 ± 0.8%) respectively, however there was only a trivial effect for SINGLE versus PLACEBO. Plasma [NO₂⁻] and [NO₃⁻] demonstrated a dose-response effect, with greater amounts of ingested NO₃⁻ leading to substantially higher concentrations (DOUBLE > SINGLE > PLACEBO). There was a moderate but insignificant correlation (r=-0.593, p=0.055) between change in plasma [NO₂⁻] and performance time. The results of this study demonstrated that for well-trained individuals to gain performance benefits, they require a
greater dose of NO$_3^-$ than what has previously been shown to be successful in lesser trained counterparts.

Finally, the effect of NO$_3^-$ supplementation on muscle contractile function was examined in a group of 18 healthy adults. In a randomized cross-over design, each participant undertook four days of supplementation with either a) nitrate-rich beetroot juice (NITRATE) or b) nitrate-depleted placebo (PLACEBO) beverage. Two hours following their last beverage on the fourth day, unilateral isometric knee extension force was measured over a series of voluntary and electrically evoked (via transcutaneous electrical nerve stimulation) tests. Maximal voluntary contraction, single twitch characteristics (half-peak time, peak tension, half-relaxation time) and the force vs. stimulation frequency relationship were not different in NITRATE compared with PLACEBO. No difference in contractile force was observed between the conditions during a fatigue test that consisted of 64 cycles of 0.8 s tetanus/ 0.8 s relaxation. However when the test was repeated with restricted blood flow to the leg, NITRATE significantly attenuated the decline in force, improving fatigue during the later stages of the test compared with PLACEBO. These results suggest that several days of NO$_3^-$ supplementation elicits peripheral responses in the musculature that attenuate muscular fatigue during hypovolemic exercise. This ergogenic action is likely attributable to improved Ca$^{2+}$ handling in the muscle, or enhanced perfusion during ischemia.

**Conclusion:** The findings of this thesis demonstrate that dietary supplementation with inorganic NO$_3^-$ may improve high-intensity endurance performance in well-trained athletes. Well-trained athletes show a more varied and typically subdued plasma [NO$_2^-$] response following NO$_3^-$ intake; a predictor of NO$_3^-$’s effect on performance. The acute dose required to elicit performance benefits is greater than what has previously been shown to be effective in lesser trained individuals. Several days of NO$_3^-$ supplementation in the lead-up to competition may be an alternate strategy than simply an acute dose, as chronic intake may evoke peripheral changes in the musculature that improve fatigue resistance.
ACKNOWLEDGEMENTS

The production of this thesis would not have been possible without the contribution of some very extraordinary people. I consider myself fortunate enough to have known and worked with so many, and would like to acknowledge each of their contributions:

Firstly I would like to thank my primary supervisor, Dr. Nathan Johnson. I must have enjoyed our time together in honours so much that I decided to sign up for three more years. Or I was blindsided by your charm? Thank you for the jovial experience you have made it and the mentoring you have provided. I’d like to think I leave slightly wiser and it is a little sad that it must come to an end. Can I sign up for three more years?

To Prof. Louise Burke, thank you for all the opportunities and guidance you have provided me. Before I started, you were a larger than life character whose reputation was a little intimidating. But as we began working together, I realised you were a very down to earth, selfless individual and it has been a pleasure working with you. I hope our paths continue to cross in the future and there are more opportunities to work together again.

To Prof. Andy Jones and Dr. Phil, thank you for all the supplementary help you each have given over the years. I have learnt so much from both of you and I’m sure I will continue to do so. It doesn’t matter if your assistance was dispensed from across the road at ACU or across the pond in Exeter, it was invaluable nonetheless and has gone a long way in helping me produce this thesis.

Thank you to all the hard working folk at the Physiology and Sports Nutrition units at the AIS, as well as the staff at the University of Sydney. Although individuals have come and gone over the years, the one thing that has remained constant is the momentous amount of help each person is willing to offer. Thank you for all the different contributions you each have made, whether it was helping me with testing, equipment or otherwise. It is a truly inspiring work ethic that is cultivated at both institutes, and I’m glad I was a part of it.

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Finally to my other friends and family, thank you for all the support you have provided outside of Uni. Having each of you in my life went a long way in keeping me sane, focused and happy over this period. I hope you are all still there with me as I step into life’s next adventure.
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## ABBREVIATIONS

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<th>Description</th>
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<tbody>
<tr>
<td>$^{31}$P-MRS</td>
<td>Phosphorus magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>Calcium</td>
</tr>
<tr>
<td>CASQ1</td>
<td>Calsequestrin 1</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CT $\frac{1}{2}$</td>
<td>Half contraction time</td>
</tr>
<tr>
<td>DHPR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>ES</td>
<td>Effect size</td>
</tr>
<tr>
<td>GXT</td>
<td>Graded Exercise Test</td>
</tr>
<tr>
<td>MVC</td>
<td>Maximum voluntary contraction</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NO$_2^{-}$</td>
<td>Nitrite</td>
</tr>
<tr>
<td>NO$_3^{-}$</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>$P_{i}$</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PCr</td>
<td>Creatine phosphate</td>
</tr>
<tr>
<td>RT $\frac{1}{2}$</td>
<td>Half relaxation time</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>TENS</td>
<td>Transcutaneous electrical nerve stimulation</td>
</tr>
<tr>
<td>TT</td>
<td>Time trial</td>
</tr>
<tr>
<td>TTE</td>
<td>Time to exhaustion</td>
</tr>
<tr>
<td>XOR</td>
<td>Xanthine oxioreductase</td>
</tr>
</tbody>
</table>
SUBMISSIONS AND PUBLICATIONS

Publications


Submitted Manuscripts


Contribution of candidate to each publication:

The Effect of Nitrate Supplementation on Exercise Performance in Healthy Individuals: A Systematic Review and Meta-Analysis: Study design, literature search and review, all statistical analysis, lead author of the manuscript.

The Effect of Variable Doses of Inorganic Nitrate-Rich Beetroot Juice on Simulated 2,000 m Rowing Performance in Trained Athletes: Study design, all subject recruitment, exercise testing, sample collection, sample preparation, all statistical analysis, lead author of the manuscript.
Nitrate Supplementation and High-intensity Performance in Competitive Cyclists: Study design, all exercise testing, sample collection, sample preparation, statistical analysis of secondary outcome measures, lead author of the manuscript.

The Effect of Nitrate Supplementation on Muscle Contractile Properties in Healthy Adults: Study design, subject recruitment, all exercise testing, all statistical analysis, lead author of the manuscript.

Conference Presentations


Nitrate Supplementation and High-Intensity Cycling Performance. Hoon MW, Jones AM, Hopkins WG, Johnson NA, Halson SL, Martin DT, West NP, Bartram J Burke LM. Poster presentation at the 2012 Annual Conference of the European College of Sport Science, Bruges, Belgium.


Dose Response of Nitrate Supplementation on 2,000m Rowing Performance. Hoon MW, Johnson NA, Jones AM, Blackwell JR, Rice AJ, Lundy B, Burke LM. Podium presentation at the 2013 Asics Conference of Science and Medicine in Sport, Phuket, Thailand.
CHAPTER I: INTRODUCTION
PHYSIOLOGICAL DETERMINANTS OF ENDURANCE PERFORMANCE

The ability to withstand fatigue and cover a fixed distance in the fastest time dictates success in many sports. A broad range of activities fall into the category of “endurance sports”, including shorter, fast-paced events such as 1,500 m track running which is covered in as little as 3.5 min and ultra-endurance events such as the ironman triathlon in which competitors travel 225 km via a range of sporting disciplines in durations of eight hours or greater. An individual’s ability to perform optimally in endurance type events is determined by numerous factors, both external (e.g. environmental conditions, competitor skill) and internal (e.g. athlete conditioning, mental tenacity) in nature.

The physiological determinants of exercise performance proposed by Joyner and Coyle (2008), is one of the most widely accepted models of endurance exercise to date. The authors suggest that the optimal physiological profile for success in endurance sports is characterized by a large aerobic power, an ability to sustain a high percentage of this capacity for prolonged periods and an economic energy cost of activity. Specifically, the model identifies three key aspects thought to primarily facilitate endurance performance: i) a large maximal oxygen consumption capacity (VO\(_{2\text{max}}\)); ii) a high lactate threshold (or sustainable % of VO\(_{2\text{max}}\)) and (iii) good mechanical efficiency. Each of these in turn, are comprised of smaller contributing factors which determine their respective capacities. An adapted model is presented in Figure 1.

Aerobic power

An individual’s maximal rate of oxygen consumption (i.e. VO\(_{2\text{max}}\)) is defined as “the highest rate at which oxygen can be taken up and utilized in the body during severe exercise”, and is thought to set the ‘upper limit’ for performance events (Bassett & Howley, 1997). Many endurance events are performed at submaximal intensities, yet VO\(_{2\text{max}}\) still heavily influences the level of aerobic respiration during exercise i.e. the ‘performance VO\(_2\)’. The performance VO\(_2\) is a function of VO\(_{2\text{max}}\) multiplied by the percent of maximum that is sustainable (i.e. ‘lactate threshold’), such that any increase in VO\(_{2\text{max}}\) directly elevates the intensity of exercise that may be sustained. Interventions that are able to elevate VO\(_{2\text{max}}\) (such as structured exercise) typically observe a concomitant improvement in performance (Smith, McNaughton, & Marshall, 1999).
VO\textsubscript{2max} is determined firstly, by the body’s ability to transport and deliver O\textsubscript{2}, and secondly its ability to utilize O\textsubscript{2} to produce Adenosine Triphosphate (ATP) (Figure 1). Cardiac output has been suggested to be the primary limitation of VO\textsubscript{2max} in a well-trained individual (Bassett & Howley, 2000; Spurway, Ekblom, Noakes, & Wagner, 2012), however local vasoconstriction in exercising muscle causing reduced blood flow and O\textsubscript{2} delivery may also present as a limitation (Amann, 2012).

![Figure 1](image.png)

**Figure 1.** A generalized model of the physiological determinants of endurance performance, adapted from Joyner and Coyle, 2008. ‘Muscular power’ (shown in red) was not in the original model proposed by Joyner and Coyle, however becomes applicable in high-intensity endurance events. X-C: excitation-contraction.

At the muscle junction, diffusion of O\textsubscript{2} through the capillary bed may also constrain O\textsubscript{2} transport in some individuals, with greater capillary density associated with improved mean transit time of red blood cells, which in turn facilitates the unloading of O\textsubscript{2} into the muscle (Saltin, 1985). Within the muscle cell, the number and capacity of the mitochondria then determine how much ATP from generated from O\textsubscript{2} as exercise approaches the upper limits of aerobic power. Although there is evidence to support the contribution of each of the aforementioned steps to aerobic respiration (Holloszy & Coyle, 1984; Spurway et al., 2012), there is contention over which is the primary limitation of VO\textsubscript{2max} (Bassett & Howley, 2000).
Threshold

The so called “lactate threshold” (also known as MLSS: Maximal Lactate Steady State or LT2: Lactate Threshold 2) is the secondary component of performance VO\(_2\) and is thought to be more indicative of performances extending beyond 10 minutes (Joyner & Coyle, 2008). The threshold represents what percentage of VO\(_{2\text{max}}\) is sustainable (also referred to as the ‘fractional utilization of VO\(_{2\text{max}}\)’). In endurance trained individuals, threshold often occurs at a higher percentage of VO\(_{2\text{max}}\) (approximately 75-90%) compared with healthy, sedentary counterparts (approximately 60% of VO\(_{2\text{max}}\)) (Farrell, Wilmore, Coyle, Billing, & Costill, 1979). Even when VO\(_{2\text{max}}\) is similar between individuals, the %VO\(_{2\text{max}}\) at which lactate threshold occurs (%VO\(_{2\text{max}}\)@LT) may vary considerably, with those exhibiting a higher %VO\(_{2\text{max}}\)@LT threshold typically outperforming counterparts with a lower %VO\(_{2\text{max}}\)@LT (Coyle, Coggan, Hopper, & Walters, 1988).

The level at which threshold occurs is thought to be primarily determined by the muscle’s oxidative capacity (Holloszy & Coyle, 1984). Principal to this is the mitochondrial content of skeletal muscle. Greater volume and number of mitochondria within the muscle provide more sites for O\(_2\) utilization in ATP resynthesis, and as such correlate with greater work rates at threshold (Ivy, Withers, Van Handel, Elger, & Costill, 1980). An associated factor that is often overlooked is the efficiency of mitochondria. During exercise, protons may enter the mitochondria through channels other than the F\(_1\)F\(_0\)-ATPase complex, and thus not contribute to ATP resynthesis, in a phenomenon known as ‘proton leak’ (Stuart, Brindle, Harper, & Brand, 1999). In skeletal muscle, the primary source of proton leak appears to be via uncoupling protein 3 (UCP-3) located on the mitochondrial membrane (Boss, Hagen, & Lowell, 2000). The physiological reason for uncoupling is unclear, but is likely to be at the detriment of aerobic energy supply (Jastroch, Divakaruni, Mookerjee, Treberg, & Brand, 2010). Accordingly, a reduction in the expression of UCP-3 may be viewed as a favourable adaptation for exercise (Schrauwen, Troost, Xia, Ravussin, & Saris, 1999).

Efficiency

Exercise efficiency/economy refers to how well aerobic energy being utilized during exercise is translated into mechanical work (efficiency) or speed/power (economy). A more efficient/ economical individual is able to sustain a similar exercise output at a lower energy cost than someone who is less efficient; or what is perhaps more applicable in
endurance sport - compete at a faster velocity for the same energy expenditure. Indeed, more efficient cyclists have been shown to sustain a significantly higher power output during time trial tests (Horowitz, Sidossis, & Coyle, 1994). Furthermore, when parameters such as VO$_{2\text{max}}$ are similar between individuals (as is often the case amongst elite competitors), differences in economy may account for a large portion of variation in performance (Conley, & Krhenbuhl, 1979). However, exercise efficiency appears to be difficult to improve, with data implying even training has limited effect (Marsh, Martin, & Foley, 2000). The principal determinant of efficiency is suggested to be the percentage of Type I fibres in exercising muscle groups (Coyle, Sidossis, Horowitz, & Beltz, 1992), however mitochondrial efficiency may also be a contributing factor, as proton leak from the mitochondria reduces ATP yield. Attenuation of proton leak may result in a greater mitochondrial P/O ratio (i.e. the amount of ATP produced per O$_2$ molecule through the electron transport chain) and thus reducing the O$_2$ cost of exercise (Fernström et al., 2007). Interestingly, the expression of UCP-3 (the principal site of proton leak) appears to be much greater in Type IIa and IIx fibres compared with Type I (Russell et al., 2003), and may explain why Type I fibres are more efficient. Alternatively, energy efficiency may also be improved by reducing the ATP cost of force production (Jones, Pringle, & Carter, 2013).

Characteristics of high intensity exercise

*Endurance performance* may cover a vast range of activities, each with their own unique combination of duration and intensity, which ultimately affect the physiological factors that determine performance. The interaction between duration and intensity is considered to be inverse; those activities of shorter duration will likely be performed at greater intensities than those of longer durations.

High-intensity exercise (which in this thesis will refer to *severe* to *extreme* intensity, i.e. workloads estimated to be near/ at VO$_{2\text{max}}$; Table 1) lies at the far end of the spectrum, requiring very high energy output over a relatively short period of time. The time exercise may be sustained at this intensity depends on the testing protocol and exercise modality, however is approximately 6 minutes (Billat & Korsalsztein, 1996; Faina et al., 1997). Energy demands at this intensity require aerobic respiration near or above maximal
capacity, performance is therefore more heavily influenced by VO\textsubscript{2}max than lactate threshold (Faude, Kindermann, & Meyer, 2009).

**Table 1. Exercise intensity domains, adapted from Burnley & Jones, (2007).**

<table>
<thead>
<tr>
<th>Domain</th>
<th>Intensity range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate</td>
<td>Lactate response remains at baseline levels (LT1)</td>
</tr>
<tr>
<td>Heavy</td>
<td>Between LT1 and MLSS</td>
</tr>
<tr>
<td>Severe</td>
<td>Between MLSS and VO\textsubscript{2}max</td>
</tr>
<tr>
<td>Extreme</td>
<td>Above VO\textsubscript{2}max</td>
</tr>
</tbody>
</table>

LT1: Lactate threshold 1 – the point at which blood lactate concentration rises above resting values; (Yoshida et al., 1987). MLSS: Maximum lactate steady state

At high intensities, oxygen delivery to the muscle is insufficient for aerobic respiration to meet energy requirements alone, and the consequent anaerobic energy production results in the accumulation of lactic acid – leaving the muscle cell in both a hypoxic and acidic state (Richardson, Noyszewski, Kendrick, Leigh, & Wagner, 1995). The accumulation of lactate and other by-products of anaerobic metabolism (including intracellular phosphate [Pi]) in turn may compromise myofibrillar force generation (Weiner, Moussavi, Baker, Boska, & Miller, 1990). Therefore, muscular power (i.e. work over time) presents as another physiological determinant of performance in intense exercise. As this was not considered in the original model proposed by Joyner and Coyle (who examined the performance of longer, steady-state exercise), it has been superimposed into the model presented in Figure 1 as a separate entity specific to high-intensity exercise.

A key factor underlying the reduction in force generation during high-intensity exercise is the impairment of Ca\textsuperscript{2+} release by the sarcoplasmic reticulum into the muscle cell. Several studies have shown a dysregulation of Ca\textsuperscript{2+} handling in the muscle cell during exhaustive exercise, reducing the number of available cross-bridge sites and therefore force generation as well (Westerblad & Allen, 1991). A three part model of this force degradation in fatigued muscle fibres was proposed by Westerblad and colleagues (1991) where: Phase 1 is signified by an initial sharp decline in force generation capability reflecting a decline in maximum Ca\textsuperscript{2+} activated force; Phase 2 is characterized by a long plateau where force generation capability is minimally changed; and Phase 3 represents the final, exhaustive
A decline in force which is attributable to diminished intracellular Ca\(^{2+}\) presence (Figure 2). A delay in the onset of Phase 3 would therefore translate into a greater resistance to fatigue/ability to maintain a higher overall level of muscular power during exercise.\(^1\)

Generally, Type I muscle fibres are preferential for endurance exercise due to their high fatigue resistance and association with economic movement (Horowitz et al., 1994). However, Type I fibres compared with all other fibre types (i.e. Type IIa and Type IIx) possess the slowest shortening velocity and lowest force generation capacity (Bottinelli, Canepari, Pellegrino, & Reggiani, 1996). As muscular power increases in accordance with exercise intensity, Type II fibres are recruited accordingly (typically Type IIa first, followed by IIx) to provide the extra force necessary (this sequential recruitment of muscle fibres is referred to as the Henneman size principle – Henneman et al., 1974). Consequently, exercise at the higher end of aerobic power (~91% \(\text{VO}_{2\text{max}}\)) has been shown to require a much greater utilization of Type II fibres than lower intensity exercise (VØLlestad & Blom, 1985). These data imply that the performance of high-intensity exercise is facilitated by a greater expression and capacity of Type II fibres in muscle; more so than steady state submaximal exercise.

**Figure 2.** Model of muscle force in response to repetitive contractions over time (units are arbitrary). The decline in force (i.e. fatigue) can be separated into three separate phases: an initial, sharp decline; a longer more consistent period and finally a rapid decline signaling near exhaustion. Adapted from Westerblad & Allen, 1991.

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\(^{1}\) It should be noted that dysregulation of Ca\(^{2+}\) handling represents only one possible mechanism contributing to muscular fatigue. The physiological basis of fatigue is a complex interaction of several biological systems; beyond the scope of this thesis. For a more detailed review, see: Allen, Lamb, & Westerblad, 2008)
**ENHANCING PERFORMANCE**

Based on the physiological determinants listed above, improving one or several of these components is expected to improve endurance exercise performance. The most established way to do so is with exercise training, with positive adaptations to VO\textsubscript{2max}, lactate threshold and efficiency/economy (to a lesser extent) known to occur following chronic endurance training (Midgley, McNaughton, & Jones, 2007). Generally, in a previously untrained individual, adaptations occur rapidly and in relatively large magnitudes following regular structured exercise (Daniels, Yarbrough, & Foster, 1978). Large gains in VO\textsubscript{2max} and capillary density may be seen in as little as eight weeks after initiating a moderate training program (Henriksson & Reitman, 1977; Klausen, Andersen, & Pelle, 1981). Changes in oxidative enzymes and exercise capacity may be even seen after two weeks of intense training (Burgomaster et al. 2005; Gibala et al. 2006).

However, given enough consistent training, adaptation responsiveness typically becomes blunted. Many well-trained athletes are suggested to reach a ‘ceiling’ - a genetically determined physiological limit (Williams & Folland, 2008). Therefore, in the well-trained athlete, the physiological parameters determining performance are often highly comparable across individuals or teams at the elite level, with competition results frequently separated by small margins (Hopkins, Hawley, & Burke, 1999).

In light of these small differences, athletes and coaches often turn to ergogenic aids to further enhance physical aptitude. Generally, ergogenic aids offer a small benefit to athletes, these may be valuable gains due to the similar levels of performance between competitors. A variety of aids are available to athletes, nutritional supplements being one in particular (Williams, 1989).

**NUTRITIONAL SUPPLEMENTS**

Nutritional supplements are designed to provide nutrients that may otherwise not be consumed in sufficient quantities from the habitual diet. There exists a broad spectrum of commercially available supplements and each may vary in their designated purpose and this thesis will focus primarily on those promoting benefits to the performance of exercise (i.e. ergogenic).
The consumption of certain foods or products to elicit physical gains is a long held concept. Records from 500-400 B.C. show that individuals would regularly ingest foods such as deer liver and lion heart in the belief it would improve strength and speed (Applegate & Grivetti, 1997). In more recent times, advances in nutritional biochemistry have allowed the easy synthesis and mass-production of supplements, which has seen their number and availability flourish. The promise of easy health gains has seen a high uptake of supplements in the population, particularly amongst those involved in sport and physical activity (Maughan, King, & Lea, 2004). This trend is perhaps greatest in competitive athletes, with cross sectional studies from North America indicating the prevalence of supplement use may range from 88% (Burns, Schiller, Merrick, & Wolf, 2004) to 94% (Kristiansen, Levy-Milne, Barr, & Flint, 2005).

Certain supplements have been supported by scientific evidence showing significant performance improvements following their use. Carbohydrate supplements (such as sports drinks and energy gels) are commonly used by athletes to support glucose utilization during exercise or resynthesize glycogen stores following. Although carbohydrate may be easily obtained through meals, supplements offer a macronutrient dense solution which may be consumed during exercise with minimal bulk. A review of studies found that carbohydrate intake through fluids during exercise was generally a more favourable intervention than drinking water alone, with 80% of included studies reporting greater performance in time trials under carbohydrate feeding (Stellingwerff & Cox, 2014). Similarly, caffeine supplementation has been shown to be an effective nutritional aid to performance. Despite originally being prohibited, the World Anti-Doping Agency approved the use of caffeine in 2004, which has seen its use amongst elite athletes proliferate (Del Coso, Muñoz, & Muñoz-Guerra, 2011). A review of available studies in 2009 found caffeine supplementation resulted in a mean improvement of 3.2% to endurance performance over a range of distances and exercise modalities, with few studies reporting any negative effects of supplementation (Ganio, Klau, Casa, Armstrong, & Maresh, 2009).

Many other available supplements exist, however the strength of scientific evidence for each varies (Juhn, 2003). Rigorous scientific investigation is required to examine a supplement’s efficacy and gauge their use as an effective ergogenic aid. Even when studies report favourable physiological effects elicited by a supplement, it is imperative the validity and applicability of each study is evaluated (Kreider et al., 2010). For instance,
while untrained or recreationally active subjects may significantly benefit from a supplement, these findings do not always translate to meaningful gains for trained individuals (Bellinger, 2014; Zanchi et al., 2011). As such, when assessing the findings of a study, the physiological characteristics of the subject group should be taken into account when interpreting results. Additionally, the specific characteristics of an exercise assessment must be considered when interpreting data. Many supplements only provide benefit to a specific metabolic pathway. For example, creatine monohydrate supplementation primarily improves creatine and phosphocreatine stores, facilitating rapid ATP regeneration via the ATP-PC energy system (Cooper, Naclerio, Allgrove, & Jimenez, 2012). Therefore, improvements to very high-intensity sprints are unlikely to be extrapolated to more aerobic reliant running performance, particularly those over 150 s in duration (Branch, 2003).

Similarly, the validity of outcome measures must be taken into account. Often, graded exercise or time to exhaustion tests are utilized to test exercise capacity and while these provide an indication of performance, they typically experience a greater degree of trial to trial variation and do not tend to correlate strongly with more ecologically valid performance tasks (Currell & Jeukendrup, 2008). The most valid assessment of performance is considered to be time trials, which require individuals to complete a set amount of work in the quickest time possible or expend the maximal amount of work possible in a set amount of time (Currell & Jeukendrup, 2008). These conditions more closely resemble the demands of competition and racing, and should therefore be employed as the gold standard for assessing performance.

Although there may be shortfalls in available scientific evidence, the use of supplements among athletes is high due to the belief they may provide that slight edge they require to succeed in competition (Kristiansen et al., 2005). As performances edge towards the elite level, differences between competitors diminish and results are separated by smaller margins. Improvements to performance as small as 0.4% may greatly increase an athlete’s chance of success at the highest levels of competition (Pyne, Trewin, & Hopkins, 2004). Even though supplements are likely to exert only a small effect, this may be all that is required for significant reward.
NITRATE SUPPLEMENTATION

Supplements containing nitrate ($\text{NO}_3^-$) have recently been identified as potential ergogenic aids. Nitrate itself, is an anion that may be organic or inorganic in nature. The organic form of $\text{NO}_3^-$ is typically found in medically synthesized products, particularly medications for cardiovascular pathologies (Omar, Artime, & Webb, 2012). The inorganic form of $\text{NO}_3^-$ can be found in chemical compounds (such as potassium nitrate and sodium nitrate) or in common food sources such as vegetables (Table 2). Both display similar physiological effects once ingested, but differ in their chemical structures and pharmacokinetics (Omar et al., 2012). Continual use of organic nitrates may result in tolerance to its effects and may increase the risk of cardiovascular complications (Abrams, 2002). Consequently organic $\text{NO}_3^-$ may not be appropriate for use as a sports supplement. This thesis will therefore focus on supplements utilizing the inorganic form of nitrate (subsequent use of the terms ‘nitrate’ or ‘$\text{NO}_3^-$’ in this thesis will refer to the inorganic form).

Table 2. Vegetables categorized by nitrate content. Adapted from Hord et al. 2009.

<table>
<thead>
<tr>
<th>NO$_3^-$ content (mg.kg$^{-1}$)</th>
<th>Vegetable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very low (&lt;200)</td>
<td>Artichoke, asparagus, eggplant, garlic, onion, green bean, mushroom, pea, potato, sweet potato, tomato</td>
</tr>
<tr>
<td>Low (200 - 500)</td>
<td>Broccoli, carrot, cauliflower, cucumber, pumpkin</td>
</tr>
<tr>
<td>Moderate (500 - 1,000)</td>
<td>Cabbage, dill, turnip</td>
</tr>
<tr>
<td>High (1,000 – 2,500)</td>
<td>Chinese cabbage, fennel, leek, parsley</td>
</tr>
<tr>
<td>Very High (&gt;2,500)</td>
<td>Beetroot, celery, lettuce, rocket, spinach</td>
</tr>
</tbody>
</table>

In the diet, the most common sources of NO$_3^-$ are vegetables and cured meats (due to the use of sodium nitrate and related products as preservatives). Root-type vegetables appear to have the highest content, due to the absorption of NO$_3^-$ from soil during planting. As such, NO$_3^-$ content in vegetable-based foods is highly sensitive to the prevailing growing conditions and can even be dissimilar across the same variety of vegetable. Factors such as the season, location and farming method all impact NO$_3^-$ content, as well as subsequent
transportation and storage (Pennington, 1998). Data on habitual intake of NO$_3^-$ are limited for these reasons, however estimates indicate the average intake of NO$_3^-$ through diet typically range from 40 – 140 mg per day in Western countries (Mensinga, Speijers, & Meulenbelt, 2003).

In the past, concerns were raised over the toxicity of NO$_3^-$, primarily due to the association of NO$_3^-$ levels in drinking water and the incidence of methemoglobinemia (“blue baby syndrome”) in infants. However, subsequent research demonstrated that complications were more likely attributed to the presence of bacteria in water sources reducing NO$_3^-$ into the more toxic nitrite (NO$_2^-$) (Avery, 1999). Additionally, NO$_3^-$ naturally occurs in human breast milk, suggesting it may actually aid in the development of infants (Berens & Bryan, 2011). Interestingly, although NO$_3^-$ is reduced to NO$_2^-$ in vivo, the human body is able to control this reduction such that exposure to higher than recommended levels of NO$_3^-$ has not been considered harmful (McKnight, Duncan, Leifert, & Golden, 1999). Typically, only 5-8% of ingested NO$_3^-$ is reduced to NO$_2^-$, with a majority of it excreted (Lundberg & Govoni, 2004).

The nitrate-nitrite-nitric oxide pathway

The NO$_3^-$ compound itself is not thought to be bio-active. Rather, it is systematically reduced into the functional compound nitric oxide (NO) in the body. The processes regulating this reversible reduction are collectively referred to as the nitrate-nitrite-nitric oxide pathway (Figure 3). This pathway is thought to compliment the nitric oxide synthase (NOS) pathway of NO generation, in which L-arginine is oxidized by the NOS enzymes to form L-citrulline and NO (Lundberg, Weitzberg, & Gladwin, 2008). When nitrate-containing foods enter the stomach following consumption, NO$_3^-$ is absorbed by the upper gastrointestinal tract and enter systemic circulation, with peak plasma concentrations occurring approximately 60 min after ingestion (McKnight et al., 1997). Whilst a majority of NO$_3^-$ is excreted in urine, approximately 25% is taken up by the salivary glands (Lundberg & Govoni, 2004). The saliva is then excreted into the oral cavity where facultative anaerobic bacteria within the mouth reduce approximately 20% of the NO$_3^-$ (or 5-8% of the original intake) to NO$_2^-$. When the saliva is swallowed, some of the NO$_2^-$ is protonated by the acidic environment of the stomach to form nitrous acid (HNO$_2$), which decomposes to release NO (Lundberg, Weitzberg, Lundberg, & Alving, 1994). A
A considerable amount of the NO₂ is absorbed and circulated in the cardiovascular system, elevating plasma [NO₂⁻], with peak concentrations occurring roughly 2-3 h after NO₃⁻ ingestion (Webb et al., 2008). However, this timing may be subject to the concentration and absolute amount of NO₃⁻ ingested, with higher concentrated products likely to induce a sharper rise in [NO₂⁻] (reducing the time to peak); and larger amounts of NO₃⁻ elevating [NO₂⁻] further increasing the time to peak (Wylie et al., 2013).

**Figure 3.** The reduction of NO₃⁻ along the nitrate-nitrite-nitric oxide pathway. *Blue lines: nitrate (NO₃⁻), red lines: nitrite (NO₂⁻). GI: gastrointestinal.*

In the circulation, systemic and in-tissue reduction to NO may occur most notably through interactions with deoxygenated haemoglobin & myoglobin, mitochondria and serum enzymes (including xanthine oxidoreductase and NOS) (Lundberg et al., 2008). The reduction of NO₂⁻ to NO through these processes appear to be facilitated under low oxygen tensions (i.e. hypoxia). For instance, when haemoglobin and myoglobin saturation is low, they display a high affinity for O₂ which is taken from NO₂⁻, resulting in the subsequent formation of NO (Huang et al., 2005; Shiva et al., 2007). Similarly, complexes of the
mitochondrial electron transport chain and the enzyme xanthine oxioreductase utilize NO$_2^-$ as an electron acceptor when O$_2$ availability is reduced (Kozlov, Staniek, & Nohl, 1999; Millar et al., 1998). These interactions indicate that the reduction of NO$_2^-$ to NO is promoted by acidic and hypoxic stimuli, which may be present in skeletal muscle during vigorous exercise (Richardson et al., 1995). In this manner, the nitrate-nitrite-nitric oxide pathway complements the traditional L-arginine-nitric oxide synthase pathway of NO production which is inhibited by hypoxic conditions (Lundberg & Govoni, 2004). The NO compound itself acts as a signaling molecule and is known to modulate numerous bodily functions including blood flow (Cannon, 1998), calcium handling and muscle contractility (Reid, 2001), neurotransmission (Garthwaite & Boulton, 1995) and mitochondrial respiration (Brown, 1995). It is through these actions that NO$_3^-$ is believed to aid exercise performance.$^2$

THE EFFECT OF NITRATE SUPPLEMENTATION ON THE PHYSIOLOGICAL DETERMINANTS OF PERFORMANCE

Emerging research has found NO$_3^-$ supplementation to modulate some of the aforementioned physiological determinants of endurance performance. Specifically, these pertain to efficiency, blood flow and force generation capabilities of the muscle. The known actions of NO$_3^-$ supplementation on these determinants are summarized in Figure 4.

Efficiency

The earliest research into the effects of NO$_3^-$ supplementation on exercise identified the ability of NO$_3^-$ to improve exercise efficiency (Larsen et al., 2011). Larsen and colleagues identified this effect after prescribing 3 days of sodium nitrate (NaNO$_3$) consumption to a

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$^2$ There is some suggestion that NO$_2^-$ itself may also regulate mitochondrial function (Shiva, 2013) and act as a endocrine messenger along multiple signalling pathways, independent of its reduction to NO (Bryan et al, 2005)

A group of healthy males. Steady state VO$_2$ was found to be 5% lower during bouts of sub-maximal cycling after supplementation, indicating alterations in exercise efficiency had occurred. This was a notable finding, as exercise efficiency had long been considered a component of exercise resistant to change. In a follow-up investigation, the researchers observed a tendency for subjects to improve their time to exhaustion during a cycling test following NO$_3^-$ supplementation; a result that was attributed to the enhanced exercise efficiency (Larsen, Weitzberg, Lundberg, & Ekblom, 2010).

Several mechanisms have been proposed to account for these observed changes to efficiency following supplementation. In vitro analysis by Larsen and colleagues found that the mitochondrial P/O ratio had been improved following supplementation (Larsen et al., 2011). Mitochondrial enzyme analysis suggested this was due to the reduced expression of adenine nucleotide translocator (ANT) and UCP-3 following three days of 0.1 mmol.kg$^{-1}$.day$^{-1}$ NaNO$_3^-$ supplementation, reducing proton leak across the inner mitochondrial membrane and thus improving oxidative phosphorylation efficiency. Alternatively, research stemming from another laboratory suggested that the overall ATP cost of exercise was reduced following NO$_3^-$ supplementation. Using Phosphorus-Magnetic
Resonance Spectroscopy ($^{31}$P-MRS) analysis, it was observed that individuals exhibited less ATP turnover for an exercise task following six days of nitrate-rich beetroot juice consumption (Bailey et al., 2010). The regulating effect of NO on the ATP consuming steps of excitation-contraction coupling (i.e. actomyosin-ATPase and Ca$^{2+}$-ATPase activity) was suggested to be responsible. It is likely that the observed improvement in exercise efficiency following NO$_3^-$ supplementation is a combination of both the greater mitochondrial efficiency shown by Larsen et al. (2007) and the enhanced mechanical efficiency demonstrated by Bailey et al. (2010).

Muscular force

Previous literature has noted the role of NO in the regulation of skeletal muscle contraction (Reid, 2001), however, these investigations have typically employed animal models and chemical NO donors, so it is unclear if these findings are applicable to NO$_3^-$ supplementation in humans. As detailed above, Bailey et al. proposed that it was the effect of NO$_3^-$-derived NO on excitation-contraction coupling that was responsible for the observed reduction in ATP cost. However, it was uncertain if this modulation of excitation-contraction processes subsequently affected muscular force production, as it was not examined.

Hernández et al. were one of the first to directly examine the effects of NO$_3^-$ supplementation on markers of muscular contraction, albeit in a rodent model (Hernández et al., 2012). In this study mice were fed 1 mM NaNO$_3$ in drinking water (equivalent to the dose used in human studies) for seven days after which whole muscles were dissected and tested under electrical stimulation. A mean 40% increase in the contraction force for low frequencies of stimulation (<50 Hz) and a faster rate of force development at higher frequencies of stimulation following supplementation was found in the extensor digitorum longus, a predominantly fast-twitch muscle. These changes to contraction were associated with an increase in intracellular Ca$^{2+}$and related proteins (calsequestrin 1 and dihydropyridine receptor), suggesting that supplementation altered Ca$^{2+}$ handling in the muscle. Interestingly, these effects and adaptations were only present in the fast-twitch muscle and not in the predominantly slow-twitch soleus. Similar results were observed in humans, although with changes were smaller in magnitude, with a 2% mean increase in contractile force under low frequency stimulation (Haider & Folland, 2014). This
discrepancy in the magnitude of effect between rodent and human studies may reflect the selective action of NO$_3^-$ on Type II fibre and the mixed proportion of muscle fibre types in human muscle. Although these data do suggest some effect on muscle contraction is present, it is unclear if this explains, in part, the observed improvements in endurance performance in humans following NO$_3^-$ supplementation.

Vasodilation

The vasodilative properties of NO$_3^-$ (more specifically its reduced forms of NO$_2^-$ and NO) have been well documented, and as such have been utilized medically to treat ischaemic conditions (Butler & Feelisch, 2008). As a consequence, several researchers have suggested that part of nitrate’s ergogenic properties may be attributable to enhanced blood flow to active muscles following artery and capillary vasodilation (Bailey et al., 2009; Muggeridge et al., 2014; Vanhatalo et al., 2011). Numerous investigations have observed a lower resting blood pressure in subjects following NO$_3^-$ intake (Kapil et al., 2010; Larsen, Weitzberg, Lundberg, & Ekblom, 2007; Vanhatalo et al., 2010; Webb et al., 2008) suggesting a vascular effect is present; however muscular perfusion during exercise has not been directly investigated. This is primarily due to limitations in the ability to measure in vivo blood flow in humans, although indirect techniques such as near-infrared spectroscopy (NIRS) may infer blood flow from measurements of local muscle oxygenation (Nioka et al., 2006). Studies utilizing this technology have reported greater regional blood flow to active muscle following supplementation (Bailey et al., 2009; Kenjale et al., 2011; Masschelein et al., 2012), implying NO$_3^-$ may facilitate hyperemia to skeletal muscle. Furthermore, it should be noted one investigation examined subjects with peripheral arterial disease (Kenjale et al., 2011) and another assessed exercise under hypoxic conditions (Masschelein et al., 2012), as these particular circumstances may exacerbate muscular perfusion and therefore intensify the vascular effects of NO$_3^-$.

To date, changes in exercising blood flow following NO$_3^-$ supplementation have not been directly examined in humans.

In a rodent model, Ferguson and colleagues observed greater muscle blood flow during exercise following NO$_3^-$ supplementation (Ferguson et al., 2013). Specifically, Spraque-Dawley rats were supplemented with beetroot juice for five days and blood flow was examined using labeled infusion isotopes during a treadmill run. The authors found not
only an increase in blood flow to the exercising limb, but greater vascular conductance (i.e. blood flow normalized to blood pressure) as well. Again, these effects favoured Type II muscle fibres, with a significant positive correlation between blood flow changes and the percentage of Type II fibres in a muscle.

NITRATE SUPPLEMENTATION FOR WELL-TRAINED ATHLETES

Although well-trained athletes are the most likely to seek performance enhancement from supplements, they may actually be less responsive to the effects of NO\textsuperscript{3−} than lesser trained individuals. Several physiological adaptations to endurance training may lessen the effect of NO\textsuperscript{3−} supplements (Bescos, Sureda, Tur, & Pons, 2012). For instance, well-trained individuals have been reported to possess higher levels of resting plasma [NO\textsubscript{2−}] and [NO\textsubscript{3−}] (Schena, Cuzzolin, Rossi, Pasetto, & Benoni, 2002) and NOS activity (Green, Maiorana, O'Driscoll, & Taylor, 2004). This greater endogenous store of NO metabolites may reduce the need and, therefore, impact of any ingested exogenous NO\textsuperscript{3−}. Additionally, endurance training typically results in greater mitochondrial expression and capillarisation in muscle (Ingjer, 1979). These adaptations serve to reduce metabolic perturbations during exercise i.e. hypoxic and acidic stress, conditions that would otherwise accentuate the reduction of NO\textsuperscript{2−} to NO (Lundberg et al., 2008). For these reasons, it is unclear if NO\textsuperscript{3−} supplementation is effective in improving exercise performance in the well-trained individual, and perhaps why the limited amount of studies have yielded mixed results. Cermak, Gibala, & van Loon (2012) found that 10-km cycling time trial performance improved in trained, male cyclists following six days of beetroot juice supplementation; however, an acute dose of beetroot juice 2.5 hours prior to exercise did not improve 1-hr cycling performance (Cermak et al. 2012b). Similarly, several days of nitrate salt ingestion did not improve 5 km running performance in junior cross-country skiers (Peacock et al.); nor cycling performance in trained cyclists (Bescos et al. 2011; Bescos et al. 2012b). These results are at odds with exiting research showing benefits to cycling (Bailey et al., 2009; Lansley et al., 2011a; Vanhatalo et al., 2011; Wilkerson et al., 2012) and running performance (Lansley et al., 2011b) in moderately trained individuals.
**Further Research**

The aforementioned studies investigating NO$_3^-$ supplementation in well-trained subjects assessed the performance of exercise in the low to middle *severe* intensity domain. Arguably, NO$_3^-$ supplementation is most applicable for higher intensities (approaching the *extreme* domain). Firstly, high-intensity events are more likely to facilitate the reduction of NO$_2^-$ to NO along NO$_3^-$ - NO$_2^-$ - NO pathway (Lundberg et al., 2008), due to greater hypoxic and acidic stress. Secondly, animal studies suggest NO$_3^-$ supplementation predominantly affects Type II fibres (Ferguson et al., 2012; Hernandez et al., 2012), which have greater activation with increasing exercise intensity. Thirdly, NO is thought to improve muscle force generation (Reid, 2001; Hernandez et al., 2012), which may be compromised at high intensities (Westerblad & Allen, 1991). Research investigating the effect of NO$_3^-$ supplementation on short, high-intensity exercise performance is currently lacking. Given these conditions may be ideal for NO$_3^-$ supplementation to elicit ergogenic benefit, further research is required - particularly in well-trained cohorts, where these affects may be diluted. This research may carry important implications for the use of NO$_3^-$ supplementation in elite sport and inform sport scientists on appropriate ways to implement supplementation.
THESIS AIMS

The aim of this thesis was to firstly examine the efficacy of NO₃⁻ as an ergogenic sports supplement in trained, competitive athletes. Secondly, the thesis examined the procedure in which to apply NO₃⁻ supplementation, with particular focus on the appropriate dose and timing, as well techniques to monitor its utilization within the body. Finally, potential physiological mechanisms by which NO₃⁻ may exert its ergogenic effect (if one is to be found) were investigated. Specifically, the aims were to:

1. Undertake a systematic review of the available literature on inorganic NO₃⁻ supplementation and quantify its effect on endurance exercise performance in healthy adults by meta-analysis;

2. Determine if acute supplementation with NO₃⁻ improves simulated track cycling performance in well-trained cyclists;

3. Examine the effect of nitrate supplementation on plasma concentrations of nitrate and nitrite ([NO₃⁻] and [NO₂⁻], respectively), and the association with changes in performance;

4. Investigate the effect of altering the dose and timing of NO₃⁻ ingestion on exercise performance and plasma [NO₃⁻] and [NO₂⁻];

5. Examine the effect of different acute NO₃⁻ doses on simulated competition performance in well-trained rowers

6. Investigate the effect of NO₃⁻ supplementation on muscular function and the role of vascular and peripheral responses in performance improvement

These aims will be addressed through one meta-analytical review and three experimental studies. For two of the experimental studies where performance of simulated competition is assessed, a magnitude-based inference statistical approach was used (Hopkins et al., 2009). This is because traditional statistical methods (particularly those requiring rejection of the null-hypothesis) may not be sensitive enough to detect the small changes in performance considered practically useful to an athlete (Hopkins & Batterham, 2005).
**THESIS OUTLINE**

Chapter 2 of the thesis is a meta-analytical review of the available literature to objectively quantify the reported effect NO3- has on exercise performance in healthy adults. A secondary goal of this meta-analysis was to identify areas of the research that require further clarification and investigation.

Chapter 3 is an original investigation of the effect of beetroot juice (containing 4-6 mmol NO3-) on the performance of repeat 4-min all out cycling time trials, in a cohort of well-trained cyclists. In a cross-over fashion, participants undertook four separate trials, where the timing and amount of NO3-ingestion varied. As a secondary outcome, the time course of NO2- appearance in plasma was examined, to get a sense of when NO3- (beetroot juice in this instance) is best prescribed to achieve maximum benefit.

Chapter 4 is another original investigation of the effect different doses of beetroot juice have on the performance of a 2,000 m rowing task in well-trained rowers. The effect of a double dose of beetroot juice (8 mmol NO3-) compared to a single dose (4 mmol NO3-) and placebo taken two hours prior to exercise was examined. The plasma response to supplementation as well as its correlation to changes in performance was also explored.

Chapter 5 is an investigative study into the effect NO3- supplementation (via beetroot juice) has on muscle contractility and how this may contribute to the reported ergogenic effect of NO3-. This study examined multiple markers of contractile function in a group of healthy adults, including force generation and fatigue resistance following four days of dietary supplementation.

Finally, Chapter 6 summarises the key findings from the thesis and the implications these may have. It will also discuss the state of currently available literature on NO3- supplementation, the major themes emanating from these studies, and how data from this thesis contribute to the current body of knowledge. Furthermore, the limitations of this thesis as well as future directions for research are also discussed.
REFERENCES


CHAPTER II: THE EFFECT OF NITRATE SUPPLEMENTATION ON EXERCISE PERFORMANCE IN HEALTHY INDIVIDUALS: A SYSTEMATIC REVIEW AND META-ANALYSIS

The following study is a copy of the accepted manuscript:


As co-authors of the paper “The Effect Of Nitrate Supplementation On Exercise Performance In Healthy Individuals: A Systematic Review And Meta-Analysis” we confirm that Matthew Hoon has made the following contributions:

- Study concept and design
- Literature search
- Data extraction, analysis and interpretation
- Manuscript preparation

Signed: Matthew Hoon Date: 01/07/2014
Signed: Date: 02/07/2014
Signed: Date: 02/07/2014
ABSTRACT

The purpose of this review was to examine the effect of nitrate supplementation on exercise performance by systematic review and meta-analysis of controlled human studies. A search of four electronic databases and cross-referencing found 17 studies investigating the effect of inorganic nitrate supplementation on exercise performance that met the inclusion criteria. Beetroot juice and sodium nitrate were the most common supplements, with doses ranging from 300 - 600 mg nitrate and prescribed in a manner ranging from a single bolus to 15 days of regular ingestion. Pooled analysis showed a significant moderate benefit (ES = 0.79, 95% CI: 0.23 to 1.35) of nitrate supplementation on performance for time to exhaustion tests (p = 0.006). There was a small but insignificant beneficial effect on performance for time trials (ES = 0.11, 95% CI: -0.16 to 0.37) and graded exercise tests (ES = 0.26, 95% CI: -0.10 to 0.62). Qualitative analysis suggested that performance benefits are more often observed in inactive to recreationally active individuals and when a chronic loading of nitrate over several days is undertaken. Overall these results suggest that nitrate supplementation is associated with a moderate improvement in constant load time to exhaustion tasks. Despite not reaching statistical significance, the small positive effect on time trial or graded exercise performance may be meaningful in an elite sport context. More data are required to clarify the effect of nitrate supplementation on exercise performance and to elucidate the optimal way to implement supplementation.

Keywords: ergogenic aids, endurance, nitric oxide, time trial, sport supplements
INTRODUCTION

Nitrate (NO\textsubscript{3}\textsuperscript{-}) is a naturally occurring anion in the human body, initially believed to be an inert by-product of nitric oxide metabolism (Lundberg, Weitzberg, & Gladwin, 2008). Although nitric oxide has various roles that are vital to normal body function including neurotransmission (Vincent, 2010), vascular control (Kelm & Schrader, 1990), mitochondrial respiration (Brown, 1999) and skeletal muscle contraction (Reid, 2001), it was originally believed that nitrate itself had little specific activity. It has since been established that nitrate may be reduced to its antecedents nitrite and nitric oxide \textit{in vivo}, particularly in environments of hypoxia and acidosis. Research investigating the physiological actions of nitrate has reported effects such as improvement of vascular compliance (Bahra, Kapil, Pearl, Ghosh, & Ahluwalia, 2012), reduction of blood pressure (Larsen, Weitzberg, Lundberg, & Ekblom, 2007) and attenuation of oxidative stress (Carlström et al., 2011) following consumption. Given these properties, nitrate is commonly used as a pharmacological agent to treat a host of cardiovascular pathologies (Butler & Feelisch, 2008).

In addition to its therapeutic use, nitrate supplementation has recently been studied for its potential to enhance exercise performance. One of the earliest investigations of the effects of inorganic nitrate ingestion on exercise physiology was conducted by Larsen and colleagues, who examined pulmonary and cardiovascular responses during progressive load cycling (Larsen, et al., 2007). They reported the novel finding that ingestion of sodium nitrate was associated with a reduction in the oxygen cost of cycling (i.e. an improvement of gross efficiency). This finding was significant in view of the traditional belief that exercise efficiency is resistant to significant change, particularly as efficiency has been found to be similar across training status (Moseley, Achten, Martin, & Jeukendrup, 2004). Given that efficiency/economy is considered a key predictor of endurance exercise performance (Joyner & Coyle, 2008), there is potential for nitrate supplements to be used as ergogenic aids in endurance based activities. However, the results of subsequent investigations of nitrate supplementation on exercise performance have been inconsistent, with some showing a benefit (Bailey et al., 2010; Bailey et al., 2009; Bond, Morton, & Braakhuis, 2012; Cermak, Gibala, & van Loon, 2012; Lansley, Winyard, Bailey, et al., 2011a; Lansley, Winyard, Fulford, et al., 2011b; Masschelein et al., 2012) but others showing no significant effect (Bescos et al., 2012a; Bescos et al., 2011; Cermak , Res, Stinkens, et al., 2012; Larsen, et al.,
2007; Larsen, et al. 2010; Murphy, Eliot, Heuertz, & Weiss, 2012; Peacock et al., 2012; Wilkerson, et al. 2012) when compared with placebo. Therefore, the purpose of this study was to systematically review the available data and evaluate the overall efficacy of nitrate supplementation on endurance exercise performance in healthy populations by meta-analysis.

METHODS

A systematic literature search was conducted by one researcher (MH) to identify studies investigating nitrate supplementation and exercise performance. Online scientific databases searched from inception to August 2012 included: Medline (Ovid), SportDiscus, Science citation index of Web of Knowledge and PubMed. The keywords employed in the search were: (nitrate) AND (exercise). Reference lists of all retrieved papers were manually searched for potentially eligible papers.

Inclusion and exclusion criteria

Inclusion and exclusion criteria were determined a priori by two researchers (MH and NJ). Only intervention studies in peer-reviewed journals were considered. Other article types such as book sections, opinion articles, observational studies and abstracts without adequate data and reviews were not included. Study participants were required to be apparently healthy adults with no reported known disease, while non-human studies were excluded. Studies were required to employ at least one trial involving nitrate supplementation and a control or placebo trial in which no active supplement was given. Only studies using inorganic nitrate were included for review, due to the reported differences in pharmokinetic properties of inorganic versus organic nitrate (Omar, Artime & Webb, 2012). Trials employing the use of additional supplements likely to affect performance were excluded. Trials were also required to employ a quantifiable measure of exercise performance.

Selection of studies

Following removal of duplicates, the title and abstract of the remaining references were screened independently against the eligibility criteria by two researchers (MH and PC). Where information was insufficient, further screening of methods and results was undertaken. Disagreements concerning the eligibility of a paper were settled by discussion or consultation with a third researcher (NJ).
Data extraction

The outcome measures assessed in this review were measures of exercise capacity or performance. This included graded exercise tests to exhaustion, time to exhaustion and time/distance trials. Data on participant characteristics (sex, training status), nutritional intervention (nitrate dose and delivery method) and exercise test data were extracted independently by two researchers (MH and PC). Where required, means and standard deviations were calculated using appropriate equations \((SE = SD/\sqrt{n})\) (Hozo, Djulbegovic, & Hozo, 2005). If studies included more than one appropriate data set (such as an additional nitrate supplementation trial or exercise assessment), these were extracted and analysed as a separate result.

Analyses and meta-analyses

The between-trial standardized mean difference (nitrate vs. placebo) and 95% confidence intervals (CI) were determined using Comprehensive Meta-analysis software (Version 2, Biostat, Englewood NJ, 2005). Given the small sample sizes in each study \((n < 20)\), Hedges’ \(g\) was selected as the measure of effect size (Hedges, 1981), with interpretations of magnitude based on Cohen’s definitions of small \((0.2)\), moderate \((0.5)\) and large \((0.8)\) effects (Cohen, 1988). Between study variability was assessed using the \(I^2\) measure of inconsistency (Higgins, Thompson, Deeks, & Altman, 2003) and pooled estimates of the effect of nitrate supplementation versus placebo on exercise performance (using effect size) were then calculated using a fixed-effects model. Sub-analysis (determined \(a priori\)) was performed based on the type of exercise assessment employed in each study.

Results

Descriptive data

The initial search of electronic databases yielded 2,776 results in total. An additional 11 studies were included following a search of reference lists in retrieved manuscripts. Following the removal of duplicates and elimination of papers based on eligibility criteria, 17 studies remained (Fig. 1). In total 184 subjects (170 male, 14 female) participated in the 17 studies (Table 1).
Nine studies recruited subjects who reported regularly participating in a structured exercise training (of these 7 were classified as “highly trained” athlete populations) and 8 recruited “recreationally fit” populations. All studies were controlled trials which employed a randomised cross-over design involving a placebo condition and at least one condition using nitrate supplementation. Two studies employed more than one exercise test in their investigations. Lansley et al. 2011 assessed performance in both a 4 km time trial and a separate 16 km time trial following intervention (Lansley, Winyard, Bailey, et al., 2011a). Vanhatalo et al. 2010 implemented a chronic supplementation strategy and assessed performance on days 1, 5 and 15 of intervention (Vanhatalo, et al., 2010). These trials were included into analysis. Multiple types of exercise assessments were employed, with five studies using a graded exercise test to exhaustion (GXT), four employing a constant work rate time to exhaustion protocol (TTE) and eight studies examining the effect of supplementation versus placebo on endurance exercise time/distance (i.e. time trial [TT]). One study employed a repeated time trial design, where 6 x 500 m time trials were performed as quickly as possible and the average of these reported. This was therefore considered as a time trial for exercise assessment sub analysis. The majority (n = 10) of the 12 studies based on TTE or TT protocols employed an aerobic exercise test involving a mean exercise time ≥ 7 min at an estimated “severe” intensity, whereas only two studies used “extreme” intensity exercise (Jones & Poole, 2005). Although a majority of studies were conducted in a normoxic environment, two studies investigated exercise capacity in hypoxia.

**Nitrate Supplementation**

A majority of studies (n = 12) utilised beetroot as the source of nitrate delivery, with 11 studies prescribing beetroot juice and one study providing whole beetroots to participants. The remainder of studies delivered nitrate through chemical means, either as sodium nitrate (n=5) or potassium nitrate (n=1). The supplementation protocol also varied across studies, with eight studies examining the effect of acute supplementation (supplement given 75 to 180 min before exercise) and ten employing a chronic dosing schedule which ranged from several boluses over a 24 hr period prior to exercise, to 15 days of nitrate loading (Table 1).

**Adverse Events**
There were few reports of adverse events as a result of nitrate supplementation. Several papers noted incidents of beeturia (discolouration of urine) following consumption of beetroot juice specifically (Bailey, et al., 2010; Bailey, et al., 2009; Vanhatalo, et al., 2010), however no major health consequences were reported in any study.

Effect of nitrate supplementation on exercise capacity and performance

Methodological heterogeneity ($I^2$) (i.e. the percentage of the total variability in the set of effect sizes due to true heterogeneity) for studies when grouped by exercise assessment (time trial, time to exhaustion and graded exercise) was low (<25%). This indicates that it is appropriate to pool study results for meta-analysis as any between study differences are likely due to sampling error rather than differences in design. The pooled corrected effect size from studies examining time trial performance was 0.11 (95% CI: -0.16 to 0.37) indicating a small effect in favour of nitrate supplementation over placebo, with six of the nine trials reporting an improvement in performance under the nitrate condition, however this was not statistically significant ($p = 0.43$) (Figure 2a). The two studies assessing exercise performance in hypoxia were not included in the pooled effect size calculation due to the dissimilarity in physiological stress. The individual effects of these studies are presented in Figure 2d. The pooled effect size for the graded exercise tests to exhaustion in normoxia was 0.26 (-0.10 to 0.62) in favour of nitrate supplementation ($p = 0.16$) (Figure 2b). The three studies that assessed time to exhaustion at a fixed work rate (in normoxia) all reported favourable results under nitrate supplementation, with a combined pooled effect size of 0.79 (0.23 to 1.35, $p = 0.006$), representing a moderate effect (Figure 2c).

**DISCUSSION**

This is the first systematic review with meta-analyses to examine the efficacy of nitrate supplementation on exercise performance in healthy populations. The results of our analyses show that when compared with a placebo control, nitrate supplementation did not significantly affect time trial performance or performance during graded exercise testing. However, pooled analysis showed that nitrate supplementation increased performance during time-to-exhaustion exercise protocols by a moderate degree.
This systematic review and meta-analyses combined 17 studies involving a total of 184 participants. Nine of the studies examined supplementation in trained cohorts while the remainder employed healthy untrained subjects. Exercise performance in these studies was examined in multiple ways, namely fixed intensity and graded exercise tests to exhaustion and time trial tasks. Overall, the literature demonstrated a wide variation in the manner nitrate was supplemented. Several sources of nitrate were employed, ranging from pharmacological (sodium and potassium nitrate) to natural products (beetroot juice, vegetables). The bioavailability of nitrate from each of these substances has yet to be investigated, so it is difficult to ascertain if the nitrate source influences its potency. It is important to note that all studies included in this analysis used the inorganic form of nitrate for supplementation. Organic nitrate is most commonly found in pharmaceutical agents (e.g. glyceryl trinitrate) which may not be appropriate for use as a sport supplement. Despite their similar physiological effects, organic and inorganic nitrate possess different chemical structures and pharmokinetics (Omar et al., 2012). Furthermore, continual use of organic nitrates may result in tolerance and may increase the risk of endothelial dysfunction (Abrams, 2002).

The dose and timing of supplementation was also highly variable across studies. A majority (n=11) implemented a chronic dosing protocol, supplementing subjects with multiple boluses of nitrate 24 hrs to 15 days before exercise. However, several studies demonstrated either an improvement in performance (Lansley, et al., 2011a) or exercise efficiency (Bescos et al., 2011; Wilkerson, et al., 2012) arising from an acute dose of nitrate 75-150 min before exercise, suggesting effects may occur in a relatively short time frame. Overall, a multiple day dosing strategy may be more efficacious for improving exercise performance, as a greater proportion of chronic supplementation trials favoured nitrate supplementation (11 of 12) compared with the acute studies (6 of 8). This notion was supported by Vanhatalo et al. 2010, who directly compared the effects of acute and chronic supplementation (Vanhatalo, et al., 2010). No detectable improvement in performance during a graded exercise test was found following an acute (2.5 hr before exercise) 0.5 L dose of beetroot juice, however following 5 and 15 days of supplementation (0.5 L of beetroot juice per day), peak power and power at gas exchange threshold improved with duration (days) of supplementation. Nevertheless, only the results at 15 days of supplementation demonstrated significance.

The studies included in our pooled effect size analysis were relatively homogenous in study design. All were conducted in a randomized cross-over fashion and used a placebo trial. Time
trial type assessments are generally accepted as the best indicator of real world performance, due to their close resemblance to the demands of competition (Hopkins, Schabort, & Hawley, 2001). The majority of these studies assessed severe intensity endurance performance with time trials lasting 15-138 min. Analysis of these studies found a mixed response to nitrate, with four of seven studies favouring supplementation. Only two studies assessed nitrate supplementation in time trials of similar or shorter duration i.e. in the extreme intensity domain (Jones & Poole, 2005). The outcomes of these include improvement of 4 km cycle time trial (Lansley, et al., 2011a) and repeated 500 m time trials on a rowing ergometer (Bond, et al., 2012) in trained participants. These high intensity events are likely to induce local (intracellular) acidosis and hypoxia, during which the reduction of nitrate to nitric oxide is greatest, having its strongest physiological effect (Lundberg, et al., 2008). However, there is currently a lack of research on the effect of nitrate on high intensity exercise and these purported physiological mechanisms remain hypothetical.

Two studies were excluded from pooled effect estimates as the exercise tests were undertaken under environmental hypoxia, presenting a distinctly different scenario to the majority of studies. A reduction in atmospheric oxygen is known to have an ergolytic effect on endurance performance (Masschelein, et al., 2012). The nitrate-nitrite-nitric oxide pathway is more active under hypoxia (Lundberg, et al., 2008) serving to offset its detrimental effects, to the extent that time to exhaustion in hypoxia following beetroot consumption was similar to a control trial conducted in normoxia (Vanhatalo et al., 2011). It is postulated that the reduction of nitrate to nitric oxide serves to improve the transport and utilization of oxygen, a key limiting factor of endurance in hypoxic conditions. Further investigation has demonstrated an increase in arterial and muscle oxygenation (as measured by near-infrared spectroscopy) during exercise at a simulated 5,000 m following nitrate supplementation. This was accompanied by a 36% greater incremental time to exhaustion compared with a hypoxic control (Masschelein, et al., 2012). To date, the two aforementioned studies are the only investigations of nitrate use in hypoxia, however positive findings from research into other vasodilative agents (Hsu et al., 2006) suggest this area to be of potential.

Therapeutic nitrates have traditionally been utilized for their vasodilative properties, relieving ischaemic-based complications in morbidities such as peripheral artery and cardiovascular disease. Additionally, nitrate has been found to improve the efficiency (i.e. reduce the energy cost) of exercise as indicated by a 4-5 % reduction in VO₂ at steady state (Bailey, et al., 2009;
Bailey, et al., 2010; Lansley, et al., 2011b; Larsen, et al., 2007). In a review by Joyner and Coyle, exercise efficiency was identified as one of three key physiological components predicting endurance exercise performance, as it directly determines the speed or power that may be maintained at a particular rate of oxygen consumption (Joyner & Coyle, 2008). The mechanism by which nitrates act to alter exercise efficiency/economy remains contentious. It is hypothesized that increased levels of nitric oxide following supplementation may reduce the ATP cost of force production (Bailey, et al., 2010). In vivo analysis of exercising muscle by 31-phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) showed a decline in muscular [Pi] and [ADP] accumulation and a reduced utilization of PCr stores following supplementation with beetroot juice (Bailey, et al., 2010), suggesting a reduction in ATP turnover at the same work rate. Based on biochemical analysis of animal muscle, researchers have suggested that the reduction of ATP use is a consequence of nitric oxide’s regulatory effect on the ATP consuming processes of sacroplasmic reticulum calcium pumping (Viner, Williams, & Schoneich, 2000) or myofibrillar actin-myosin interaction (Galler, Hilber, & Gobesberger, 1997) in force production. A recent investigation found increases in myoplasmic [Ca$^{2+}$] and Ca$^{2+}$ handling proteins (accompanied by an increase in contractile force of fast-twitch muscle fibres) following seven days of nitrate treatment in mice (Hernandez et al., 2012), supporting the idea nitrate may elicit its effect in this manner. Alternatively, the nitrate-nitrite-nitric oxide pathway may directly influence mitochondrial efficiency. Three days of nitrate supplementation reduced the P/O ratio (i.e. the amount of oxygen consumed per ATP molecule produced) of isolated mitochondria from the vastus lateralis, which explained much of the variance in the reduced oxygen cost of exercise (Larsen et al., 2011). The authors suggested that this effect is attributable to the reduction in proton leakage through the mitochondrial membrane (i.e. mitochondrial coupling) possibly due to reduced expression of adenine nucleotide translocase.

The literature suggests an interaction between training status and the ergogenic effect of nitrate supplementation. All of the eleven trials conducted in untrained individuals reported a favorable result with nitrate supplementation, compared with seven of ten trials in trained subjects. The reason for this phenomenon has not been established, however adaptations to endurance training may play a role (Bescos, et al., 2012b). Fitter individuals have been found to possess superior vascular control as characterized by a greater activity and presence of eNOS (endothelial nitric oxide synthase), the enzyme responsible for endogenous generation of nitric oxide (Green, Maiorana, O'Driscoll, & Taylor, 2004). An increase in
eNOS activity may diminish the reliance on nitrate-derived nitric oxide thereby reducing the potency of nitrate supplementation. Similarly, when compared with an untrained individual, a trained athlete may be less likely to experience the physiological stimuli (low muscle oxygenation and muscle acidosis) favourable for nitrate reduction at a given work rate (Wilkerson, et al., 2012). Six of the nine studies which employed a trained subject cohort reported a favourable result for nitrate supplementation, however none reached significance. Further research is required to elucidate the factors influencing individual response to nitrate supplementation.

Limitations
The heterogeneity in study design across included studies restricted pooled effect estimate of the combined total data set and sub-analyses of parameters likely to influence the potency of nitrate supplementation (i.e. training status and dosage strategy). We chose *a priori* to differentiate studies only on the basis of exercise test protocol as we have justified previously (Temesi, Johnson, Raymond, Burdon, & O'Connor, 2011). We acknowledge that differences existed in subject characteristics and nitrate supplementation regimens within these sub-analyses, but the heterogeneity of these was low ($I^2 < 25\%$) supporting the use of our pooled approach. Studies were generally characterized by small sample sizes, further warranting pooling of data to increase statistical power. A limitation of our analyses, which were based on effect size calculations, is that small but meaningful performance effects may have been undetected. The expressions of “small”, “moderate” and “large” used by Cohen to describe effect size measures have been noted to be rather arbitrary in nature, and should rather be viewed as “relative” terms (Cohen, 1988). As discussed by Hopkins, traditional statistical methods may not be sensitive enough to detect the small changes in performance considered practically useful to an athlete (Hopkins & Batterham, 2005).

Future directions
At present, the research of nitrate as an ergogenic aid for exercise performance is in its infancy. The bulk of evidence shows promise, with a majority of studies reporting a favorable result for nitrate supplementation. However, as is often the case with nutritional supplements, protocols of best practice need to be developed for optimal usage. This includes elucidating the sports and environments where nitrate supplements may be most useful and refining the procedure on how it is applied, with particular focus on the quantity, timing and quality (i.e. nitrate source) of supplementation.
Conclusion
The current meta-analysis of available research suggests a small benefit to performance may be afforded by taking nitrate-based supplements. Despite the small effect size, these gains may be considered extremely meaningful in a sports performance context. Across studies measuring time trial performance in trained cohorts, there was an approximately 0.9% improvement following nitrate supplementation. To put this in context, the measured difference between first and fourth place for elite swimming performance has been calculated to be 0.6% (Trewin, Hopkins et al., 2004), and improvements as little as 0.3% have been noted to be valuable to elite track and field athletes (Hopkins, 2005). At the recent UCI world track cycling championships, first and third place in both the men’s individual sprint and pursuit events were separated by <0.5% (http://www.cyclingnews.com). As is the nature with all sports supplements, the risk to reward ratio should be considered. Given nitrate may be ingested through natural forms such as beetroot juice and vegetables, this may limit the risk of prohibited substance contamination (Maughan, King, & Lea, 2004) and carry complementary health benefits associated with increased dietary nitrate intake (Lidder & Webb, 2012). In conjunction with the low number of studies reporting negative effects, nitrate supplements present as a low risk intervention that may aid endurance exercise performance.
REFERENCES


FIGURES AND TABLES

Figure 1. Search strategy and results.
Figure 2a. Effect of nitrate supplementation on time trial performance

Figure 2b. Effect of nitrate supplementation on graded exercise test performance
Figure 2c. Effect of nitrate supplementation on time to exhaustion tasks.

Figure 2d. Effect size estimates of nitrate supplementation versus a placebo control in studies assessing exercise capacity in hypoxia. GXT – graded exercise test; TTE – Time to exhaustion.
Table 1. Summary of included studies examining the effect of nitrate supplementation versus placebo on exercise test performance.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Subjects, n (male)</th>
<th>Nitrate protocol</th>
<th>Exercise assessment</th>
<th>Trial result ± SD</th>
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<tr>
<td><strong>Time Trials</strong></td>
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<tr>
<td>Bescos et al. 2012a</td>
<td>Well trained cyclists 13 (13)</td>
<td>10 mg nitrate per kg body mass a day (as NaNO\textsubscript{3}) for 3 days</td>
<td>40 min cycle distance trial</td>
<td>N: 26.4 ± 1.1 km *</td>
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<td>P: 26.3 ± 1.2 km</td>
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<tr>
<td>Bond et al. 2012</td>
<td>Well trained junior rowers 14 (14)</td>
<td>500ml beetroot juice (~340 mg NO\textsubscript{3}) per day for 6 days</td>
<td>6 × 500m rowing ergometer trials</td>
<td>N: 0.4% ± 1.0% (95% CI) improvement vs P</td>
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<tr>
<td>Cermak et al. 2012a</td>
<td>Well trained cyclists 13 (13)</td>
<td>140ml beetroot juice concentrate/day (~500mg NO\textsubscript{3}) for 6 days</td>
<td>10 km cycle time trial</td>
<td>N: 953 ± 18 s</td>
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<td>P: 965 ± 18 s</td>
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<tr>
<td>Cermak et al. 2012b</td>
<td>Well trained cyclists 20 (20)</td>
<td>140ml beetroot juice concentrate (~550mg NO\textsubscript{3}), 150 min before exercise</td>
<td>Energy expenditure based time trial</td>
<td>N: 65.5 ± 1.1 min</td>
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<td>P: 65.0 ± 1.1 min</td>
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<td>Lansley et al. 2011a (i)</td>
<td>Moderately trained cyclists 9 (9)</td>
<td>500ml beetroot juice (~350mg NO\textsubscript{3}), 150min before exercise</td>
<td>4 km cycle time trial</td>
<td>N: 6.27 ± 0.35 min</td>
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<td>P: 6.45 ± 0.42 min</td>
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<td>Lansley et al. 2011a (ii)</td>
<td>as above</td>
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<td>16 km cycle time trial</td>
<td>N: 26.9 ± 1.8 min</td>
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<td>P: 27.7 ± 2.1 min</td>
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<tr>
<td>Murphy et al. 2012</td>
<td>Healthy, recreationally fit 11 (15)</td>
<td>200g whole beetroot (~500mg NO\textsubscript{3}), 75min before exercise</td>
<td>5km treadmill time trial</td>
<td>N: 12.3 ± 2.7 km/h *</td>
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<td>P: 11.9 ± 2.6 km/h</td>
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* – greater number denotes improved performance
### Reference

**Time Trials (Continued.)**

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<th>Subjects, n (male)</th>
<th>Nitrate protocol</th>
<th>Exercise assessment</th>
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<tr>
<td>Peacock et al. 2012</td>
<td>Well trained cross country skiers 10 (10)</td>
<td>1 g KNO(_3) (614 mg NO(_3^-)), 150 min before exercise</td>
<td>5 km running time trial</td>
<td>N: 1005 ±53 s</td>
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<td>P: 996 ± 49 s</td>
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<tr>
<td>Wilkerson et al. 2012</td>
<td>Well trained cyclists 13 (13)</td>
<td>500 ml beetroot juice (~380 mg NO(_3^-)) , 150 min before exercise</td>
<td>50 mi cycle time trial</td>
<td>N: 136.7 ± 5.6 min</td>
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<td>P: 137.9 ± 6.4 min</td>
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### Time to Exhaustion Tests

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<th>Reference</th>
<th>Subjects, n (male)</th>
<th>Nitrate protocol</th>
<th>Exercise assessment</th>
<th>Trial result ± SD</th>
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<tr>
<td>Bailey et al. 2009</td>
<td>Healthy, recreationally fit 8 (8)</td>
<td>500 ml beetroot juice (~340 mg NO(_3^-)) a day for 6 days</td>
<td>cycling TTE @ 70% between GET and VO(_2)(\text{max})</td>
<td>N: 675 ± 203 s</td>
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<td>P: 583 ± 145 s</td>
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<td>Bailey et al. 2010</td>
<td>Healthy, recreationally fit 7 (7)</td>
<td>500 ml beetroot juice (~300 mg NO(_3^-)) a day for 6 days</td>
<td>leg extension TTE @ 30% MVC</td>
<td>N: 734 ± 109 s</td>
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<td>P: 586 ± 80 s</td>
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<td>Lansley et al. 2011b</td>
<td>Healthy, recreationally fit 9 (9)</td>
<td>500 ml beetroot juice (~380 mg NO(_3^-)) a day for 6 days</td>
<td>treadmill time to exhaustion @ 75% between GET and VO(_2)(\text{max})</td>
<td>N: 8.7 ± 1.8 min</td>
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<td>P: 7.6 ± 1.5 min</td>
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<td>Vanahatlo et al. 2011</td>
<td>Healthy, recreationally fit 9 (7)</td>
<td>750 ml beetroot juice (~580 mg NO(_3^-)), 24 h prior, with last 250 ml 2.5 h pre exercise</td>
<td>leg extension TTE @ 14.5% O(_2)</td>
<td>N: 477 ± 200 s</td>
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<td>P: 393 ± 169 s</td>
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<td>Nitrate protocol</td>
<td>Exercise assessment</td>
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<td><strong>Graded exercise tests</strong></td>
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<td>Bescos et al. 2011</td>
<td>Well trained cyclists 11 (11)</td>
<td>10 mg nitrate per kg body mass (as NaNO(_3)) 3 hr before exercise</td>
<td>cycle incremental TTE</td>
<td>N: 416 ± 32 s</td>
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<td>P: 409 ± 27 s</td>
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<td>Larsen et al. 2007</td>
<td>Moderately trained cyclists 9 (9)</td>
<td>0.1 mmol (6.2 mg) NO(_3) per kg body mass per day (as NaNO(_3)) for 3 days, last dose 60 min before exercise</td>
<td>cycle incremental time to exhaustion</td>
<td>N: 360.6 ± 32.8 Wmax</td>
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<td>P: 358.9 ± 32.3 Wmax</td>
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<td>Larsen et al. 2010</td>
<td>Healthy 9 (7)</td>
<td>0.1 mmol (6.2 mg) NO(_3) per kg body mass per day (as NaNO(_3)) for 2 days</td>
<td>Arm crank and cycle incremental TTE</td>
<td>N: 563 ± 30 s</td>
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<td>P: 524 ± 31 s</td>
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<tr>
<td>Masschelein et al. 2012</td>
<td>Healthy, recreationally fit 15 (15)</td>
<td>0.07 mmol (4.4 mg) NO(_3) per kg body mass per day (as beetroot juice) for 6 days</td>
<td>cycle incremental TTE @ simulated 5,000m altitude</td>
<td>N: 597 ± 22 s</td>
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<td></td>
<td></td>
<td>P: 568 ± 23 s</td>
</tr>
<tr>
<td>Vanahatlo et al. 2010</td>
<td>Healthy, recreationally fit 9 (5)</td>
<td>500 ml beetroot juice (~300 mg NO(_3)) 2.5 h before exercise</td>
<td>cycle incremental TTE</td>
<td>N: 325 ± 71 Wmax</td>
</tr>
<tr>
<td></td>
<td>as above</td>
<td>500 ml beetroot juice (~300 mg NO(_3)) per day for 5 days</td>
<td>as above</td>
<td>N: 328 ± 68 Wmax</td>
</tr>
<tr>
<td></td>
<td>as above</td>
<td>500 ml beetroot juice (~300 mg NO(_3)) per day for 15 days</td>
<td>as above</td>
<td>N: 331 ± 68 Wmax</td>
</tr>
</tbody>
</table>

*Note. TTE – time to exhaustion; Wmax – peak power; N – nitrate trial; P – placebo trial; NR – not reported; GET – gas exchange threshold; MVC – maximal voluntary contraction;*
CHAPTER III: NITRATE SUPPLEMENTATION AND HIGH-INTENSITY PERFORMANCE IN COMPETITIVE CYCLISTS

The following study is a copy of the accepted manuscript:


As co-authors of the paper “Nitrate Supplementation and High-Intensity Performance in Competitive Cyclists” we confirm that Matthew Hoon has made the following contributions:

- Study concept and design
- Significant data collection
- Data analysis and interpretation
- Manuscript preparation

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ABSTRACT

Consumption of inorganic nitrate (NO$_3^-$) is known to enhance endurance exercise performance in recreationally trained subjects. Here we report the effect on a high-intensity performance task in national-level cyclists. The performance test consisted of two cycle-ergometer time trials of 4 min duration with 75 min between trials. In a randomized crossover design, 26 cyclists performed the test under the following 4 conditions, each separated by a 6 day washout: consumption of 70 ml of nitrate-rich beetroot juice at 150 min or 75 min before the first time trial, addition of a 35 ml “top-up dose” following the first time trial in the 150 min condition, and consumption of a placebo. A linear mixed model with adjustments for learning effects and athlete fitness (peak incremental power) was used to estimate effects on mean power, with probabilistic inferences based on a smallest important effect of 1.0%. Peak plasma nitrite (NO$_2^-$) concentration was greatest when nitrate was taken 75 min before the first time trial. Relative to placebo, the mean effect of all 3 nitrate treatments was unclear in the first time trial (1.3%, 90% confidence limits: ±1.7%), but possibly harmful in the second time trial (-0.3%, ±1.6%). Differences between nitrate treatments were unclear, as was the estimate of any consistent individual response to the treatments. Allowing for sampling uncertainty, the effect of nitrate on performance was less than in previous studies. Under the conditions of our experiment, nitrate supplementation may be ineffective in facilitating high-intensity exercise in competitive athletes.

Keywords:
Ergogenic aid, Athlete, Beetroot, Nitrite
INTRODUCTION

The consumption of inorganic nitrate (NO$_3^-$) is currently advocated as a means of improving several facets of cardiovascular health. Recent literature has reported improvements to hypertension (Lundberg et al. 2008), peripheral arterial disease (Pattillo et al. 2010) and metabolic dysfunction (Carlstrom et al. 2010) following doses of nitrate. A currently evolving area of research is now examining the application of nitrate supplementation outside of a clinical setting, focusing on its possible ergogenic effect on exercise performance. Of note, Larsen and colleagues reported that supplementation with sodium nitrate was able to enhance cycling mechanical efficiency (Larsen et al. 2007). Additional studies investigating supplementation with beetroot juice (a rich source of NO$_3^-$) have reported similar changes in efficiency (Bailey et al. 2009), as well as improvements in running endurance (Lansley et al. 2011b), power output at ventilatory threshold and ramp test peak power (Vanhatalo et al. 2010).

The benefits reported in these studies are not attributable to the NO$_3^-$ compound per se, but rather its reduced states: nitrite (NO$_2^-$) and nitric oxide (NO) (Bailey et al. 2011). Ingested NO$_3^-$ is quickly absorbed by the upper gastrointestinal tract before being taken up by the salivary glands and secreted into the mouth, where anaerobic bacteria reduce NO$_3^-$ to bioactive NO$_2^-$. Nitrite may then be reduced further into NO in the stomach or reabsorbed to increase plasma NO$_2^-$ concentration ([NO$_2^-$]). Nitric oxide is known to be an important intermediate in numerous physiological pathways, regulating key functions such as blood flow (Umans and Levi 1995), glucose uptake (Merry et al. 2010), muscle contraction (Maréchal and Gailly 1999) and mitochondrial respiration (Moncada and Erusalimsky 2002). The precise mechanism by which nitrogen derivatives enhance exercise efficiency is not yet fully understood, making it difficult to determine the most practical and applicable use for NO$_3^-$ supplementation.

Although several studies have documented improvements in the performance of exercise lasting 7–20 min (Lansley et al. 2011b; Cermak et al. 2012a; Murphy et al. 2012), there is little research focusing on shorter, more intense activities. High-intensity efforts such as those seen in competitive track cycling have different physiological characteristics to the activities in the aforementioned research, with a much greater rate of energy demand (Jeukendrup et al. 2000). As a consequence of this energy demand the body is placed under a greater hypoxic and acidic stress, conditions favourable for the reduction of NO$_2^-$ to NO.
Recently, nitrate supplementation was reported to improve endurance during a fixed-load, time-to-exhaustion task at severe intensity in healthy males (Kelly et al. 2013). However, it is unclear if this ergogenic effect is apparent in an athletic cohort performing more sport-specific tasks. Furthermore, the competition requirements of typical track cycling events such as the team pursuit require multiple efforts over the course of a day, with competitors usually required to perform qualifying races before the finals. Presently, it is not known whether nitrate can be beneficial to performance when maximal bouts are repeated in the same day or what the optimal dosing regimen might be.

Accordingly, the primary purpose of this study was to examine the effect of NO₃⁻ supplementation on the performance of repeated bouts of high-intensity cycling performance simulating the team pursuit track cycling event. A secondary purpose of the study was to investigate different protocols of supplementation, varying in the timing of nitrate intake in relation to the cycling bouts.

METHODS

Participants

A total of 28 trained male cyclists (Mean ± SD; Age: 20.3 ± 1.4 years; Body Mass: 72.3 ± 6.4 kg; Maximum Aerobic Power: 356 ± 35 W) volunteered to participate in the study. All participants were involved in a 6 week training camp at the Australian Institute of Sport. Written informed consent was obtained from each individual following explanation of the experimental procedures and associated risks, which were approved by the Australian Institute of Sport Ethics Committee.

Pre-experimental trial procedures

Before undertaking experimental trials all subjects underwent a graded exercise test on a cycle ergometer (Wattbike Ltd, Nottingham, UK) to determine maximal aerobic power. After a 10 min warm-up at 100 W, the test protocol began at 150 W and increased by 50 W every 5 min until volitional exhaustion. The method used to determine maximal aerobic power is described elsewhere (Quod et al. 2008). All tests were conducted under controlled laboratory conditions (23 ± 2°C, Barometric pressure (P₀) = 702 mmHg).
Within 1 week of the graded exercise test, subjects performed two separate familiarization trials similar to the experimental protocol to acquaint them with the repeated 4 minute time trials. Subjects were then assigned a trial order based on their maximal aerobic power and body mass, such that there were minimal differences in these characteristics for each trial order (Hopkins 2010).

**Experimental protocol**

The study employed a placebo-controlled, cross-over design which was counter-balanced in a Latin square arrangement. On 4 separate occasions all subjects completed a trial involving 2 bouts of 4 min cycling time trials (TT1 and TT2) which were separated by 75 min. This protocol of repeated 4 min time trials has been previously shown to be a reproducible and reliable performance test, with typical day to day variation approximately 2.6% (Driller et al. 2013). The time trials were performed on the same ergometers used in the pre-experimental procedures (i.e. Wattbike).

In all experimental trials, subjects consumed a beverage (70 ml of either nitrate-rich beetroot juice or nitrate-depleted placebo) at 150 min and 75 min prior to TT1 and an additional half dose 75 min prior to TT2 (Webb et al. 2008). Briefly, combinations of NO$_3^-$ rich and placebo beetroot juice were organised to achieve the following 4 treatments: supplementation to achieve the usual 150 min pre-trial ingestion prior to TT1 (150-PRE); supplementation to achieve 150 min pre-trial ingestion before TT2, occurring 75 min before TT1 (75-PRE); supplementation 150 min before TT1 and an additional dose 75 min before TT2 (TOP UP); placebo treatments at all time points. The study design is summarised in Figure 1. The nitrate-rich beverage was 70 ml of commercially available beetroot concentrate (Beet-it, James White Drinks, Ipswich, UK) which contained 4.1 mmol of NO$_3^-$. The placebo beverage was identical in taste, texture and packaging but contained negligible amounts of NO$_3^-$ (0.03 mmol).

All trials were performed at the same time of day (±1 hr) with 6 days washout between each trial. On the morning of experimental trials, subjects reported to the laboratory 3 hr before TT1 in an overnight-fasted state (*ad libitum* water consumption). Following baseline measures of weight, resting blood pressure and a venous blood sample, subjects were provided with a standardized breakfast (detailed subsequently). Thirty minutes prior to TT1, subjects began a controlled warm-up involving cycling for 10 min at 60% $HR_{\text{max}}$, 5 min at 70% $HR_{\text{max}}$, 3 min at 80% $HR_{\text{max}}$ and 1 min at 90% $HR_{\text{max}}$, followed by 5 min of easy
pedalling. For TT1, under the instruction to achieve the highest average power possible over 4 min. During the time trials, participants received no feedback about their external power output or cadence but could view elapsed time. Following TT1, subjects were instructed to cool-down with freely selected pedal cadence for 10 min. Subjects repeated the same warm-up before TT2 and were again instructed to achieve the highest average power possible. Mean power and energy expenditure over the 4 min were recorded as the performance measures.

**Blood Sampling**

Blood samples were collected at 0, 75, 150 (just before TT1) and 225 min (just before TT2). Samples were collected via indwelling cannula inserted into an antecubital vein using a Vacutainer system (Becton, Dickson and Company, Franklin Lakes, N.J., USA). A 5 ml sample was collected into a lithium-heparin tube which was immediately centrifuged (4000 rcf) at 4°C for 5 min. Plasma was decanted into 500 uL aliquots and placed in a -80°C freezer for later analysis of plasma nitrite concentration [NO₂⁻] via chemiluminescence as described elsewhere (Bailey et al. 2009). All assays were performed in duplicate with the mean reported.

**Dietary Control**

In view of the large number of subjects participating in the study, and their common living environment and on-site food availability, we undertook dietary standardization prior to each trial using the ‘dietary prescription aided by education tools’ method (Jeacocke and Burke 2010). Specifically, an Accredited Sports Dietician who was responsible for the dietary standardization section of the study instructed the subjects on a protocol designed to meet a minimum carbohydrate target of 6 g.kg⁻¹ body mass each day for the 48 hr prior to each trial. After receiving verbal and written education on carbohydrate content, subjects were free to select the type and quantity of food and fluids to meet this target from the options available in their common living environment. Subjects were instructed to keep a detailed food diary over the 2 days. Food diaries allowed both subjects and researchers to track progress toward meeting target intakes. Subjects were also instructed to avoid using chewing gum and mouthwash for the duration of the study to preserve oral bacteria pertinent to nitrate reduction. They were also requested to refrain from the intake of alcohol and other known ergogenic aids during each period of dietary standardization. The dietician lived in
the same environment as the subjects for the duration of the trial and was able to monitor and assist with dietary compliance.

Each morning of testing, subjects arrived at the laboratory after an overnight fast. They provided food diaries to the dietician to check for dietary compliance and were subsequently cleared to undertake the trial. Approximately 2 h before to the commencement of the first time trial, subjects were provided with a standardized breakfast containing carbohydrate = 2.5 g.kg\(^{-1}\) body mass. Throughout the warm up and time trial period, subjects consumed sports products to provide an additional carbohydrate = 2 g.kg\(^{-1}\) body mass according to the following protocol: during the warm up preceding each time trial, subjects consumed a sports gel containing 26 g carbohydrate (PowerBar Gel, PowerBar Oceania, NSW, Australia). Between time trials, subjects were provided with a recovery drink containing 44 g carbohydrate and 15 g protein (PowerBar Protein Plus, PowerBar Oceania, NSW, Australia) and 600-900 ml of sports drink (Gatorade, Cadbury Schweppes, Victoria, Australia) depending on body mass to meet the total carbohydrate target of 2 g.kg\(^{-1}\). Water was consumed ad libitum during testing. No other food or fluids were consumed during the testing period apart from the beetroot juice treatments.

Statistical Analysis

Each cyclist's mean power output in each time trial was the dependent variable in a mixed linear model using the Proc Mixed procedure in SAS (version 9.2, SAS Institute, Cary, N.C., USA). The fixed effects were: the week of testing (4 levels, first to fourth), to adjust for training and habituation effects and a treatment x trial interaction (8 levels), to estimate mean effects of each of the 4 treatments in the 2 time trials. The random effects were: athlete identity, to account for repeated measurement of athletes; the interaction of athlete and treatment identities, to account for repeated measurements on athletes between weeks; the interaction of athlete identity with placebo and nitrate treatments (i.e. 150-PRE, 75-PRE and TOP UP), to estimate individual responses consistent across the 3 nitrate treatments; and the residual, representing test-to-test variation. In a separate mechanisms analysis, a linear fixed effect for [NO\(_2\)-] was included in the model to estimate and adjust for the mean effects of each treatment on [NO\(_2\)-] immediately before to the 2 time trials.

All data are presented as means ± SD unless otherwise stated. Mean power was log-transformed before analysis, and percent effects were estimated via back transformation.
Uncertainty in the estimates of effects on performance are expressed as 90% confidence limits and as probabilities that the true value of the effect was beneficial, trivial or harmful in relation to threshold values for benefit and harm of ±1.0% (Paton and Hopkins, 2006). These probabilities are not presented quantitatively but were used to make a qualitative probabilistic clinical inference about the effect in preference to a statistical inference based on a null-hypothesis test (Hopkins et al. 2009). Briefly, the effect was deemed unclear when the chance of benefit was sufficiently high to warrant use of the treatment but the risk of harm was unacceptable. Such unclear effects were identified as those with an odds ratio of benefit-harm of <66, a ratio that corresponds to an effect that is borderline possibly beneficial (25% chance of benefit) and borderline most unlikely harmful (0.5% risk of harm). All other effects were deemed clinically clear and expressed as the chance of the true effect being trivial, beneficial or harmful with the following scale: 25-75%, possibly; 75-95%, likely; 95-99.5%, very likely; >99.5%, most likely (Hopkins, 2007). Magnitudes of covariates and effects on measures other than performance were evaluated mechanistically (Hopkins et al. 2009): if the confidence interval overlapped thresholds for substantial positive and negative values (±0.20 of the between-subject SD in the placebo condition), the effect was deemed unclear; all other effects were deemed clear and were evaluated probabilistically as described above. The relationship between plasma [NO₂⁻] and performance was examined by mechanistically evaluating the effect of a 2 SD change in [NO₂⁻] from baseline (PLACEBO) on mean power.

RESULTS

One participant elected to withdraw from the study due to intolerance to the beetroot juice and another participant suffered a training injury following the first time trial and could not complete any more testing. Their data was removed from statistical analysis. Two participants elected not to have blood testing performed; therefore [NO₂⁻] analysis was conducted with an n=24. Analysis of participant food diaries determined that any differences between carbohydrate intake (g.kg⁻¹ of body weight) during each trial were likely trivial (99% clear) and therefore unlikely to contribute to any performance effects.

Plasma [NO₂⁻]
The plasma nitrite kinetics for each trial are shown in Figure 2. Baseline $[\text{NO}_2^-]$ was (mean ± SD): 238 ± 87 nM, 254 ± 85 nM, 229 ± 92 nM, and 253 ± 85 nM for PLACEBO, 150-PRE, 75-PRE and TOPUP, respectively. From baseline to 150 min, there was a general increase in plasma $[\text{NO}_2^-]$ across all trials. The rise in the 75-PRE (mean, ±90% confidence limits; 70, ±62 %) was likely greater than the rise in TOPUP (38, ±36 %), which itself was likely greater than the rise in 150-PRE (22, ±31 %) and PLACEBO (13, ±34 %). By 225 min, $[\text{NO}_2^-]$ had increased further in 75-PRE (88, ±85% of baseline), which was likely greater than the increase in TOPUP (51, ±46 %), which in turn was very likely greater that the rise in 150-PRE (24, ±26 %) and PLACEBO (22, ±31 %).

**Cycling Performance**

The mean power of each 4 min time trial is presented in Table 1, and the estimated effects of supplementation on performance are presented in Table 2. Briefly, there was an unclear effect on performance of TT1 when each of the nitrate trials (i.e. 150-PRE, 75-PRE, TOPUP) was compared to PLACEBO, with a combined mean improvement to power of 1.3% (90% confidence limits ±1.7%). There were unclear differences between nitrate conditions (data not shown). There was a “possibly harmful” effect on power in TT2 for each nitrate condition, with an average change of -0.3, ±1.7%. There were unclear differences in the magnitude of effect between the nitrate conditions on TT2 (data not shown). With nitrate supplementation, 9 participants improved in at least 4 of 6 time trials, whereas 3 participants did not improve in any time trials.

$[\text{NO}_2^-]$ and Performance

Changes in $[\text{NO}_2^-]$ are plotted against changes in performance in Figure 3. From the mechanistic analysis, the observed effect of $[\text{NO}_2^-]$ on performance was trivial, with a +2 SD change in $[\text{NO}_2^-]$ associated with a +0.1% change in performance. However, because of the large uncertainty (90% confidence limits ±2.5%) this effect was unclear.

**Random Effects**
From the analysis of the random effects, time trial-to-time trial SEM (expressed as a coefficient of variation) was 2.1% (90% confidence limits ×/÷1.13), whereas week-to-week error was 4.0% (×/÷1.29). The CV for individual responses (in performance) to NO$_3^-$ trials was estimated to be -0.9% (±3.9%).

**DISCUSSION**

The principal finding from our investigation was that nitrate supplementation (via a 70-105 ml dose of commercially available concentrated beetroot juice) had an unclear effect on the performance of a maximal 4 min bout of cycling. Performance of a subsequent bout 75 min later appeared to be worse with NO$_3^-$ supplementation compared to a placebo; however the possibility of the positive (yet unclear) performance boost of NO$_3^-$ in TT1 resulting in residual fatigue during TT2 cannot be excluded. These results are not in agreement with previous research; however the current study is one of the first to examine the effect of NO$_3^-$ supplementation by higher calibre athletes on repeat performance of high-intensity cycling.

*Effect of nitrate dose and timing on performance*

No discernible differences in performance of the initial time trial were detected from staggering the timing of nitrate ingestion. It was hypothesized that 150-PRE would result in a greater power output than 75-PRE for TT1, but that the latter would exhibit greater performance during the TT2; however, both had similar outcomes. Both dosing regimens were found to have a possibly negative effect on the second time trial, and an additional half dose of nitrate in the TOPUP trial was unable to alter this. Although the magnitude of effect on TT2 (-0.3, ±1.7%) was smaller than the unclear effect for TT1 (+1.3, ±1.7%), supplementation was still deemed to be possibly negative. This is due to the lower threshold for substantial chance of harm (0.5%) compared to benefit (25%) in the statistical analysis and given the 90% CL extend into positive effects, it must be considered that nitrate may be beneficial in a secondary time trial for some. Furthermore we cannot conclude that NO$_3^-$ is harmful to secondary bouts of high-intensity exercise, as the interaction between time trials must be considered. It is possible that the small positive effect of NO$_3^-$ on TT1 resulted in greater post test fatigue causing poorer performance in TT2. Despite the net effect over both time trials leaning slightly in favour of nitrate supplementation (0.5, ±1.6%), the effect falls
below the smallest meaningful change as described by Paton and Hopkins (Paton and Hopkins 2006). Overall the reported variation in individual response to NO$\textsuperscript{3-}$ (-0.9%) represents no substantial differences among participants; however in light of the large sampling uncertainty in the present study (±3.9%) we cannot rule out the possibility of a substantial individual response being found in a larger sample.

Although previous studies have reported significant improvements in time-to-exhaustion tasks and related performance measures, the populations under investigation have generally been recreationally active individuals or club-level athletes. In contrast, the cohort of well-trained athletes in the current investigation were training approximately 20 hr per week and competing at the highest level of national racing. There has been a recent suggestion that the physiological effects elicited by NO$\textsuperscript{3-}$ supplementation is blunted in highly fit populations (Bescós et al. 2012). This notion is supported by other studies of trained individuals which have failed to find an improvement to exercise performance associated with acute nitrate supplementation (Cermak et al. 2012b; Peacock et al. 2012; Bescos et al. 2012). This phenomenon may be attributable to fitter individuals having greater capacity for producing NO through the ‘conventional’ pathway in which L-arginine is oxidised in a reaction catalysed by nitric oxide synthase (NOS) (Vassalle et al. 2003). A better developed NOS system may result in a diminished need for the NO$\textsuperscript{3-}$NO$\textsuperscript{2-}$ pathway to synthesize NO, thereby moderating the effects of NO$\textsuperscript{3-}$ supplementation. Additionally, physiological adaptations to endurance training (i.e. increased capillary density) serve to reduce local muscular hypoxia and acidosis, conditions that would facilitate the reduction of NO$\textsuperscript{2-}$ to NO (Lundberg et al. 2008).

The amount of nitrate prescribed in the current study may have been inadequate. There has been a suggestion that endurance trained athletes may require a greater acute NO$\textsuperscript{3-}$ dose to elicit performance benefits compared to non-trained counterparts (Hoon et al. 2013a). Indeed, a 8.4 mmol NO$\textsuperscript{3-}$ bolus improved 2,000 m rowing time in a group of well-trained rowers, whereas a 4.2 mmol dose (similar to the present study) did not (Hoon et al. 2013b). In a similar study, time to completion of multiple bouts of 500 m rowing were improved in well-trained juniors following consumption of 500 ml of beetroot juice per day for 6 days (Bond et al. 2012). Coupled with other results (Vanhatalo et al. 2010), these findings
suggest that a moderate to large dose of NO$_3^-$ may be required for performance benefits to be seen in trained athletes, and a chronic supplementation strategy (i.e. supplementing with NO$_3^-$ for several days preceding exercise) may be a practical and effective means of providing these quantities. Although the overall low number of responders in the present study may be attributable to the small dose of NO$_3^-$, some participants were able to improve most time trials following a relatively small serving of nitrate. In contrast, 3 participants were unable to improve regardless of NO$_3^-$ dose or time. This *responder and non-responder* trait has also been noted in previous literature (Wilkerson et al. 2012; Wylie et al. 2013a), suggesting that some individuals may be more sensitive to the effects of NO$_3^-$ than others. The physiological mechanisms responsible for the differences in inter-individual response to NO$_3^-$ supplementation remain to be determined and require further investigation.

Nitrate supplementation is hypothesized to be more effective for improving short, high-intensity exercise performance compared to longer, less intense activities (Hoon et al. 2013b; Wilkerson et al. 2012). Higher intensities are more likely to subject working muscles to a hypoxic and acidic environment, facilitating the reduction of NO$_2^-$ to NO (Lundberg et al. 2008). Although the extreme intensity (Jones and Poole 2005) of the 4 min time trials likely elicited these favourable conditions, the present study was unable to show any substantial improvement to performance following NO$_3^-$ supplementation. As discussed previously, this was most likely attributable to the small prescribed dose of NO$_3^-$ relative to the high fitness of subjects. Because of these considerations, we cannot draw any conclusions regarding the effect of exercise intensity on the efficacy of nitrate supplementation from the present study.

**Effects of NO$_3^-$ supplementation on [NO$_2^-$]**

In the present study, all NO$_3^-$ trials successfully elevated plasma [NO$_2^-$]; however this was not accompanied by a detectable improvement in performance. The peak concentrations in [NO$_2^-$] (339 ± 89, 384 ± 136, and 372 ± 105 for 150-PRE, 75-PRE and TOP UP, respectively) occurred between 75-150 min post-ingestion and are comparable to findings in investigations employing similar design and analysis (Wylie et al. 2013b). However, despite similar peak concentrations, the relative increase in [NO$_2^-$] was considerably smaller in the present investigation, likely due to an elevated baseline [NO$_2^-$]. Fitter individuals have been noted to have a greater resting [NO$_2^-$] compared to lesser trained counterparts (Jungersten et
al. 1997). Indeed, the mean [NO₂⁻] baseline value in the present study (244 ± 83 nM) was greater than those reported in studies utilizing untrained participants and similar NO₂⁻ analysis techniques (Bailey et al. 2009; Bailey et al. 2010; Lansley et al. 2011b; Wylie et al. 2013b). The relative change in [NO₂⁻] may be a principal determinant of the effectiveness of NO₃⁻ supplementation and perhaps why performance changes were not detectable in the current study.

Previous investigations have reported a positive correlation between the change in [NO₂⁻] and change in exercise capacity (Wilkerson et al. 2012; Wylie et al. 2013b). This relationship was not confirmed in the present study perhaps because of the large SD of [NO₂⁻] and the small changes in performance. The large variation in [NO₂⁻] (even in this cohort of similarly trained cyclists) is not uncommon and has been observed in other investigations, where up to a ~50% SD in baseline [NO₂⁻] has been reported (Lansley et al. 2011a; Lansley et al. 2011b; Wylie et al. 2013a). Discrepancies in [NO₂⁻] are further amplified when the individual response to supplementation is considered, as evident by the increasing SD over time following NO₃⁻ ingestion. Further research is required to elucidate the particular factors that may influence an individual’s ability to reduce NO₃⁻ to NO₂⁻, and how this aspect may impact exercise performance.

Limitations and future perspectives

Strengths of this study included the Latin square balanced crossover design and large sample size compared to most other studies; however, a major limitation was the large week-to-week variation in performance (4%) which resulted in greater uncertainty about the ergogenic effect of nitrate. Despite this, the between time trial error of 2% was typical for this cohort and performance test (Driller et al. 2013), indicating that testing was performed to its highest standard. In addition, although dietary records were kept over the duration of the study, subjects were able to eat ad libitum without restriction on food types, and it must be considered that there was a background level of nitrate consumption, estimated to be 100-150 mg in a typical Western diet (Zeegers et al. 2006). However, allowing subjects to freely select food presents a more ecologically valid examination of the efficacy of nitrate supplementation. Other studies have restricted dietary nitrate consumption for the course of
the study (Bescos et al. 2011; Larsen et al. 2011; Larsen et al. 2007), which may have amplified the effect of the nitrate supplement given.

Further research should seek to refine guidelines surrounding the use of nitrate supplements, identifying the appropriate populations and situations for its use. Although the present study was unable to confirm its applicability to high intensity cycling, previous studies have demonstrated that, given the right conditions, nitrate is able to elicit positive improvements in exercise capacity. Conversely, the present study indicates that the potential for a negative effect on performance must also be considered.

**Conclusion**

The primary finding from the present study was that NO$_3^-$ supplementation administered as beetroot juice concentrate was unable to elicit performance gains to short duration, high intensity. A subsequent effort appears to be negatively affected; however this may be attributable to a small improvement in performance of the initial bout (unclear in the present study) leaving residual fatigue. This was found to be true up to an amount of ~500 mg of NO$_3^-$ ingested 75-150 min before exercise. These results occurred despite supplementation causing a rise in plasma [NO$_2^-$], hypothetically increasing the amount of available NO to support physiological processes during exercise. This finding is contradictory to previous research, but may be explained, in part, by the highly-trained nature of the subjects. Under the conditions of our experiment, nitrate supplementation may not be effective in facilitating high intensity exercise in well-trained athletes.

**Conflict of interest statement**

All authors declare that there are no conflicts of interest present.

**Acknowledgements**

Funding was provided by the Australian Institute of Sport (AIS) Sports Supplement Program. The authors would like to thank all the subjects for participating and all the research assistants from the AIS involved with the study.
REFERENCES


Figure 1. Trial schedule for each experimental condition. 150-PRE trial consumed 1 bottle (70 ml; 4.1 mmol NO$_3^-$) nitrate-rich beetroot juice 150 min before the onset of TT1, 75-PRE took beetroot juice 75 min before TT1 and in TOPUP condition beetroot juice was taken 150 min prior to TT1 with an additional half dose immediately after. No beetroot juice was taken in the placebo condition (PLA). The placebo beverage contained 0.03 mmol NO$_3^-$.

Figure 2. Mean plasma nitrite concentration ([NO$_2^-$]) for each supplementation condition with SD (error bars). Time trial 1 (TT1) occurs at 150 min and time trial 2 (TT2) occurs at 225 min. * likely greater than 150-PRE; ** likely greater than TOPUP; + very likely greater than 150-PRE. 75-PRE and 150-PRE time points have been offset (+2 min) for clarity.
Figure 3. The relationship between changes in plasma nitrite ($\text{NO}_2^-$) concentration (%) and changes in time trial mean power (%). Change scores are calculated by comparing nitrate ingesting trials to the PLACEBO condition, which was used as baseline results. Results for both time trials are shown.
Table 1. Mean power (±SD) for each 4 min time trial performance in all supplementation conditions.

<table>
<thead>
<tr>
<th></th>
<th>Time trial 1 Mean Power ± SD (W)</th>
<th>Time trial 2 Mean Power ± SD (W)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLACEBO</td>
<td>396 ± 57</td>
<td>397 ± 56</td>
</tr>
<tr>
<td>150-PRE</td>
<td>402 ± 47</td>
<td>396 ± 46</td>
</tr>
<tr>
<td>75-PRE</td>
<td>403 ± 52</td>
<td>396 ± 54</td>
</tr>
<tr>
<td>TOPUP</td>
<td>400 ± 48</td>
<td>396 ± 45</td>
</tr>
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</table>
Table 2. Effect of nitrate supplementation on 4-min time-trial performance in all cyclists.

<table>
<thead>
<tr>
<th>Time trial 1</th>
<th>Difference in mean vs. PLACEBO (%) ±90%CL</th>
<th>Practical inference a</th>
</tr>
</thead>
<tbody>
<tr>
<td>150-PRE</td>
<td>1.2, ±2.1</td>
<td>Unclear</td>
</tr>
<tr>
<td>75-PRE</td>
<td>1.7, ±2.1</td>
<td>Unclear</td>
</tr>
<tr>
<td>TOPUP</td>
<td>1.1, ±2.1</td>
<td>Unclear</td>
</tr>
<tr>
<td>ALL</td>
<td>1.3, ±1.7</td>
<td>Unclear</td>
</tr>
<tr>
<td>Time trial 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150-PRE</td>
<td>-0.4, ±2.1</td>
<td>Possibly harmful</td>
</tr>
<tr>
<td>75-PRE</td>
<td>-0.5, ±2.1</td>
<td>Possibly harmful</td>
</tr>
<tr>
<td>TOPUP</td>
<td>-0.1, ±2.1</td>
<td>Unlikely harmful</td>
</tr>
<tr>
<td>ALL</td>
<td>-0.3, ±1.7</td>
<td>Possibly harmful</td>
</tr>
<tr>
<td>Both time trials</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150-PRE</td>
<td>0.4, ±2.0</td>
<td>Unclear</td>
</tr>
<tr>
<td>75-PRE</td>
<td>0.6, ±2.0</td>
<td>Unclear</td>
</tr>
<tr>
<td>TOPUP</td>
<td>0.5, ±2.0</td>
<td>Unclear</td>
</tr>
<tr>
<td>ALL</td>
<td>0.5, ±1.6</td>
<td>Unclear</td>
</tr>
</tbody>
</table>

aBased on a smallest worthwhile change of 1%. Unclear effects are defined by a benefit-harm odds ratio of <66, corresponding to an effect that is borderline possibly beneficial (25% chance of benefit) and borderline most unlikely harmful (0.5% risk of harm). Other effects are deemed clinically clear and expressed as the chance of the effect being trivial, beneficial or harmful with the following scale: 25-75%, possibly; 75-95%, likely; 95-99.5%, very likely; >99.5%, most likely.
CHAPTER IV: THE EFFECT OF DIFFERENT DOSES OF INORGANIC NITRATE-RICH BEEFROOT JUICE ON SIMULATED 2,000 M ROWING PERFORMANCE IN TRAINED ATHLETES

The following study is a copy of the accepted manuscript:


As co-authors of the paper “The Effect of Variable Doses of Inorganic Nitrate-Rich Beetroot Juice on Simulated 2000-m Rowing Performance in Trained Athletes” we confirm that Matthew Hoon has made the following contributions:

- Study concept and design
- Significant data collection
- Data analysis and interpretation
- Manuscript preparation

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ABSTRACT

Context: Beetroot juice is a naturally rich source of inorganic nitrate (NO₃⁻), a compound hypothesized to enhance endurance performance by improving exercise efficiency. Purpose: This study investigated the effect of different doses of beetroot juice on 2,000 m ergometer rowing performance in highly trained athletes. Methods: Ten highly trained male rowers volunteered to participate in a placebo controlled, double blinded cross–over study. Two hours before undertaking a 2,000 m rowing ergometer test, subjects consumed beetroot juice containing 0 mmol (PLACEBO), 4.2 mmol (SINGLE) or 8.4 mmol (DOUBLE) NO₃⁻. Blood samples were taken before supplement ingestion and immediately before the rowing test for analysis of plasma [NO₃⁻] and [nitrite (NO₂⁻)]. Results: The SINGLE dose demonstrated a trivial effect on time to complete 2,000 m compared to PLACEBO (mean difference: 0.2 ± 2.5 s). A possibly beneficial effect was found with DOUBLE compared to SINGLE (mean difference: -1.8 ± 2.1 s) and PLACEBO (-1.6 ± 1.6 s) respectively. Plasma [NO₂⁻] and [NO₃⁻] demonstrated a dose-response effect, with greater amounts of ingested nitrate leading to substantially higher concentrations (DOUBLE > SINGLE > PLACEBO). There was a moderate but insignificant correlation (r=-0.593, p=0.055) between change in plasma [NO₂⁻] and performance time. Conclusion: When compared with nitrate-depleted beetroot juice, a high (8.4 mmol NO₃⁻) but not moderate (4.2 mmol NO₃⁻) dose of NO₃⁻ in beetroot juice, consumed 2 hours before exercise, may improve 2,000m rowing performance in highly trained athletes.

Keywords: supplements, ergogenic aid, endurance, nitric oxide
INTRODUCTION

Supplements purported to increase nitric oxide (NO) have gained considerable research attention as ergogenic aids for sports and exercise. Nitric oxide is known to mediate numerous physiological processes that facilitate the maintenance of exercise including glucose uptake, blood flow distribution, muscle contraction and neurotransmission. It has been hypothesized that increasing the pools of bio-available NO may enhance the capacity of these processes to accommodate improved exercise performance. Since endogenous NO is produced from the oxidization of L-arginine by the NO synthase (NOS) enzyme family, supplementation strategies have focused on increasing concentrations of L-arginine or its antecedent L-citrulline. Indeed, some studies in healthy populations have reported improvements in muscular strength, cycling time to exhaustion and total work performed following the use of such supplements.

Recently it has been discovered there exists a NOS-independent method of NO production, the nitrate-nitrite-nitric oxide pathway. Along this pathway, nitrate (NO$_3^-$) may be reduced to nitrite (NO$_2^-$), which may then be reduced to NO. In this way endogenous stores of NO$_3^-$ and NO$_2^-$ may be effectively viewed as NO “reservoirs” and thus strategies to boost these may present as alternative means of enhancing exercise performance. Of these, attempts to increase NO$_3^-$ appear to be most practical as inorganic NO$_3^-$ is easily consumed through a targeted diet and does not appear to carry the same health risks as NO$_2^-$ supplementation.

Early studies of inorganic nitrate supplementation reported improvement in gross efficiency during exercise, a known predictor of endurance performance, following several days of supplementation with sodium nitrate or increased consumption of natural food sources such as beetroot juice. Recent work has built upon these results by examining the effect of supplementation on direct measures of exercise performance. Cermak et al. and Lansley et al. both reported 2-3% improvements in cycling time trial performance following consumption of beetroot juice across distances ranging from 4-16 km in trained cyclists. In the latter study subjects were supplemented just 2.5 hrs prior to the onset of exercise, indicating that ergogenic effects may be apparent shortly after administration.

Few studies have examined the efficacy of nitrate supplementation in highly trained athletes. A recent review suggested that the performance benefit of supplements which increase NO may be blunted in trained individuals whose cardiovascular system is nearing its physiological capacity and thus has little scope for further enhancement. Furthermore,
baseline plasma NO\textsubscript{2} and NO\textsubscript{3} levels have been reported to be higher in athletes compared with non-athletic individuals,\textsuperscript{17,18} potentially reducing the effectiveness of inorganic NO\textsubscript{3}\textsuperscript{−} supplementation. In this vein, a greater dose of NO\textsubscript{3}\textsuperscript{−} may be required in athletic cohorts than those employed in earlier studies to elicit ergogenic effects. Indeed, in a recent study of trained cyclists who were supplemented with approximately twice the amount of NO\textsubscript{3}\textsuperscript{−} (~8 mmol NO\textsubscript{3}\textsuperscript{−} via concentrated beetroot juice) used in previous investigations, 10 km cycling time trial performance was significantly improved compared with a placebo-supplemented condition.\textsuperscript{15}

Competitive 2,000 m rowing typically requires continuous whole-body work at ~90% of VO\textsubscript{2}max for approximately 6-7 min,\textsuperscript{19} an ideal environment for the use of nitrate as an ergogenic aid. These sustained high-intensity efforts are likely to place the body into an acidic and hypoxic state,\textsuperscript{20} where the nitrate reduction pathway becomes more active in the supply of NO.\textsuperscript{9} Therefore, the purpose of the current study was to investigate the effect of high and moderate doses of nitrate supplementation (using nitrate-rich beetroot juice) compared to a placebo control on the performance of a 2,000 m rowing ergometer test in highly trained rowers. We hypothesized that a high dose of nitrate-rich beetroot juice would be required for observable performance improvement in trained athletes.

METHODS

Subjects

Ten highly trained males, whose characteristics are presented in Table 1, were recruited for the study. The group included 3 lightweight and 7 heavyweight rowers who were all competitive rowers racing at a sub-elite competition level. Written informed consent was obtained from each individual following explanation of the experimental procedures and their associated risks. The study was approved by the Australian Institute of Sport Ethics Committee.

Design

Participants completed 3 supplementation trials in a counterbalanced cross-over design, using a Latin-square arrangement, with each trial separated by at least 48 hours. Each trial required the consumption of beetroot juice two hours prior to a 2,000 m rowing ergometer
time trial, with the amount of nitrate ingested varying between trials. The desired nitrate dose was achieved by the intake of two 70 ml bottles of beetroot juice that were either nitrate-rich or nitrate-depleted (placebo). The nitrate-rich beetroot juice was a commercially available product (Beet-it, James White Drinks, Ipswich, UK) which contained 4.2 mmol of nitrate per bottle. The placebo-control beverage was identical in taste, appearance and packaging to the treatment beverage with nitrate removed by the manufacturer to negligible levels. The treatments were organized so that participants ingested two bottles of nitrate-rich beetroot juice prior to the time trial in the high nitrate treatment (= DOUBLE; 8.4 mmol nitrate), one bottle each of the nitrate-rich and nitrate-depleted juice in the moderate nitrate trial (= SINGLE; 4.2 mmol nitrate) and two bottles of the nitrate-depleted juice in the control trial (CONTROL; 0 mmol nitrate).

**Methodology**

All trials were performed between 0600 and 0800. We collected baseline measures of body mass as well as a venous blood sample following 10 min of passive rest (pre). Subjects then consumed two bottles of beetroot juice according to their trial allocation. Following 90 min of passive rest, a second blood sample and was then obtained before subjects initiated a 30 min self-administered warm-up routine which consisted of stretching and light to moderate intensity rowing.

All rowing was completed in a laboratory setting (21 °C, 60% humidity) on a Concept II ergometer (Model D, Vermont, USA) which was mounted on slides (Concept2, Concept, Vermont, USA) according to the national rowing body’s guidelines. Researchers conducting performance testing were unaware of the supplementation trial each subject was assigned to. For the 2,000 m time trial participants were instructed to “obtain the best time possible.” Participants were allowed to view concurrent data on the display unit (time elapsed, stroke rate, heart rate, 500 m pace) and received no other feedback. All participants were familiar with the exercise test as they were required to complete one each month during their regular training as part of the national rowing selection protocol.

**Blood Sampling**

Blood samples were collected by venepuncture from an antecubital vein using a vacutainer system (Becton, Dickson and Company, New Jersey, USA) as the subject lay supine. A 9 ml sample was collected into a lithium-heparin tube which was immediately centrifuged (4,500 rpm) at 4°C for 8 min. Plasma was decanted into 500 uL aliquots and placed in a -80°C
freezer for later analysis of [NO$_2$] and [NO$_3$] via chemiluminescence. The procedure for plasma [NO$_2$] analysis has been detailed elsewhere. For [NO$_3$] analysis, plasma samples were deproteinised in an aqueous solution of zinc sulphate (5% w/v) and 0.5M sodium hydroxide, prior to analysis. NO$_3$ was reduced to NO in a solution of vanadium (III) chloride in 1 M hydrochloric acid (0.8% w/v) at 95°C. The spectral emission of electronically excited nitrogen dioxide product from the NO reaction with ozone is detected by a thermoelectrically cooled, red-sensitive photomultiplier tube housed in a Sievers gas-phase chemiluminescence NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The [NO$_3$] was determined by plotting signal (mV) area against a calibration plot of 100nM to 10µM sodium nitrate. All assays were conducted by a laboratory technician blinded to the trial allocation of subjects. Analyses were performed in duplicate with the mean reported. A blood sample was also collected from the ear lobe one minute after the completion of each time trial for determination of blood lactate concentration using a Lactate Pro Blood Analyzer (Arkray, Kyoto, Japan).

**Dietary and Exercise Training Standardisation**

Training during the 24-hour period prior to each performance trial was standardised for each individual by scheduling each testing day for an identical time of the training week. Dietary intake was standardized during the 24-hour period before each trial using a combination of replication of usual diet and standardized diet techniques. Specifically, subjects were instructed to follow their normal training diet and fluid intake for 24 hours prior to each trial but were asked to refrain from all caffeine and alcohol during this period. Subjects were also instructed to avoid using chewing gum and mouth wash (to preserve oral bacteria that may facilitate nitrate reduction) and refrain from all other ergogenic supplements before testing. Instructions on how to keep a food diary were provided. A copy of the food diary kept prior to the first trial was provided to subjects with the instructions to replicate this eating pattern as closely as possible before subsequent trials, with a further record being kept to assess compliance. To aid glycogen repletion following the training session undertaken on the afternoon prior to each trial, subjects were provided with a standardized snack providing 82 g of carbohydrate, to be consumed immediately after the session. On arrival to the laboratory on a trial day, the subject’s diary was qualitatively assessed for compliance to these dietary instructions. Semi-quantitative analysis of these dietary records revealed that all subjects complied with the study requirements by consuming the post-exercise carbohydrate-rich snack, avoiding the specified dietary constituents and consuming
a carbohydrate intake within 0.5 g/kg BM of their trial 1 intake during the 24-hour period prior to subsequent trials.

**Post-test questionnaire**

After each trial subjects were asked to report any gastric symptoms that may have arisen from the administered treatment/placebo juice. Following completion of the third and final trial, subjects were asked: to guess the order of treatment they were given; if they believed nitrate supplementation had any effect on their performance; and if they would chose to use it in a real race situation.

**Statistical Analysis**

The post-only crossover spreadsheet developed by Hopkins\(^{23}\) was used to compare differences between trials with pairwise comparisons made between all trials. For rowing performance, the mean effect (±90% confidence limits) was calculated and a clinical inference was then made based on a smallest worthwhile change of 0.5%, and the probability that the effect was beneficial, trivial or harmful\(^{23,24}\) Briefly, the probabilistic terms were set at: 0.5%, almost certainly not; 0.5–5%, very unlikely; 5–25%, unlikely or probably not; 25–75%, possibly; 75–95%, likely or probably; 95–99.5%, very likely; >99.5%, almost certainly.

For other variables (change in plasma [NO\(_2^-\)] and [NO\(_3^-\)]; post exercise blood [La\(^+\)]; average heart rate), a mechanistic inference was calculated i.e. if the 90% confidence interval overlapped thresholds for substantial positive and negative values (±0.20 standardized units, i.e., 0.20 of the between-subject SD in the placebo condition\(^{25}\)), the effect was deemed unclear; all other effects were deemed clear (either positive or negative) and were evaluated probabilistically as described above.

A Pearson product-moment correlation was also conducted (IBM SPSS statistics 19.0.0) to examine the relationship between differences in plasma [NO\(_2^-\)] and the change in 2,000 m time, with CONTROL results used as the baseline and confidence set at \(P<0.05\). All data are presented as mean ± SD unless otherwise stated.
RESULTS

Of 10 participants, only 3 were able to correctly guess the order of treatment indicating the blinding of the beetroot juice had been successful. No major adverse reactions were reported by any participant, however one subject described slight gastrointestinal symptoms immediately after beverage consumption across trials, whilst another reported slight gastrointestinal discomfort following consumption during the SINGLE trial; all of which had resolved by the onset of the 2,000 m test. One participant elected not to have blood samples taken during the study.

Effect of nitrate supplementation

The results of each supplementation trial are presented in Table 2. Compared with the PLACEBO trial, differences in 2000 m performance in the SINGLE trial were likely trivial, with a mean difference of 0.2 s (90% confidence limits ± 2.5 s). The DOUBLE dose was possibly beneficial to performance compared with both PLACEBO (-1.6 ± 2.5 s) and SINGLE (-1.8 ± 2.1 s) (Table 2a).

Two of the plasma samples were uncollected and two were found to be haemolysed (2 = PLACEBO; 1 = SINGLE; 1= DOUBLE). One sample was found to contain [NO$_2^-$] and [NO$_3^-$] >30 SD greater than the mean. These were excluded from analysis. The change in baseline to post supplement plasma [NO$_2^-$] and [NO$_3^-$] was greater in both SINGLE and DOUBLE versus PLACEBO, with greater changes in both plasma [NO$_2^-$] and [NO$_3^-$] in DOUBLE compared to SINGLE (Table 2b). There was a moderate correlation (r = -0.593) between change in plasma [NO$_2^-$] and change in time to completion which almost reached statistical significance (p = 0.055, Figure 1). The effect of SINGLE or DOUBLE versus PLACEBO on blood [La$^-$] was unclear (Table 2b).

DISCUSSION

The primary findings of the current study was that a high dose (140 ml) of nitrate-rich beetroot juice (containing approximately ~550 mg NO$_3^-$) may improve 2,000 m rowing performance in highly trained rowers, however a standard dose (70 ml) is unlikely to elicit an ergogenic effect. This is the first study to report possible benefits from acute NO$_3^-$ supplementation using a laboratory measure frequently used to simulate race performance in
rowing. Our findings support previous data from Cermak and colleagues showing that beetroot juice supplementation can enhance performance in highly trained athletes. From the available literature, the efficacy of using inorganic NO\textsuperscript{3−} supplementation to enhance exercise performance in highly trained athletes is unclear. In contrast to our findings and those of Cermak et al., other researchers have failed to detect an improvement in performance following NO\textsuperscript{3−} supplementation. For example, supplementation with a nitrate salt was shown to have no measurable effect on 5 km treadmill time in elite cross-country skiers\textsuperscript{26} or 40 min cycle distance in trained cyclists.\textsuperscript{27} The difference in exercise intensity between these studies and the 2,000 m rowing task employed in the present study may partly explain the difference in results. The mean time for completion of the 2,000 m by participants in the current study was 6:23 min, compared with the >20 min required to complete the performance trials in the aforementioned investigations.\textsuperscript{26,27} The reduction of NO\textsubscript{2} to NO (the agents associated with the physiological benefit of supplementation)\textsuperscript{5} is known to be more active in hypoxic and acidic environments which would be more likely observed during intensive exercise.\textsuperscript{9} This may suggest that nitrate supplementation is most efficacious for supramaximal or perimaximal endurance exercise. Indeed, a recent paper by Bond and colleagues reported improvement in repeated sets of supramaximal 500 m rowing efforts following beetroot juice supplementation.\textsuperscript{28} Overall, the magnitude of benefit from NO\textsuperscript{3−} supplementation in individuals appears to be much more variable in studies employing highly trained or elite cohorts compared with those using recreationally trained populations in whom the benefits appear consistent.\textsuperscript{13,14,21} This may be due to the greater levels of baseline plasma NO\textsuperscript{3−} and NO\textsubscript{2} reported in some elite athletes\textsuperscript{18} and/or a highly adapted cardiorespiratory system diluting the physiological impact of the NO\textsuperscript{3−} supplementation.\textsuperscript{5} Therefore, a primary objective of the present study was to determine if beneficial effects could be elicited after consuming larger amounts of NO\textsuperscript{3−}, in light of potential training adaptations in highly trained athletes. Confirming our initial hypothesis, the single serving, moderate dose of nitrate-rich beetroot juice led to no detectable change in exercise performance, despite previous studies demonstrating a benefit of a similar dose in lesser trained individuals.\textsuperscript{16} In contrast, the high dose of NO\textsuperscript{3−} was associated with a probable improvement in 2,000 m rowing time. The mechanism underpinning this benefit is likely related to the increase in NO levels following the chemical reduction of the ingested NO\textsuperscript{3−}. However, the correlation between NO levels and physiological enhancement remains theoretical as NO is difficult to measure.
in vivo during exercise. As a consequence plasma \([\text{NO}_2^-]\) has been adopted as a surrogate measure. Our analysis found a dose-response effect, with greater amounts of ingested \(\text{NO}_3^-\) leading to higher concentrations of \([\text{NO}_2^-]\) and \([\text{NO}_3^-]\) in the plasma (DOUBLE > SINGLE > PLACEBO respectively). Changes in \([\text{NO}_2^-]\) reached a near-significant correlation with 2,000 m performance \((p = 0.055)\), such that greater levels of plasma \([\text{NO}_2^-]\) were associated with reductions in time to completion, similar to the findings of Wylie et al.\(^{29}\) and Wilkerson et al.\(^{30}\) These results suggest the ergogenic effect of nitrate supplementation and performance improvement may be related to an individual’s ability to elevate \([\text{NO}_2^-]\). However, it must be noted that \([\text{NO}_2^-]\) can vary significantly between individuals, even in apparently homogenous cohorts. This has been observed in a majority of investigations, often with a \(\sim 50\%\) standard deviation in \([\text{NO}_2^-]\) at baseline.\(^{16,21,29}\) Furthermore there appears to be a varied intra-individual response to nitrate supplementation, where in some instances \([\text{NO}_2^-]\) was raised significantly following supplementation, and in other cases it remained unaltered.\(^{30}\) The determinants of individual \([\text{NO}_2^-]\) and response to nitrate supplementation require further research.

Our study suggested that a threshold dose of nitrate (\(\sim 8.4\) mmol in this case) must be ingested to observe notable improvements in exercise performance. This is consistent with recent work which also reported that a \(\sim 8.4\) mmol dose of nitrate was required to see improvement in a time to exhaustion exercise task.\(^{31}\) However, despite the positive relationship between ingested \(\text{NO}_3^-\) and plasma \([\text{NO}_2^-]\), and the correlation between plasma \([\text{NO}_2^-]\) and exercise capacity or performance, the study also showed evidence of a maximum dose of acute \(\text{NO}_3^-\) ingestion beyond which no further improvements in exercise outcomes can be consistently detected. Specifically, although a 16.8 mmol \(\text{NO}_3^-\) dose elevated plasma \([\text{NO}_2^-]\) to a greater extent than an 8.4 mmol \(\text{NO}_3^-\) dose, no additional performance benefits were evident.\(^{31}\)

A more effective strategy for nitrate ingestion may be to chronically supplement athletes over several days prior to competition. Previous work has demonstrated greater improvements to \(\text{VO}_2\text{max}\), peak power and gas exchange threshold following 5 and 15 days of nitrate supplementation compared to an acute dose 2.5 hours before exercise.\(^{32}\) Furthermore, several days of supplementation may induce changes to mitochondrial protein expression, an effect unlikely to be seen after a single bolus.\(^{33}\) The optimal source of \(\text{NO}_3^-\) for ergogenic effect on endurance exercise performance has yet to be established. A wide variety of sources have been employed across studies, which makes collective interpretation...
of results difficult. For instance, no improvement in 5 km run time was seen in a cohort of highly trained cross-country skiers following ingestion of potassium nitrate.\(^{26}\) Although the amount of nitrate delivered by the potassium nitrate (614 mg NO\(_3^-\)) was greater than the beetroot juice (520 mg NO\(_3^-\)) in the present study, the bio-availability of the NO\(_3^-\) may vary. Other sources of NO\(_3^-\) investigated elsewhere include sodium nitrate\(^{14,27}\) and whole beetroots.\(^{34}\) Although chemical sources of NO\(_3^-\) (potassium nitrate and sodium nitrate) offer a convenient method of controlling the exact dose of nitrate, its use is regulated in certain countries. Given that beetroot juice is a natural product, it may be a preferable source of NO\(_3^-\). However, studies should be careful to measure the actual NO\(_3^-\) present in juice products as it will vary between batches, due to factors such as growth conditions and storage and transport.\(^{35}\) It should be also noted that beetroot juice may also carry additional ingredients that may enhance health and performance including polyphenols, quercitin and anti-oxidants.\(^{21}\)

**Conclusion**

Consumption of 140 ml of a commercially available beetroot juice concentrate (providing 8.4 mmol of NO\(_3^-\)) 2 hours before exercise may have an ergogenic effect on 2,000 m rowing ergometer performance in highly trained athletes. However, consumption of 70 ml of the same juice (providing 4.2 mmol of NO\(_3^-\)) did not lead to detectable changes in performance compared with a placebo control. Highly trained athletes may require greater doses of NO\(_3^-\) than those prescribed to moderately trained individuals in previous studies to elicit performance benefit. On the basis of these results, nitrate supplementation with beetroot juice may present as a naturally available and low-risk method to enhance high intensity exercise performance.

**Acknowledgements**

We would like to acknowledge the help and support provided by several groups including the NSW Institute of Sport, WA Institute of Sport, ACT Academy of Sport. We would also like to thank the time and efforts of the participants involved in the study. The project was funded by the Australian Institute of Sport Sports Supplement Program. The authors declare
there are no conflicts of interest that would inappropriately influence the outcome or quality of our research.
REFERENCES


FIGURES AND TABLES

Figure 1. Pearson product-moment correlation between the Δ plasma [NO$_2^-$] and Δ 2,000 m ergometer time for nitrate trials (DOUBLE; SINGLE) compared to PLACEBO. n=11

Table 1. Subject characteristics (n = 10 males)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD</th>
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<tr>
<td>Age (yrs)</td>
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</tr>
<tr>
<td>Body mass (kg)</td>
<td>85.5 ± 7.8</td>
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<tr>
<td>Training hours per week (h/wk)</td>
<td>16.9 ± 3.4</td>
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<td>2,000 m personal best (mm:ss)</td>
<td>6:17 ± 10 s</td>
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Table 2a. Effect of nitrate supplementation on 2,000 m rowing measures

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>Mean ± SD</th>
<th>Mean difference vs. placebo ± 90% CL</th>
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<tr>
<td><strong>2,000m time to completion (mm:ss)</strong></td>
<td>06:23.5 ± 9.0 s</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Average heart rate (bpm)</strong></td>
<td>185 ± 11</td>
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<td>-</td>
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</tr>
<tr>
<td><strong>Post test [La'] (mmol/L)</strong></td>
<td>13.5 ± 1.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Δ Plasma [NO₂⁻] (nM)</strong></td>
<td>-23.9 ± 17.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td><strong>Δ Plasma [NO₃⁻] ( M)</strong></td>
<td>1.0 ± 6.8</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Single</strong></td>
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<td></td>
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<td><strong>2,000m time to completion (mm:ss)</strong></td>
<td>06:23.4 ± 8.7 s</td>
<td>0.2 ± 2.5 s</td>
<td>Likely trivial</td>
<td></td>
</tr>
<tr>
<td><strong>Average heart rate (bpm)</strong></td>
<td>181 ± 14</td>
<td>-2 ± 3</td>
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<td><strong>Post test [La'] (mmol/L)</strong></td>
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<td>-0.6 ± 2.0</td>
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<td><strong>Δ Plasma [NO₂⁻] (nM)</strong></td>
<td>54.9 ± 61.8</td>
<td>78.7 ± 52.8</td>
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<tr>
<td><strong>Δ Plasma [NO₃⁻] ( M)</strong></td>
<td>99.2 ± 12.7</td>
<td>98.1 ± 12.0</td>
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</tr>
<tr>
<td><strong>Double</strong></td>
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<td></td>
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<td><strong>2,000m time to completion (mm:ss)</strong></td>
<td>06:21.9 ± 9.0 s</td>
<td>-1.6 ± 1.6 s</td>
<td>Possibly beneficial</td>
<td></td>
</tr>
<tr>
<td><strong>Average heart rate (bpm)</strong></td>
<td>183 ± 13</td>
<td>-2 ± 1</td>
<td>Very likely trivial</td>
<td></td>
</tr>
<tr>
<td><strong>Post test [La'] (mmol/L)</strong></td>
<td>13.9 ± 2.0</td>
<td>0.5 ± 2.8</td>
<td>Unclear</td>
<td></td>
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<tr>
<td><strong>Δ Plasma [NO₂⁻] (nM)</strong></td>
<td>83.3 ± 80.7</td>
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<tr>
<td><strong>Δ Plasma [NO₃⁻] ( M)</strong></td>
<td>180.6 ± 46.2</td>
<td>179.5 ± 31.6</td>
<td>Almost certainly positive</td>
<td></td>
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*Based on a smallest worthwhile change of 0.5% in performance or 0.20 of the between-subject SD in PLACEBO for other measures. Δ Plasma [NO₂⁻] and [NO₃⁻] are calculated from 2 h post beverage consumption samples minus the trial baseline values.
**Table 2b. Effect of nitrate supplementation on 2,000 m rowing measures**

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>Mean difference vs. Single ± 90% CL</th>
<th>Inference</th>
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<tr>
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<tr>
<td>2,000m time to completion (mm:ss)</td>
<td>06:21.9 ± 9.0 s</td>
<td>-1.8 ± 2.1 s</td>
<td>Possibly beneficial (^a)</td>
</tr>
<tr>
<td>Average heart rate (bpm)</td>
<td>183 ± 13</td>
<td>-1 ± 3</td>
<td>Likely trivial</td>
</tr>
<tr>
<td>Post test [La(^-)] (mmol/L)</td>
<td>13.9 ± 2.0</td>
<td>1.1 ± 1.0</td>
<td>Likely positive</td>
</tr>
<tr>
<td>(\Delta) Plasma [NO(_2^-)] (nM)</td>
<td>83.3 ± 80.7</td>
<td>28.4 ± 72.6</td>
<td>Unclear</td>
</tr>
<tr>
<td>(\Delta) Plasma [NO(_3^-)] (μM)</td>
<td>180.6 ± 46.2</td>
<td>91.4 ± 31.7</td>
<td>Almost certainly positive</td>
</tr>
</tbody>
</table>

\(^a\) Based on a smallest worthwhile change of 0.5% in performance or 0.20 of the between-subject SD in PLACEBO for other measures. \(\Delta\) Plasma [NO\(_2^-\)] and [NO\(_3^-\)] are calculated from 2 h post beverage consumption samples minus the trial baseline values.
CHAPTER V: THE EFFECT OF NITRATE SUPPLEMENTATION ON MUSCLE CONTRACTILE PROPERTIES IN HEALTHY ADULTS

The following paper is a copy of the submitted manuscript:

Hoon MW, Fornusek C, Chapman PG, Johnson NA. The Effect of Nitrate Supplementation on Muscle Contractile Properties in Healthy Adults.


As co-authors of the paper “The Effect of Nitrate Supplementation on Muscle Contractile Properties in Healthy Adults” we confirm that Matthew Hoon has made the following contributions:

- Study concept and design
- Significant data collection
- Data extraction, analysis and interpretation
- Manuscript preparation

Signed: Date: 16/09/2014
Signed: Date: 09/09/2014
Signed: Date: 02/09/2014
ABSTRACT

This study examined the effect of dietary supplementation with inorganic nitrate (NO$_3^-$) on markers of contractile function in human knee extensors. In a double-blinded, randomized cross-over design, eighteen (12 M) healthy participants undertook four days of supplementation with either nitrate-rich beetroot juice (NITRATE; days 1-3: 525 mg NO$_3^-$, day 4: 1050 mg NO$_3^-$) or nitrate-depleted beetroot juice (PLACEBO). On the fourth day, isometric knee extension force was assessed during a series of voluntary and electrically evoked (stimulation) tests. In addition, muscular fatigue was examined in two separate continuous-stimulation fatigue tests, one with and one without blood flow restriction. There were no differences for maximum voluntary contraction, peak twitch force, half-relaxation time and the force-frequency relationship for stimulations up to 100 Hz between the NITRATE and PLACEBO trials. No differences between trials were observed in the non-restricted fatigue test, however NITRATE was found to attenuate the decline in force during the blood flow restricted test, such that the force at the 80 s mark (PLACEBO: 66 ± 11 vs. NITRATE 74 ± 9 % of initial force; $P$=0.01) and 102 s mark (PLACEBO: 47 ± 8 % vs. NITRATE 55 ± 8 %; $P$<0.01) were significantly higher. These results suggest that four days of NO$_3^-$ supplementation elicits peripheral responses in muscle that attenuate muscular fatigue during exhaustive exercise under hypovolemic conditions. This ergogenic action is likely attributable to improved Ca$^{2+}$ handling in the muscle, or enhanced perfusion during ischemia.

Keywords: beetroot juice; excitation-contraction coupling; fatigue; muscle force; transcutaneous electrical nerve stimulation
INTRODUCTION

A growing body of evidence demonstrates that dietary supplementation with inorganic nitrate (NO$_3^-$) can enhance exercise capacity in healthy adults and trained individuals (Hoon et al., 2013). Improvements in cycling, running and rowing performance have all been reported following ingestion of NO$_3^-$ rich beetroot juice (Vanhatalo et al., 2010; Lansley et al., 2011; Hoon et al., 2014). The physiological adaptations following supplementation are not attributable to the NO$_3^-$ compound per se, but more likely due to its reduction into the multi-purpose cell signaling molecule nitric oxide (NO) molecule. Following ingestion in whole food or otherwise, the NO$_3^-$ compound is systematically reduced to nitrite (NO$_2^-$) and then NO through a host of reactions along the O$_2$ independent NO$_3^-$ - NO$_2^-$ - NO pathway (Lundberg, Weitzberg, & Gladwin, 2008). This process is known to be further facilitated under hypoxia and acidosis, as can occur during intense exercise.

The NO compound is known to mediate several physiological pathways (Bailey et al., 2011). One particular extramusscular effect of NO that may aid exercise performance is vasodilation and subsequent hyperaemia to active skeletal muscle (Joyner, 1997). This vasodilation has been suggested to be responsible for a portion of nitrate’s ergogenic effect (Bailey et al., 2009), in theory improving blood flow and oxygen transport to working muscle when metabolic rate is high. Indeed, Ferguson and colleagues reported greater blood flow and vascular conductance in exercising hind limb muscles in rats fed NO$_3^-$ rich beetroot juice (Ferguson et al., 2013), however the role of blood flow changes following supplementation is yet to be established in humans.

Nitric Oxide has also been shown to modulate a host of physiological actions directly within the muscle cell, independent of blood flow effects. This includes the regulation of muscle contraction (Reid, 1998) through the alteration of Ca$^{2+}$ release and uptake by the sarcoplasmic reticulum (SR) (Mészáros et al., 1996, Viner et al., 2000). The effect of NO on these processes may consequently improve exercise efficiency following supplementation (Bailey et al., 2010). However, it is unclear if these effects concomitantly result in alterations to the contractile properties of skeletal muscle. Recent rodent research found that NO$_3^-$ supplementation increased the rate of force development and contractile force in fast twitch muscle fibres (Hernández et al., 2012). In contrast, supplementation studies in human cohorts have reported mixed findings on contractile properties. In one
investigation, 15 days of NO$_3^-$ rich beetroot juice consumption (~5.1 mmol NO$_3^-$ per day) did not alter peak force output or fatigue resistance in physically active males during an exhaustive leg extension task (Fulford et al., 2013). However, recent work utilizing transcutaneous electrical nerve stimulation (TENS) reported alterations in contractile function following a similar protocol of beetroot juice intake (Haider & Folland, 2014). Seven days of beetroot juice consumption (9.7 mmol NO$_3^-$ per day) were found to increase peak force at low levels of electrically evoked contraction (1-20 Hz) and the rate of force development at 300 Hz.

Given these inconsistent findings, the aim of the current investigation was to examine the effect of dietary inorganic nitrate supplementation on muscular contractile properties in adults. We hypothesized that supplementation would improve submaximal contractile force and attenuate muscular fatigue. A secondary aim was to examine these effects during blood flow restriction, to investigate if NO$_3^-$ supplementation exerts a peripheral effect on muscle function that is independent of blood flow changes. Here, we hypothesized NO$_3^-$ supplementation would attenuate the decline in contractile force under blood flow restriction due to intramuscular effects elicited by NO$_3^-$.

**METHODS**

**Participants**

Nineteen adults (13 males, 6 females; mean ± SD; age: 29 ± 6 years; body mass: 72 ± 11 kg) volunteered to participate in the study. All participants presented with ≤1 cardiovascular or metabolic risk factor (Pescatello, 2014) and following a written informed consent were admitted into the study. All participants were involved in a low-moderate amount of habitual physical activity, in which they were instructed to keep consistent during the course of the study. Five individuals reported currently taking either (a) fish oil (b) multi-vitamins (c) whey protein powder or combination of all. All participants were asked to maintain their current intake during their involvement with the study. Physical activity and dietary intake were recorded for the 48 h preceding each testing block and qualitatively assessed by researchers for consistency. The study was approved by the University of Sydney Human Research Ethics Committee (Project No. 2014/252).
Experimental protocol

The study was carried out in a double blinded, crossover manner. Participants underwent three trials: a familiarisation trial, one NITRATE trial and one PLACEBO trial; with the order of the latter two being randomized using a digital sequence. During their initial visit, participants were familiarized with all muscle function testing procedures (detailed below). For the following visits to the laboratory, a dietary intervention was prescribed. During the NITRATE trial, participants undertook four days of nitrate-rich beetroot juice supplementation. In NITRATE, one bottle (Nitrate Max, UpBeat Sports Drinks, Australia; 525mg NO₃⁻ per 250 mL) of beetroot juice was consumed each of the three days preceding muscle function testing, and two bottles on the day of testing (4 and 2 h prior to testing). The supplementation protocol in PLACEBO was identical, however a ‘placebo’ beetroot juice (in bottles of identical appearance) was used. The placebo beetroot juice had been passed through an ion exchange resin, so only negligible levels of NO₃⁻ (25 mg NO₃⁻ per 250 mL) remained. Experimental testing was completed at the same time of day ±1 h to minimize any effect of circadian rhythm on muscular contraction (Martin et al., 1999). A 7 day washout period was used between trials. Participants were asked to refrain from using chewing gum or mouthwash during their involvement with the study, to preserve oral bacteria necessary for the reduction of NO₃⁻. Individuals were also instructed to maintain their current level of physical activity, but avoid strenuous exercise the day before and the day of testing.

Muscle function testing

Participants sat on a custom-built adjustable chair and were secured at the chest and waist, with the hips and knees flexed at 90°. The ankle was strapped securely to a force transducer (XTran Load Cell S1 W, Applied Measurement, Sydney, Australia) which was calibrated prior to and following the experimental sessions using standard weights. For all tests, participants were asked to keep their arms folded on their chest. Upon being affixed to the chair, the skin above each individual’s quadriceps femoris was prepared for electrode placement by shaving, sanding, and alcohol swabbing. A 13 cm wide blood pressure cuff connected to a rapid inflator source (Hokanson E20 cuff inflator; Bellevue, WA) was placed at the proximal end of the leg, immediately below the inguinal crease. Two oval, pre-gelled surface electrodes (8x13cm) coated in ECG preparation gel were placed distal to the cuff (one immediately inferior to the cuff with the second approximately 8 cm away) over the belly of the muscle. Their anatomical location was
recorded and replicated in subsequent trials. Electrodes were connected to a pulse generator (Digitimer DS7A, Welwyn Garden City, England; 50 μs, 400 V). A five minute rest period was given between each of the individual tests.

**Maximum Voluntary Contraction (MVC)**
Participants underwent a short warm up of 3 s submaximal knee extensions, (three contractions at a 50% estimated maximal effort, three at 75% and two at 90% estimated effort). Following, participants performed four attempts at 100% effort, with the greatest force (Newtons; N) over 0.9 s recorded as the maximum (in line with previous investigations: Fulford et al., 2013). Each effort was separated by 30 s of rest and strong verbal encouragement was provided during contraction.

**Passive twitch test**
Single pulses at increasing amperage were administered until 75% of MVC was reached or the subject reported reaching their maximum pain tolerance. Stimulation commenced at an intensity of 100 mA and increased in 50 mA increments with 15 s relaxation time between pulses. The maximal tolerated twitch was then administered four times with 30 s rest between each, with force data recorded at 1 Khz. The mean instantaneous peak force, half contraction time (CT ½) and half relaxation time (RT ½) of all twitches were recorded. The CT ½ was defined as the time taken to reach peak tension from half- peak tension; while RT ½ was defined as the time between peak tension and half- peak tension in the relaxation phase.

**Force Frequency**
A familiarisation protocol similar to the initial phase of the passive twitch test was then performed to determine appropriate amperage for the force-frequency test. Stimulations of 2 s @ 20 Hz commenced at 90 mA, increasing 30 mA each increment until maximum pain tolerance was reached (pilot testing indicated that stimulations at this frequency were the most tolerable across a 10-100 Hz range). The maximal amperage achieved was then used in a train of 2 s pulses to test the force-frequency relationship of the muscle. Briefly, six stimulations at increasing frequency (10, 20, 40, 60, 80 and 100 Hz) lasting 2 s each and separated by 10 s were administered. The same test was repeated after a 5 min rest period and the mean peak force for each frequency was recorded. The peak force was defined as
the greatest instantaneous force during the 10 Hz contraction or the mean over the last 300 ms of the force plateau for 20 - 100 Hz contractions. Values were then normalized to 100 Hz prior to analysis.

**Fatigue Tests**

Using the maximum current attained in the force frequency test, participants then underwent a protocol eliciting a 0.8 s tetanus @ 20 Hz followed by 0.8 s relaxation time, repeated 64 times (total time of 102.4 seconds). Short, intermittent electrical stimulations of this nature have been shown to fatigue the muscle at a greater rate than longer, more continuous contractions (Bergstrom, 1988). The peak force (greatest 300 ms mean) at 20, 40, 60, 80 and 102 s were recorded during the test.

Following a 5 min period of rest, the blood pressure cuff was inflated and sustained at 250 mmHg in order to restrict blood flow to the quadriceps. After 30 s of initiating the occlusion, the fatigue protocol of a 0.8 s/0.8 s tetanus-relaxation was repeated as described above. The greatest 300 ms mean force at 20, 40, 60, 80 and 102 s were recorded.

**Statistical Analysis**

Statistical analyses were completed using SPSS version 22 (SPSS Inc, Chicago, USA), with significance set at \( P<0.05 \). Differences in MVC, peak twitch force, CT \( \frac{1}{2} \) and RT \( \frac{1}{2} \) between NITRATE and PLACEBO conditions were compared using paired samples t-tests. A two-way, repeated measures ANOVA was used to assess treatment x frequency interactions for the force-frequency test and treatment x time interactions for the fatigue tests. If a significant interaction effect was found, Bonferroni corrected paired t-tests were conducted to determine where difference between treatments occurred. Where Mauchly’s Test of Sphericity was found to be significant, a Greenhouse-Geisser correction was applied to the analysis. All data are presented as mean ± SD.
RESULTS

One participant withdrew from the study for personal reasons before completing both interventions. Their data has been removed from the study, leaving a sample size of n=18 (12 M, 6 F). One participant was unable to avoid voluntary contractions during the fatigue tests; their data has been removed from analyses of these outcomes.

Maximal voluntary contraction
There was no significant differences observed in MVC for PLACEBO vs. NITRATE trials (492 ± 141 vs. 482 ± 127 N; P>0.05).

Passive Twitch test
No significant differences were found between treatments for CT ½ (PLACEBO vs. NITRATE; 6.7 ± 0.4 vs. 6.7 ± 0.5 ms; P>0.05), peak force (86 ± 21 vs. 86 ± 27 N, P>0.05) or RT ½ (7.6 ± 0.6 ms; P>0.05).

Force-Frequency Relationship
The normalized muscle force-stimulation frequency relationship is shown in Figure 1. No treatment x frequency effect was found between PLACEBO and NITRATE trials (P>0.05).

Fatigue Tests
The force profile of the non-restricted fatigue test is shown in Figure 2. The initial force for each treatment (PLACEBO: 83 ± 40 N vs. NITRATE 85 ± 44 N) was not significantly different (P>0.05). No differences in normalized force (i.e. % of initial force) were found between treatments (PLACEBO vs. NITRATE) at each time point (20, 40, 60, 80 and 102 s).

The force profile during the restricted fatigue test is presented in Figure 3. The initial force for each treatment (PLACEBO: 63 ± 28 N vs. NITRATE 64 ± 31 N) again, was not significantly different (P>0.05). A significant treatment x time effect however, was observed (P= 0.03). The decline in the % of initial force was less in NITRATE versus PLACEBO at 40, 60, 80 and 102 s (P= 0.04, 0.06, 0.01 and <0.01, respectively); however following Bonferroni correction only 80 s (PLACEBO: 66 ± 11 % vs. NITRATE 74 ± 9%);
P=0.01) and 102 s (PLACEBO: 47 ± 8 % vs. NITRATE 55 ± 8 %; P<0.01) were deemed to be significantly different.

**DISCUSSION**

The primary finding of the present investigation was that dietary supplementation with nitrate-rich beetroot juice did not improve maximal force, submaximal contractile force or the force-frequency relationship of the quadriceps muscles in adults. However, when compared with a placebo, NO$_3^-$ supplementation prior to exercise attenuated skeletal muscle fatigue during exhaustive quadriceps contraction when blood flow was restricted. This finding suggests that several days of NO$_3^-$ supplementation may induce muscular adaptation, resulting in greater fatigue resistance.

This is the first study to demonstrate that several days of NO$_3^-$ supplementation exerts an ergogenic effect under hypovolemic conditions in human muscle. This finding is similar to previous work that has reported greater stamina following NO$_3^-$ supplementation (Bailey et al., 2010, Larsen et al., 2010). While the present study did not identify specific adaptations within the musculature, the most likely contributor to the observed reduction in fatigue would be an increase in SR Ca$^{2+}$ release during excitation-contraction coupling. Supplementation with NO$_3^-$ reduced fatigue during the later stages of repeated tetani-induced contraction in the current study, where a decline in intracellular [Ca$^{2+}$] is primarily responsible for a reduction in force generation (Westerblad and Allen, 1991; Place et al., 2009). Interventions known to induce SR Ca$^{2+}$ release (such as the addition of caffeine to muscle) have been shown to restore muscle force following exhaustive exercise (Allen and Westerblad, 1995) and there is strong evidence to suggest NO$_3^-$ supplementation may work along similar pathways. For example, an increase in intracellular [Ca$^{2+}$], Ca$^{2+}$ binding protein calsequestrin 1 (CASQ1) and dihydropyridine receptor (DHPR) has been previously reported in mice following several days of NO$_3^-$ supplementation (Hernández et al., 2012). The greater CASQ1 expression and intracellular [Ca$^{2+}$] likely increase the amount of releasable Ca$^{2+}$ from the SR during excitation-contraction coupling (producing more actin-mysoin binding sites), whilst an increase in DHPR would stimulate Ca$^{2+}$ release more readily during contraction. Additionally, an increase in metabolic efficiency has also been documented following NO$_3^-$ supplementation, reducing the accumulation of
intracellular phosphate ([P$_i$]) during contraction (Bailey et al., 2010). This may further contribute to the preservation of muscle force observed during exhaustive exercise, given that increased [P$_i$] has previously been shown to reduce Ca$^{2+}$ sensitivity and SR Ca$^{2+}$ release (Duke and Steele, 2001).

However, it must be acknowledged that the ischemic conditions induced by restricting blood flow to the muscles are likely to favour the reduction of NO$_2^-$ to NO (Lundberg et al., 2008), which may have actually facilitated NO-mediated perfusion. This would mean that, against our hypothesis, muscle function changes were not solely a result of peripherally mediated mechanisms. Documented improvements to muscle oxygenation (and consequently exercise tolerance) in subjects with Peripheral Arterial Disease (PAD) following NO$_3^-$ supplementation suggest this may be the case (Kenjale et al., 2011). However, it is unclear if the ischemic conditions induced by the current study are comparable to the pathophysiology of PAD, and if both are governed by the same mechanisms.

Although our study showed a benefit of NO$_3^-$ supplementation on fatigue resistance during muscle contraction performed under blood flow restriction, no effect was observed during the non-restricted fatigue test. These results seem to conflict with a previous investigation, which found time to task failure during an exhaustive leg exercise test improved following NO$_3^-$ supplementation (Bailey et al., 2010). Although it is difficult to compare different outcome measures, in the current study it was expected that muscular force during the non-restricted test would be higher following NO$_3^-$, based on the observation that supplementation improved muscular stamina in the previous study. However, the different intensities of each task should be considered. Despite a slight and gradual decline in force, it is most likely that the intensity and duration of the non-restricted test were not enough to compromise Ca$^{2+}$ handling, negating the aforementioned intracellular adaptations. The contractions at the end of the non-restricted test had reduced approximately 25% of the initial force, suggesting that only ‘Phase I’ of fatigue had been achieved, during which the decline in force is not explained by reduced Ca$^{2+}$ release (Westerblad & Allen, 1991). Again, while it is difficult to compare the results of different tests, it is likely the exercise test utilized in the study of Bailey et al. (2010) was more intensive and therefore challenging to Ca$^{2+}$ handling, given it resulted in complete exhaustion.
The force-frequency relationship of stimulated muscle and contractile characteristics also remained largely unchanged by NO$_3^-$- supplementation. The effect of supplementation on markers such as CT $\frac{1}{2}$, RT $\frac{1}{2}$ and peak stimulated force has been inconsistent among the few papers that have examined contractile function. In dissected mice fast-twitch muscle fibres, a large increase in peak force (~40%) and a decrease in time to achieve 50% maximum force (~35%) across 1-50 Hz stimulations was found following seven days of NO$_3^-$-supplementation (Hernández et al., 2012). Slow twitch fibres taken from the same mice did not demonstrate any changes, suggesting adaptations resulting from NO$_3^-$ intake occur predominantly in fast-twitch fibres. The fibre-specific adaptations to NO$_3^-$ supplementation may explain why only a small effect on knee extension force at a low stimulation frequency (approximately 2%) was observed in human extensor muscles (Haider & Folland, 2014), which typically display a large proportion of slow twitch fibres (Lexell and Taylor, 1991). In the same study, time to peak force or half-relaxation time were not affected by supplementation either. It is possible that the participants in the present study had a greater distribution of slow twitch fibres than these studies, such that any effects elicited by NO$_3^-$- on twitch characteristics were diluted and hence undetected. In addition, the studies of Haider & Folland (2014) and Hernández et al. (2012) employed a seven day supplementation protocol in contrast to the four day protocol utilised in the present study, and it is possible that the expression of Ca$^{2+}$ handling proteins and receptors that contribute to muscle excitability may be increased by additional days of supplementation. Although NO has been known to shift the force-frequency curve right in animal models (Reid, 2001), the ability of NO$_3^-$-supplementation to elicit this effect in a human cohort remains equivocal.

While there is conflicting evidence concerning the force-frequency relationship of the muscle, the effect of NO$_3^-$-supplementation on MVC force appears to be more consistent throughout the literature. Similar to the results in this current study, additional investigations have also reported no change to MVC force following NO$_3^-$-supplementation (Fulford et al., 2013; Haider and Folland, 2014). Although NO$_3^-$ may improve contractile force under fatigue, it appears to have little to no effect on maximal force generation.

*Study limitations*
The inability to measure blood flow within the muscle restrains the applicability of our findings, as we cannot definitively show that all vascular effects were nullified during the blood flow restricted fatigue test. However, previous work utilizing a similar sized occlusion cuff has found femoral blood flow was reduced by almost 90% at a pressure of 250 mmHg (Iida et al., 2007), suggesting the targeted muscles in the present study were also considerably deprived of blood and that the impact of any NO₃⁻ mediated changes to blood flow were minimised. On this note, we cannot exclude the possibility that NO₃⁻ supplementation resulted in individuals experiencing varying levels of hypovolemia (and related performance changes). In a separate matter, participant plasma [NO₂⁻] and [NO₃⁻] analysis may have provided more insight into our results, in light of the documented link between Δ[NO₂⁻] and performance (Hoon et al., 2014, Wilkerson et al., 2012, Wylie et al., 2013). However, given that the hypothesized adaptations to intramuscular Ca²⁺ handling work independent of blood flow, it is uncertain if the relationship between plasma [NO₂⁻] and performance would be applicable to the results of our study.

Conclusion

Four days of dietary supplementation with nitrate-rich beetroot juice did not alter traditional measures of muscle contractile function (including maximal voluntary force, force-frequency relationship and passive twitch characteristics) in humans, but reduced muscular fatigue during sustained muscular contraction performed under blood flow restriction. This finding suggests that peripheral alterations which enhance muscular endurance may occur following several days of NO₃⁻ supplementation, and, in part, may contribute to the performance enhancing benefit of NO₃⁻ in humans.

Acknowledgements and Conflicts of Interest

The authors would like to thank Upbeat Natural Beverages who provided the beetroot juice utilized in the study. The views expressed in this article do not necessarily reflect those of the company. The authors declare there are no conflicts of interest in the work presented.
REFERENCES


**Figures**

**Figure 1.** Isometric force vs. Electrical stimulation frequency relationship of the knee extensors. Force is normalized to the peak force at 100 Hz. Data are mean ± SD; n = 18.

**Figure 2.** Force during the non-occluded fatigue test, which consisted of 64 cycles of 0.8 s tetanus at 20 Hz and 0.8 s rest. Force is normalized to peak force of the initial contraction. Data are mean ± SD; n = 17.
Figure 3. Force during the occluded fatigue test, which consisted of 64 cycles of 0.8 s tetanus at 20 Hz and 0.8 s rest. A cuff inflated to 250 mmHg was placed superior to the knee extensors during the test. Force is normalized to peak force of the initial contraction. *Bonferoni NITRATE>PLACEBO ($P<0.01$). Data are mean ± SD; n= 17.
CHAPTER SIX: SUMMARY
SUMMARY OF FINDINGS

A growing number of studies have found that dietary supplementation with inorganic nitrate (NO$_3^-$) may enhance exercise capacity in healthy adults. While not all studies report an improvement in exercise performance following supplementation, a majority show at least one marker of exercise to improve (such as submaximal efficiency). Whether these enhancements translated into meaningful improvements in endurance performance for well-trained, competitive athletes was yet to be established. Preexisting studies on NO$_3^-$ supplementation had generally utilized exercise tests with low ecological validity (e.g. graded exercise and time to exhaustion tests) or non-athletic populations. Often, performances between athletes at the elite level are separated by such miniscule margins that slight gains elicited by ergogenic aids may yield significant reward. Given these possible benefits, NO$_3^-$ supplement use in well-trained populations warranted examination. From the limited amount of available research, it appeared short, high intensity exercise may benefit most from supplementation. Furthermore, the optimal protocol of supplementation had not been elucidated, and no recommendations concerning the most effective amount or time to prescribe NO$_3^-$ were available. This may have been, in part, due to the physiological mechanisms and adaptations resulting from supplementation not being completely understood. Therefore, the aim of this thesis was to address these unresolved issues in the scientific literature by assessing the effect of NO$_3^-$ supplementation on high-intensity exercise (i.e. exercise conducted at or near VO$_{2\text{max}}$) in trained athletes; provide new data concerning the effect of NO$_3^-$ dose and timing on performance; and shed light on mechanisms underlying an ergogenic effect.

Specifically, the aims were to:

1. Undertake a systematic review of the available literature on inorganic NO$_3^-$ supplementation and quantify its effect on endurance exercise performance in healthy adults by meta-analysis;
2. Determine if acute supplementation with NO$_3^-$ improves simulated track cycling performance in well-trained cyclists;
3. Examine the effect of NO$_3^-$ supplementation on plasma [NO$_3^-$] and nitrite [NO$_2^-$], respectively, and the association with changes in performance;
4. Investigate the effect of altering the dose and timing of nitrate ingestion on exercise performance and plasma $[\text{NO}_3^-]$ and $[\text{NO}_2^-]$;
5. Examine the effect of different acute nitrate doses on simulated competition performance in well-trained rowers;
6. Investigate the effect of nitrate supplementation on skeletal muscle function and the role of vascular and peripheral responses in performance improvement.

The results of the systematic review and analysis showed that inorganic $\text{NO}_3^-$ supplementation had a favourable effect on exercise capacity in a majority of studies available to date, however few reached statistical significance. When collectively assessed by meta-analysis, it was determined that $\text{NO}_3^-$ supplementation had an overall likely ergogenic effect on exercise performance, most notably in fixed-rate time to exhaustion tasks. Across the seventeen studies included in the analysis there were numerous methods of supplementation, each varying the prescribed $\text{NO}_3^-$ dose, timing and product. This lack of consistency demonstrated that the optimal protocol of $\text{NO}_3^-$ supplementation had not yet been established. The review also highlighted a lack of studies investigating the effects of supplementation in well-trained participants, particularly on time trial performance which is considered to most closely resemble real-world performance. Of these, only two investigated exercise of extreme intensity. The physiological demands of exercise of this intensity arguably lend themselves to the utilization of $\text{NO}_3^-$, due to the hypoxic and acidic stimulated reduction of $\text{NO}_3^-$ to the functional NO compound.

In light of these findings, the second study of the thesis (Chapter 3) used a randomized control trial to examine the effect of $\text{NO}_3^-$ supplementation on high-intensity cycling time trial performance in a group of well-trained cyclists. Over four separate trials, different timing (75 min or 150 min before exercise) and amounts (0.03; 4.1; 6.2 mmol $\text{NO}_3^-$) of nitrate-rich or nitrate-depleted beetroot juice were ingested, and the subsequent effect on two bouts of 4 minute time trials was examined. Nitrate supplementation was found to have a trivial effect on the performance of the first time trial regardless of when the beetroot juice was taken (mean ±95% CL; 1.3% ±1.7%). Performance in the second time trial was slightly worse following nitrate supplementation (−0.3%, ±1.6%), with no clear effect of varying the amount and timing of $\text{NO}_3^-$ ingestion. The interaction between time trials must be considered i.e. it is
possible that the small improvement in performance in the first time trial resulted in additional fatigue which compromised the performance of the second time trial. Plasma analysis of $[\text{NO}_2^-]$ indicated that the NO$_3^-$ had been successfully absorbed, with peak levels of $[\text{NO}_2^-]$ occurring roughly 75-150min following consumption. However, the $\Delta[\text{NO}_2^-]$ was smaller than what has been documented in previous investigations, possibly a result of the elevated training status of participants in the study. Given the correlation between $\Delta[\text{NO}_2^-]$ and performance postulated by previous research, the lack of performance improvement may have been due to the failure of the NO$_3^-$ dose to adequately elevate $[\text{NO}_2^-]$ in this well-trained cohort.

To determine if a higher dose of NO$_3^-$ elicits a performance benefit in well-trained athletes, the effect of multiple doses on rowing performance was subsequently examined in a group of ten well-trained rowers (Chapter 4). It was hypothesized that athletes would require a ‘double dose’ (8.4 mmol NO$_3^-$) of nitrate-rich beetroot juice to improve exercise performance and the ‘single dose’ (4.2 mmol NO$_3^-$) utilized in previous investigations would be inadequate. In a randomized crossover design, participants ingested either a double dose, single dose or placebo 120 min before a 2,000 m time trial. The ‘double dose’ of beetroot juice reduced (improved) the time to complete 2,000m by (mean difference ±90% CL) –1.6 ±1.6 s compared with a placebo beverage (corresponding to a 0.4 ± 0.8% improvement); while performance following the single, lower dose was found to be no different from the placebo trial. The double dose also increased plasma $[\text{NO}_2^-]$ to a greater degree than the single dose, and subsequently there was a near-significant correlation found between $\Delta[\text{NO}_2^-]$ and reduction in 2,000 m time ($r = -.593, P = .055$). From these results, it can be suggested that inorganic NO$_3^-$ supplementation may improve rowing performance in well-trained individuals if a sufficient dose is ingested.

The final investigation of the thesis aimed to examine the effect of NO$_3^-$ supplementation on muscle contractile function and possible physiological mechanisms underlying a benefit. Available literature had raised the suggestion that increased blood flow to active muscle and augmentation of muscular contractile properties are likely candidates to explain the ergogenic effects of NO$_3^-$. To explore these mechanisms, a combination of transcutaneous electrical nerve stimulation (TENS) and blood flow occlusion techniques were employed to study muscle function following NO$_3^-$


supplementation. Using a placebo-controlled cross-over design, eighteen healthy adults consumed either nitrate-rich beetroot juice or a nitrate-depleted placebo beverage for four days before undertaking tests of maximum voluntary contraction force and muscle contractile function under stimulation. Additionally, leg extension force during continuous intermittent stimulation was examined under both normal (non-restricted) and blood flow restricted conditions. NO³⁻ supplementation was found to have no significant effect on the maximum voluntary contraction force or other contractile characteristics (peak stimulated force, half-relaxation time, half-peak time or force-frequency relationship) compared with placebo. NO³⁻ supplementation had no effect on muscular force or fatigue during the non-occluded fatigue test, however significantly improved the force of contraction during the later stages of the occluded fatigue test. These results show that several days of inorganic NO³⁻ supplementation improves muscular endurance when blood flow to exercising muscle is restricted. This adaptation is potentially mediated by improvements in Ca²⁺ handling during excitation-coupling in the muscle.

**DISCUSSION**

*The efficacy of NO³⁻ supplementation for improving exercise performance in well-trained athletes*

Currently available literature suggests that well-trained individuals are not as likely to benefit from NO³⁻ supplementation as lesser trained counterparts (Bescos et al., 2012b; Jones, 2014). This is likely a result of physiological adaptations to regular exercise which reduce the impact of exogenous NO³⁻. For instance, adaptations synonymous with exercise training include the increase in number and size of mitochondria and capillaries in muscle (Ingjer, 1979); decreasing the appearance of lactic acid and improving oxygen supply (Holloszy & Coyle, 1984). While these are typically desirable attributes in the context of sports performance, they inhibit the reduction of NO₂⁻ to the biologically active NO allowing the NO³⁻-NO₂⁻NO pathway (Lundberg et al., 2008). Similarly, well-trained individuals typically exhibit elevated plasma [NO₂⁻] and [NO³⁻] (Schena, Cuzzolin, Rossi, Pasetto, & Benoni, 2002) and NOS activity (Green, Maiorana, O'Driscoll, & Taylor, 2004), potentially decreasing the need for NO derived from exogenous NO³⁻. These adaptations may explain why several
investigations have reported no improvement to performance following NO$_3^-$ supplementation in groups of well-trained participants (Bescos 2012a; Boorsma et al., 2014; Cermak et al., 2012b; Christensen et al., 2013; Lane et al., 2013; Peacock et al., 2012; Wilkerson et al., 2012; Table 1). However, the specific conditions of an exercise task may also be highly influential on the effectiveness of NO$_3^-$ supplementation, such that highly trained individuals may still benefit under certain circumstances. High-intensity events are more likely to elicit favourable physiological conditions for the generation of NO through the NO$_3^-$ - NO$_2^-$ - NO pathway, with key reductive pathways of NO$_2^-$ amplified when O$_2$ supply is limited (Jones, 2014). Furthermore, research suggests NO$_3^-$ supplementation affects Type II fibres more so than Type I (Ferguson et al., 2012; Hernández et al. 2012). The greater contributions of Type II fibres to high-intensity exercise compared to low-to-moderate intensity exercise (VØLlestad, & Blom, 1985), again, suggest the effects of NO$_3^-$ supplementation may be most potent during very high-intensity exercise. In the studies previously mentioned where no effect of NO$_3^-$ supplementation was noted, the duration of exercise ranged from approximately 17 min (Christensen et al., 2013; Peacock et al., 2012) up to 40 and 60 min (Bescos et al., 2012a; Cermak et al., 2012b; Lane et al., 2013) and one study using a 50 mile cycling time trial that lasted over 2 hours (Wilkerson et al., 2012). These intensities would be at an estimated low-mid severe intensity (Jones & Poole, 2005). This contrasts to the rowing task assessed in Chapter 4, which was completed on average in 6.5 min (therefore at an estimated higher intensity), with a benefit from supplementation found. Similarly, additional studies have also shown improvements in the performance of short events, ranging from 1.5 to 4 min in duration (Bond, Morton, & Braakhuis, 2012; Peeling, Cox, Bullock, & Burke, 2014). Our data from Chapter 3 also found a dose of NO$_3^-$ improved an initial time trial performance by 1.3%, although this was deemed trivial due to the large variation in response. A subsequent performance appeared to be impaired under NO$_3^-$ supplementation, with a -0.3% change in performance of a secondary time trial (although smaller than the 1.3%, this was still deemed “possibly harmful” due to the negative weighting of the statistical approach). However, this may be due to the small improvement in the first time trial resulting in greater post test fatigue causing poorer performance in the second time trial. Interpretation of the performance data is
confounded by this interaction effect. Collectively, these results support the assertion that NO\textsuperscript{3} supplementation is best applied to short, intense endurance events, and may have limited effect for longer duration events in well-trained populations. In contrast to this hypothesis, Boorsma and colleagues found no improvement to 1,500 m performance (approximately 4 min exercise time) in a group of elite runners (Boorsma et al., 2014). This, however, may be partially explained by the use of mostly lower body muscles in running, compared to the large contribution of upper-body muscles in the kayaking (Peeling et al., 2014) and rowing (Chapter 4; Bond et al., 2012) tasks in studies reporting a benefit with NO\textsuperscript{3} supplementation. Studies using a predominantly upper-body exercise task seem to have greater success in improving performance with NO\textsuperscript{3} supplementation than others (Jones, 2014), and may partially explain why we were able to report an improvement in a rowing (Chapter 4) but not cycling (Chapter 3). The reason for this phenomenon has not yet been elucidated, but may be due greater competition for cardiac output in upper-body exercise (Toner, Glickman, & McArdle, 1990), reducing O\textsubscript{2} supply to active muscle and therefore accentuating the vasodilative and hyperaemic role NO plays – however, this is speculative. Therefore our finding that NO\textsuperscript{3} supplementation improves high-intensity rowing in well-trained athletes may not be applicable to all modalities of exercise. It is unclear if the performance requirements of rowing are more receptive to supplementation than other modalities such as cycling or running, even if intensity and duration are similar.

A common observation from studies is the existence of non-responsive subjects (i.e. those who do not improve performance) to NO\textsuperscript{3} supplementation, which arguably contribute to the inconsistent results across the literature. It is difficult to quantify the typical percentage of non-responders to supplementation, as a majority of studies do not report these statistics nor individual subject data, and few studies have acknowledged this trend. From the limited data, the occurrence of non-responders appears to be lower in recreationally trained individuals (20-30%; Wilkerson et al., 2012; Wylie et al., 2013a & 2013b) compared to highly trained (75%; Christensen et al. 2013; Boorsma et al. 2013). Our data from Chapter 3, showed that 62% did not improve a majority of their cycling time trials. In Chapter 4, 70% of participants did not improve their performance following a 4.2 mmol dose of NO\textsuperscript{3}; however, this number reduced to 50% when an 8.4 mmol dose was taken. Likewise, Wylie and colleagues (2013a) also reported the number of non-responders declined with increasing NO\textsuperscript{3}
dose. The reason for inter-individual variation in response to NO₃⁻ supplementation is currently unexplained, although appears to be moderately influenced by fitness status (Porcelli et al., 2014) - presumably due to the aforementioned training adaptations reducing the role of exogenous NO₃⁻. Other possible factors affecting individual response to NO₃⁻ supplementation are discussed in Future Research later on in this chapter; however, overall, research in this area is limited.

The exact magnitude of performance gains that may be brought about by NO₃⁻ supplementation is difficult to quantify. As discussed, the effect of supplementation is subject to several different factors such as the type of exercise, the intensity of exercise and the fitness of the individual. If high-intensity, upper-body dominant events are the best conditions for NO₃⁻ supplementation (for reasons previously discussed), there appears to be a 0.4 – 1.7% improvement in performance for trained individuals (Chapter 4; Bond et al. 2012; Peeling et al., 2014). This compares similarly to other popular ergogenic supplements and their effect on performance in similar conditions. For upper-body exercises lasting 3 – 7 minutes, supplements have been reported to elicit improvements in performance in the order of: 0.7 – 1.3% (Anderson et al., 2000; Bruce et al., 2000) for caffeine, 1.5% and 0.8% for beta-alanine and sodium bicarbonate, respectively (Hobson et al., 2013) and 1.1% for creatine monohydrate (Rossiter, Cannell, & Jakeman, 1996). Overall, all appear efficacious given that performances at the elite level are often separated by very small margins and improvements as small as 0.4% may be considered highly beneficial (Pyne, Trewn, & Hopkins, 2004). As the inorganic form of NO₃⁻ can be readily consumed from natural, dietary sources, the risk of adverse events (such as contamination with prohibited substances) is low. Together, these elements make NO₃⁻ supplementation an appealing strategy for coaches and athletes to maximize race performance. Additionally, the previously stated 0.4% improvement in performance in Chapter 4 reflects an average across subjects, covering both ‘responders’ and ‘non-responders’. Therefore, the ‘responders’ to supplementation are likely to see even greater than 0.4% improvements, as evidenced in other investigations (Christensen et al., 2012; Boorsma et al., 2014).

NO₃⁻ supplementation protocols
At commencement of this thesis, limited data were available concerning the timing of NO$_3^-$ supplement ingestion. One of the few available studies which examined plasma NO$_3^-$ and NO$_2^-$ kinetics showed that a bolus of beetroot juice caused peak [NO$_2^-$] to occur approximately 2.5 hours post ingestion (Webb et al., 2008). This peak in [NO$_2^-$] was used as a surrogate measure for NO activity (which is difficult to measure due to its short half-life *in vivo*; Ghasemi, & Zahediasl, 2011), theoretically representing the point where the physiological actions of NO were most potent. Subsequent studies have shown that changes in [NO$_2^-$] correlate well with improvements in performance (Wilkerson et al, 2012; Wylie et al., 2013a), supporting the idea that the onset of exercise is best coincided with peak [NO$_2^-$] (with supporting data from our study in Chapter 4). Data from the study conducted in Chapter 3 of this thesis found peak [NO$_2^-$] may actually occur earlier than 2.5 hours, particularly if a more concentrated NO$_3^-$ supplement is used. Webb and colleagues prescribed a 500 mL bolus to participants, whereas in Chapter 3, the same amount was provided in just 70 mL of liquid – 7 times the concentration. In several of the experimental conditions in our Chapter 3 study, [NO$_2^-$] 150 min post ingestion was no different to the [NO$_2^-$] at 75 min post ingestion (in the 150-PRE trial it was even lower); suggesting that [NO$_2^-$] had either plateaued or peaked somewhere between the two time points. A later study confirmed that beetroot juice of similar concentration caused [NO$_2^-$] to peak earlier than 2.5 hours post ingestion, somewhere closer to the 2 hour mark (Wylie et al, 2013a).

Research concerning the most effective amount of NO$_3^-$ to elicit performance gains has also developed considerably during the course of this thesis. An early study had reported quantities of approximately 350 mg were effective in improving cycling performance in recreationally trained individuals (Lansley et al., 2011a). However, our results from Chapter 3 found a similar dose (~300 mg) did not improve performance in well-trained cyclists. Expanding upon literature suggesting training adaptations blunted responsiveness to NO$_3^-$ supplementation (Bescos et al., 2012b), we hypothesized that a greater dose was required to elicit performance improvements in well-trained individuals. In chapter 4, we prescribed two different doses, a 4.2 mmol (280 mg) and 8.4 mmol (560 mg), to well-trained rowers 2 hours before a 2,000 m time trial. We showed that a 8.4 mmol dose of NO$_3^-$ was able to improve performance, while a 4.2 mmol dose did not; confirming our hypothesis that well-trained individuals require
larger doses of NO₃⁻ to improve performance than what has previously been shown effective in lesser trained. These findings were also replicated in subsequent study: 4.2 mmol was not enough to improve cycling performance in moderately trained participants; however, 8.4 mmol was (Wylie et al., 2013). Interestingly, additional quantities of NO₃⁻ may not elicit further gains, with the same study showing that a 16.8 mmol dose did not improve performance to a greater degree than the 8.4 mmol dose. This suggests that there is a maximum effect of NO₃⁻ supplementation around 8.4 mmol. However, as mentioned, the study utilized a cohort of moderately trained subjects and it is unclear if these findings extrapolate to well-trained individuals. What is most important for NO₃⁻ supplementation to elicit improvements in performance, it seems, is that an acute dose of NO₃⁻ must elevate plasma [NO₂⁻]. In chapter 4, our data reported a near significant relationship between Δ [NO₂⁻] and change in performance, a correlation that has been acknowledged in other studies (Wilkerson et al, 2012; Wylie et al., 2013a). This suggests that the ability of NO₃⁻ to elicit ergogenic benefits is tied to the individual’s ability to reduce it to NO₂⁻; at least when Δ[NO₂⁻] is measured in absolute terms. The exact Δ [NO₂⁻] required to improve performance, however, is not known. Currently, it is unclear if this association is influenced by the pre-supplementation [NO₂⁻] i.e. are performance improvements better explained by relative changes in [NO₂⁻] (rather than absolute amounts)? If so, this may partially explain why athletes, who typically display a higher resting [NO₂⁻], may not be as susceptible to the effects of supplementation. Unfortunately these correlations were not performed in our analysis, so this relationship remains speculative. Furthermore, the [NO₂⁻] response following a NO₃⁻ load appears to vary greatly between individuals (for reasons previously discussed), making it difficult to establish clear guidelines regarding the use of NO₃⁻ supplements.

Recently, mounting research has suggested that several days of NO₃⁻ supplementation may elicit physiological adaptations unlikely to be seen with acute doses. Larsen et al. (2011) and Hernández et al. (2012) both reported favourable changes in the expression of particular proteins following 3-7 days of supplementation, adaptations unlikely to occur in the typical 2-3 hours between an acute dose and the onset of exercise. Building on the observation that several days of NO₃⁻ intake may increase Ca²⁺ handling proteins in the muscle (Hernández et al. 2012), in Chapter 5 we showed that 4 days of NO₃⁻ supplementation improved muscular contractile force during an exhaustive task
(although it is uncertain if these improvements would have been observed following a single acute dose). The exact number of days required for supplementation prior to competition is debatable, and may depend on the time course of the various adaptations suggested to occur. For instance, Larsen et al. (2011) reported a significant reduction in the expression of mitochondrial proteins UCP-3 and Adenine Nucleotide Translocator (ANT) following just 3 days of supplementation; which the authors suggested lead to improved mitochondrial efficiency. Alternatively, greater expression of Ca\(^{2+}\) handling proteins (specifically calsequestrin 1 and dihydropyridine receptor) in rats was seen after 7 days of supplementation, which the authors suggested lead to improved mitochondrial efficiency. Alternatively, greater expression of Ca\(^{2+}\) handling proteins (specifically calsequestrin 1 and dihydropyridine receptor) in rats was seen after 7 days of supplementation, which the authors attributed to greater excitation-contraction coupling (Hernández et al. 2012). However, it is unclear if these changes could be observed within 7 days, or if UCP-3 and ANT expression could be further reduced with more than 3 days supplementation. In the Chapter 5 of the thesis, we showed that 4 days of supplementation was enough to improve muscular fatigue resistance (nb: In this study, the final dose of beetroot juice was consumed 2 hours prior to exercise. In essence, this approach incorporated both chronic and acute supplementation protocols). Although there are limited studies directly comparing the effects of acute and chronic supplementation, multiple days of supplementation may be optimal based on the aforementioned research, and given that no adverse effects of chronic supplementation have been reported to date, may be the ideal approach.

Presently, there is insufficient data on the best source of NO\(_3^-\). The two most commonly used NO\(_3^-\) supplements in the available literature have been beetroot juice and sodium nitrate (NaNO\(_3^-\)). While there is evidence to suggest both may improve exercise performance in moderately-trained individuals, those studies reporting improvement in exercise performance of well-trained subjects have typically used beetroot juice. Few studies have explored NaNO\(_3^-\) use in athletes, with the only two currently available studies showing no effect on maximal aerobic power during a graded exercise test (Bescos et al., 2011) or a 40 min cycling time trial (Bescos et al., 2012a). These results may be more linked to the exercise intensity and modality not being ideal for NO\(_3^-\) supplementation (as described earlier), rather than reflect the ergogenic potential of
NaNO$_3$. However, natural sources such as beetroot juice and spinach have additional components such as polyphenols and antioxidants which may contribute to exercise performance independent of NO$_3^-$ (Lansley et al., 2011b), perhaps making it a more appealing option.

**Physiological mechanisms**

Presently, there are many hypotheses as to how NO$_3^-$ supplementation exerts an ergogenic effect. Most researchers agree that, first and foremost, this effect is most likely mediated by NO$_3^-$ reduction to the more biologically active NO (Bailey, Vanhatalo, Winyard, & Jones, 2012). However, given the many metabolic pathways NO is thought to signal (Dawson & Dawson, 1995), the exact mechanism by which it acts is uncertain. While it was beyond the scope of this thesis to explore this, in Chapter 5 we were able to show that peripheral muscular endurance was improved following 4 days of supplementation. We believe this was likely a result of improved Ca$^{2+}$ handling during excitation-contraction coupling, based on the findings of Haider & Folland (2014). Following 7 days of NO$_3^-$ supplementation, the researchers found a greater expression of calsequestrin 1 and the dihydropyridine receptor proteins; an adaptation that would explain the preservation of muscle force during exercise observed in our study. Conversely, although we restricted blood flow to the limb to try and isolate this effect, it is possible that we facilitated the vasodilative role of NO, which becomes prominent in hypoxic conditions (Lundberg et al, 2008). This vasodilation and the consequent hyperaemia to muscle has been suggested as a possible contributor to the ergogenic effect NO$_3^-$ supplementation (Bailey et al, 2010). Greater blood flow and vascular conductance have been observed in rat hindlimb muscle following 7 days of NO$_3^-$ supplementation (Fergusson et al., 2013), suggesting this may be a functional adaptation to chronic NO$_3^-$ supplementation. In humans, it is more difficult to investigate these vascular changes due to limitations in being able to directly measure blood flow. From those studies in humans which have estimated blood flow using near-infrared spectroscopy (NIRS), several have reported greater regional blood flow to active muscle following supplementation (Bailey et al., 2009; Kenjale et al., 2011; Masschelein et al., 2012). However, it should be noted one investigation examined subjects with peripheral arterial disease (Kenjale et al., 2011) and another assessed
exercise under hypoxic conditions (Masschelein et al., 2012). These particular circumstances likely intensify the vascular effects of NO, and may not be applicable to the performance of endurance sport. Further work, with specific attention to blood flow during exercise, is required in well-trained participants to confirm this mechanism of NO$_3^-$ in the context of sports performance.

NO$_3^-$ supplementation has also been shown to improve exercise efficiency, by modulating mechanical or mitochondrial efficiency, either by reducing the ATP cost of force production (Bailey et al., 2010) or the O$_2$ cost of ATP resynthesis (Larsen et al. 2011), NO$_3^-$ supplementation may facilitate performance by allowing individuals to compete at a higher intensity with lower metabolic cost. It is likely that all the aforementioned functions (and possibly other undiscovered ones) act in synergy to produce the ergogenic effect of NO$_3^-$ supplementation. However, as noted previously, certain effects (namely Ca$^{2+}$ handling proteins and increased blood flow) appear to be fiber-type specific, with data suggesting Type II fibers are the ones predominantly affected (Hernandez et al., 2012; Ferguson et al., 2013). Therefore, the various mechanisms underpinning the ergogenic effect of NO$_3^-$ supplementation may be intensity dependent, with the ratio of Type I: Type II muscle fiber activation dictating which are most prevalent in aiding exercise performance.
**PRACTICAL APPLICATIONS**

- Inorganic NO$_3^-$ supplementation may improve high-intensity upper-body exercise performance by 0.4% in well-trained athletes.

- Whilst an acute ~5.0 mmol/350 mg dose of NO$_3^-$ may improve performance in recreationally trained individuals, well-trained athletes may require a larger (e.g. 8.4 mmol/520 mg) NO$_3^-$ dose for improvements in performance.

- This acute dose of NO$_3^-$ should be taken approximately 2 hours prior to the onset of exercise, to coincide with peak plasma [NO$_2^-$].

- High-intensity endurance events (those performed near VO$_2$max) appear more likely to benefit from NO$_3^-$ supplementation than longer, less intense events.

- Upper-body dominant exercises such as rowing and kayaking may be more sensitive to the effects of NO$_3^-$ supplementation than lower-body exercise.

- There is a large between-athlete variation in the response to NO$_3^-$ supplementation, for which athlete fitness only accounts for part.

- Taking NO$_3^-$ for several days may induce changes in the expression of certain proteins aiding exercise capacity. After 4 days, muscular force is preserved during exhaustive activity.

- There is little data available comparing the effectiveness of the various sources of NO$_3^-$, however natural sources such as beetroot juice also provide additional compounds such as polyphenols and antioxidants which may have additional health benefits and may carry a lower risk of containing prohibited substances.
THESIS LIMITATIONS

Our systematic review in Chapter 2 highlighted a high degree of variation in the supplementation protocol, subject cohort and outcome measures utilized in existing published research concerning NO₃⁻ supplementation and exercise performance. These discrepancies restricted meta-analytical sub-analysis of potential moderators of supplementation. Analysis grouped by participant training status or supplementation procedure (i.e. the amount and duration of supplementation) may have provided insight into the interaction effects of these factors, however this was inappropriate given the diverse nature of the exercise assessments. Instead, the authors made the decision to group studies according to the type of exercise assessment and qualitatively assess other variables accordingly.

The intervention study presented in Chapter 3 was confounded by a relatively large week-to-week variation in performance, which likely contributed to a greater uncertainty in the results (as denoted by the confidence intervals). This was arguably a consequence of the concurrent training participants were completing during the course of the study. Nevertheless, we employed a randomized cross-over design and used mixed modeling statistical analysis to minimize the impact of this on the main effect. From a practical perspective, the testing protocol utilized in the study was designed to emulate the demands of real world track cycling, where multiple bouts of exercise are required (such as qualifying rounds followed by the semi-finals and finals). However, repeat tests introduced a time effect that further confounded interpretation of the results. Moreover, it is common for competitors to ‘pace’ their first race (such as a Semi-Final) in order to save their efforts and peak in the following (Finals). In contrast, in this study participants were instructed to obtain the best results in each time trial. Although the methodologies applied in the study were intended to obtain the most valid results, the findings may therefore lose some applicability to elite competition. Perceptual measures and analysis of pacing strategies during testing may have provided further insight into the physiological effects of NO₃⁻ supplementation; however were not collected at the time. This additional information may have provided greater context for interpreting the study findings and applying them practically.
The primary drawback of our study in Chapter 4 was the low number of subjects in the investigation. Restricting inclusion to only well-trained rowers significantly limited the pool of eligible participants; however, ensured that a) subjects were familiar with the testing protocol to reduce possible learning effects b) the sample group was relatively homogenous and c) the results were valid and specific for trained athletes. In a separate issue, the number of plasma $[\text{NO}_2^-]$ data points was reduced due to some errors during the blood collection process. Whilst the correlation between $\Delta\text{NO}_2^-$ and performance reached near significance, more data may have improved the power of analysis. With only 11 data points, interpretation of this relationship must be treated with caution. Analysis was further confounded by the high degree of variation in $\text{NO}_2^-$ kinetics between individuals; however, this disparity is apparent in similar studies and may represent natural biological variation.

The findings from Chapter 5 indicate that several days of $\text{NO}_3^-$ supplementation induces peripheral adaptations that improve the fatigue resistance of muscle. Although these data contribute to the scientific body of literature and may explain part of the ergogenic effect of supplementation, some inconsistencies may limit the applicability of the results to the previous chapters. Firstly, the subjects recruited in the study were varied in their level of fitness. Similar to the previous chapter, well-trained subjects were difficult to recruit due to their limited number and demanding training schedules. Therefore, it is not certain if the observed improvements to fatigue resistance translate to a more highly trained population. Similarly, the $\text{NO}_3^-$ supplementation protocol utilized differed to those used in previous chapters. If the observed improvement in muscular force following chronic $\text{NO}_3^-$ supplementation is due to protein expression changes as postulated, these are unlikely to explain the results of the previous chapters where only an acute dose was used. Secondly, the isometric, single-joint movement assessed in the study was not an exact representation of the skeletal contraction seen in rowing or cycling. The constrained and electrically evoked contraction likely utilizes the knee extensor muscles in a different manner to rowing and cycling. However, by tightly controlling the contraction, internally valid and precise measurements were possible. We were limited in our ability to examine directly within the muscle, which may have provided insight into the cellular events underlying the attenuation of muscular fatigue observed following supplementation. Our hypothesis that $\text{Ca}^{2+}$
handling had been altered in the muscle was drawn from similar research in a rodent model, as these findings have yet to be confirmed in humans. *In vitro* analysis of muscle for changes in Ca\(^{2+}\) and related proteins may confirm this hypothesis. Similarly, being able to measure blood flow in the limb may have confirmed that any vasodilative effects of NO\(_3^-\) had truly been removed.

Although the supplementation protocol evolved and adapted with concurrent research over the course of the thesis, the changing nature of it from chapter to chapter made it more difficult to elucidate the optimal dosing protocol and tease out the effects of timing and dose. We used what we believed to be the model of best practice at the time of each study, however by doing so, the ability of certain chapters to inform the other parts of thesis has been limited.

**FUTURE RESEARCH**

Although data from this thesis confirm that inorganic NO\(_3^-\) supplementation may improve endurance exercise in well-trained athletes, the prevalence of ‘non-responders’ appears to be higher in this cohort than lesser trained populations. In conjunction with other available literature (Wilkerson et al., 2012, Wylie et al., 2013a), our data (Chapter 4) suggest that the ability of NO\(_3^-\) to elicit ergogenic benefits is tied to the individual’s ability to reduce it to NO\(_2^-\), such that there is a Δ[NO\(_2^-\)] and Δperformance correlation; at least when Δ[NO\(_2^-\)] is measured in absolute terms. Currently, it is unclear if this association is influenced by the pre-supplementation [NO\(_2^-\)] i.e. are performance improvements better explained by relative changes in [NO\(_2^-\)] (rather than absolute amounts)? If so, this may partially explain why athletes, who typically display a higher resting [NO\(_2^-\)], may not be as susceptible to the effects of supplementation. Unfortunately these correlations were not performed in our analysis, so this relationship remains speculative.

A more complex problem perhaps, is determining why individuals, even those of similar fitness levels, express a varied [NO\(_2^-\)] response following NO\(_3^-\) ingestion. The
within-individual factors that moderate this response require further investigation to better inform the practices of sport dietitians, coaches and athletes. Habitual intake of NO$_3^-$ is likely a key influence, but has not been critically evaluated to date. A greater background intake of NO$_3^-$ is likely to contribute to an elevated baseline plasma [NO$_2^-$] and reduce the impact of supplementation. Research into this topic is currently hampered due to few resources cataloging the NO$_3^-$ content of foods. However, a listing of NO$_3^-$ content may not be feasible as NO$_3^-$ levels in food vary greatly and are highly subject to both growing and storage conditions (Hord, Yang, & Bryan, 2009). Alternatively, the recent proliferation of epigenetic research and technologies may provide opportunities to identify key genetic traits mediating the response to supplementation. For instance, recent work has found that particular genetic variation at the uncoupling protein 2/3 locus (UCP2/UCP3) may favour efficiency adaptation (Dhamrait et al., 2012). Given that UCP3 expression is associated with an improvement in exercise efficiency and performance following supplementation (Larsen et al., 2011), individuals displaying a favourable genotype may be more responsive to NO$_3^-$.

Extrinsic factors that may influence the efficacy of NO$_3^-$ supplementation also require further examination. Although results from this thesis have shown NO$_3^-$ may significantly improve short and intense exercise performance, there is a paucity of research on activities of extended duration. While the reduction of NO$_3^-$ is aided by the acidic and hypoxic stress associated with short and intense exercise, these physiological stimuli become less prominent as the duration of exercise increases and subsequently, intensity reduces. To date, the longest exercise test investigated has been just over two hours (Wilkerson et al., 2012). Certain endurance events may last >8 hours (such as the ironman triathlon) and it is unknown if NO$_3^-$ supplementation can aid these situations. In addition, environmental factors may not only alter the potency of supplementation, but also the mechanism by which it acts. For instance, exercise in hot conditions is known to impart a heavy cardiac strain due to loss of plasma through sweat and conflicting blood flow demand from muscle and skin (González-Alonso, Crandall, & Johnson, 2008). Greater vasculature control following NO$_3^-$ supplementation (Ferguson et al., 2013) may alleviate some of these effects and facilitate performance in thermoregulatory-challenging conditions in this manner. Similarly, diminished performance in hypoxic environments (characterized by reduced O$_2$ supply to muscle)
may be aided by the lower O\textsubscript{2} cost of exercise following supplementation (Larsen, Weitzberg, Lundberg, & Ekblom, 2007). Of the few studies performed to date, several have already shown that NO\textsuperscript{3−} supplementation may partially reduce the deleterious effects hypoxia has on exercise (Masschelein et al., 2012, Muggeridge et al., 2014a, Vanhatalo et al., 2011). Given the multitude of conditions sport competition may be conducted in and the unique physiological challenges they impart, further work is required to quantify the effect NO\textsuperscript{3−} supplements in each.

Gradually, the scientific data in favour of NO\textsuperscript{3−} supplementation is mounting, with new insights on the mediating physiological mechanisms. The results from our study presented in Chapter 5 suggest that peripheral muscle adaptations occur following several days of supplementation, contributing to fatigue resistance when O\textsubscript{2} delivery is compromised. We suggest, based on research in rodent models, that adaptations are related to Ca\textsuperscript{2+} handling during excitation-contraction coupling; however, further research is required to confirm this in human tissue. Subsequent research should also seek to determine the time course of adaptation (including when favourable changes begin to rescind) and if larger doses of NO\textsuperscript{3−} elicit further structural adaptation. With greater insight into the mechanisms underpinning supplementation, research may then begin to elucidate synergistic supplements or elements that may be additive to the effect of NO\textsuperscript{3−}. Caffeine is one supplement that has already been investigated, as it is known to elicit a similar effect on muscular contraction. However, the limited studies available to date have reported mixed findings (Handzlik and Gleeson, 2013, Lane et al., 2013). Additive therapies may extend beyond dietary means, with a recent intervention combining novel ultra-violet light (UV-A specifically) exposure with beetroot juice consumption (Muggeridge et al., 2014b). The UV-A light is thought to stimulate endogenous NO production and initial results suggest both interventions may be complementary.

In addition to work focusing on additive interventions, more research is required to quantify the effects of the numerous NO\textsuperscript{3−} supplements currently available, which range from pharmaceutical (such as sodium nitrate and potassium nitrate) to nutraceutical (e.g. beetroot and pomegranate juice concentrates) in nature. Although NO\textsuperscript{3−} content can be measured and standardised, it is unknown if the bio-availability and potency of
Nitrate-rich beetroot juice was selected as the predominant method of supplementation throughout this thesis due to its natural source. Even though the results of this thesis and other published work (Bailey et al. 2010; Cermak et al. 2012, Lansley et al. 2011a) have demonstrated beetroot juice can be ergogenic, it is unclear if it is more or less effective than other agents such as sodium nitrate (NaNO₃), which have also been shown to be effective in improving exercise performance (Larsen et al., 2011, Larsen et al., 2007). More recent products to become commercially available include beetroot bars and nitrate-containing sports gels which have the added benefit of being easily consumed during exercising. The convenience of these products make them appealing to athletes, despite their efficacy not being scientifically proven.

Although the literature pertaining to inorganic NO₃⁻ as a sports supplement has gained traction in recent years, the topic still has many unanswered aspects. Whilst the adoption of interventions such as beetroot juice supplementation has proved popular amongst elite performers (Jones, 2014), more work is required to optimize the way NO₃⁻ is applied to improve endurance exercise performance.

**CONCLUSION**

The findings of this thesis demonstrate that dietary supplementation with inorganic NO₃⁻ has the potential to improve endurance performance during high-intensity exercise in well-trained athletes, however, only if a sufficient dose is prescribed (Chapters 2 & 3). The acute dose required for benefit appears to be greater than what has previously been shown to be effective in lesser trained individuals (Chapter 2), as trained athletes tend to display a blunted plasma [NO₂⁻] response following NO₃⁻ intake, indicating similar doses of NO₃⁻ are not as effective (Chapters 3 & 4). This may be potentially overcome by prescribing greater amounts of NO₃⁻ pre-exercise (Chapter 4). Alternatively, NO₃⁻ supplements may be prescribed over several days in the lead up to exercise/competition, which may provide fatigue-reducing responses in the musculature (Chapter 5).
REFERENCES


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<td>Cermak et al., 2012a</td>
<td>Trained cyclists/triathletes 12 (12)</td>
<td>6 days ~8.0 mmol NO₃/d</td>
<td>BJ concentrate</td>
<td>10 km cycling TT</td>
<td>N: 953 ± 18 s P: 965 ± 18 s</td>
<td>1.2%</td>
</tr>
<tr>
<td>Peeling et al., 2014</td>
<td>Elite kayakers 5 (0)</td>
<td>~ 9.6 mmol NO₃; 2 h before exercise</td>
<td>BJ concentrate</td>
<td>500 m on-water TT</td>
<td>N: 114.6 ± 1.5 s P: 116.7 ± 2.2 s</td>
<td>1.7%</td>
</tr>
</tbody>
</table>

**NB:** All studies were randomized, placebo-controlled and double-blinded. N: Nitrate trial; P: Placebo trial; BJ: beetroot juice; NaNO₃: sodium nitrate; TT: time trial
<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Supplementation protocol</th>
<th>Nitrate supplement</th>
<th>Performance measure</th>
<th>Study results</th>
<th>Performance following NO₃⁻ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bescos et al., 2012a</td>
<td>Trained cyclists/triathletes 13 (13)</td>
<td>3 days of 10 mg/kg/d NO₃⁻</td>
<td>NaNO₃</td>
<td>40 min cycle distance trial</td>
<td>N: 26.4 ± 1.1 km P: 26.3 ± 1.2 km</td>
<td>-0.4%</td>
</tr>
<tr>
<td>Boorsma et al., 2014</td>
<td>Elite runners 8 (8)</td>
<td>8 days of 13.0 – 19.5 mmol NO₃⁻/d</td>
<td>BJ concentrate</td>
<td>1500m running TT</td>
<td>N: 250.5 ± 6.2 s P: 251.4 ± 7.6 s</td>
<td>0.4%</td>
</tr>
<tr>
<td>Cermak et al., 2012b</td>
<td>Trained cyclists/triathletes 20 (20)</td>
<td>~8.7 mmol NO₃⁻ 2.5 h before exercise</td>
<td>BJ concentrate</td>
<td>~1 h cycling TT</td>
<td>N: 65.5 ± 1.1 min P: 65.0 ± 1.1 min</td>
<td>-0.7%</td>
</tr>
<tr>
<td>Christensen et al., 2013</td>
<td>Trained cyclists 8 (8)</td>
<td>6 days of 500 mg (~8 mmol) NO₃⁻/d</td>
<td>BJ</td>
<td>~20 min cycling TT</td>
<td>N: 18:20 min P: 18:37 min (± not provided)</td>
<td>1.5%</td>
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<tr>
<td>Lane et al., 2014</td>
<td>Trained cyclists/triathletes 24 (12)</td>
<td>2 days of 8.4 mmol NO₃⁻/d</td>
<td>BJ concentrate</td>
<td>Males: 43.83 km cycling TT Females: 29.35 km cycling TT</td>
<td>N: 64:05 ± 2:50 min P: 63:30 ± 3:16 min</td>
<td>-0.8%</td>
</tr>
<tr>
<td>Peacock et al., 2012</td>
<td>Trained junior x-country skiers 10 (10)</td>
<td>614 mg NO₃⁻ , 2.5 h before exercise</td>
<td>KNO₃</td>
<td>5km running TT</td>
<td>N: 1005 ±53 s P: 996 ± 49 s</td>
<td>-0.9%</td>
</tr>
<tr>
<td>Peeling et al., 2014</td>
<td>Elite kayakers 6 (6)</td>
<td>~ 4.8 mmol NO₃⁻ , 2 h before exercise</td>
<td>BJ concentrate</td>
<td>2 x 4 min ergometer TT</td>
<td>N: 989 ± 31 m P: 982 ± 36 m</td>
<td>0.7%</td>
</tr>
<tr>
<td>Wilkerson et al., 2012</td>
<td>Trained cyclists 8 (8)</td>
<td>6.2 mmol NO₃⁻ , 2.5 h before exercise</td>
<td>BJ</td>
<td>50 mile cycling TT</td>
<td>N: 136.7 ± 5.6 min P: 137.9 ± 6.4 min</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

NB: All studies were randomized, placebo-controlled and double-blinded. N: Nitrate trial; P: Placebo trial; BJ: beetroot juice; NaNO₃: sodium nitrate; KNO₃: potassium nitrate; TT: time trial.
APPENDICES
APPENDIX A: CHAPTER 3 SUPPLEMENTARY MATERIALS
Plain Language statement:

The effect of beetroot juice supplementation on team pursuit performance in the London Olympics Games

The background
The team pursuit is a key race for Australian track cyclists. The schedule for the London Olympic Games track cycling competition provides an interesting challenge, with the mens timetable involving a semi final and final on the same day of competition, with 75 minutes between races. We will be undertaking a concentrated series of investigations to allow the Australian track pursuit team to fine tune their race day nutrition strategies for this schedule, including the use of well-established sports supplements. Things that we would like better information about with supplement use include scenarios in which

- individually effective supplements are used in combination
- specific timing and dose protocols are organised to suit a novel race program.

The current study involves an investigation of the potential benefits of using a beetroot juice drink before the team pursuit event to enhance cycling efficiency. This new idea comes from work of Professor Andy Jones, a British Sports Scientist, and is based on the knowledge that beetroot juice is a significant source of dietary nitrate. Professor Jones has recently conducted a series of studies showing that a single dose of the beetroot juice, taken 2.5 hours before exercise, increases the efficiency (reduces the oxygen cost) of some exercise protocols. This is likely to allow an athlete to sustain optimal pace or work intensity for a longer time before fatiguing. Further work that needs to be undertaken includes:

- Confirmation that this effect occurs in well-trained individuals
- Confirmation that this transfers into better performance in a protocol simulating the demands of the track pursuit event
- Experimentation to find the best way to achieve the effect in a repeated race protocol simulating the London Olympic Games track cycling schedule.

Why am I being invited to participate?
A monitored training program provides a great opportunity to throw some trials of various interventions. We have standardised your training, your diet, your surroundings and your training partners. So, it should be easier than usual to detect the effects of trying a supplement on a particular performance measure.

We have built a series of “London Olympic Games team pursuit simulations” into the program of your 6 week training block. You are invited to participate in a series of 4 supplement trials on these training days to help us answer these questions regarding beetroot juice. Each testing day will focus on your performance of a 4 minute “all out” effort on the laboratory Watt bike (called the MMP4). This protocol is known to mimic the demands of the team pursuit race. You will undertake two “races”, preceded by a 30 minute warm up on rollers, with 75 minutes between the races. Involvement in this study will require you to consume a serve of beetroot juice on these testing days which may aid in your performance of this exercise protocol. The information we gain from this study may provide you with a new tactic to use in your own racing pursuits, but will providing a starting point for our development of the LOG nutrition program for the Australian Track Cycling team.

What will the study actually involve?
We will study a commercially produced vegetable juice drink from the UK (Beet it) which offers a range of useful nutrients in addition to its dietary nitrate content. This drink is in the form of a 70 ml “shot” of concentrated beetroot juice which is equivalent to a normal commercial bottle of beetroot juice (250-300 ml). We have chosen this because although beetroot juice might be considered a normal dietary item for people who love beetroots, it is an acquired taste for some Australians. Therefore, the same nutritional content can be consumed in a smaller portion size. Given that this is a normal food product consumed in normal amounts, we do not anticipate any side-effects occurring from the supplementation protocol.
course, if you are not a regular beetroot eater, you might not know that it can make your wee turn pink for a couple of hours)

Our study protocol will use beetroot juice shots containing nitrate (= normal beetroot juice) and an identical product in which the dietary nitrate has been removed. This will allow us to determine the best timing of intake of the nitrate in relation to the two races to achieve the best final outcome. Our trials will involve

- One shot of nitrate-containing beetroot juice (“Beet it”) consumed 2.5 hours before the second team pursuit event
- One shot of nitrate-containing beetroot juice consumed 2.5 hours before the first team pursuit
- One shot of nitrate-containing beetroot juice consumed 2.5 hours before the first team pursuit event with a top up (half a shot) before the second event
- Beetroot juice containing no nitrate

In each trial you will consume beetroot juice on three occasions, and we will switch the order of the nitrate-containing and the nitrate-removed juices so that you will be unaware of what juice you have consumed. The only preparation that you will need to undertake, outside the conditions of the general training study, is to refrain from using an anti-bacterial mouth wash in the 2 days leading into a trial. The normal bacteria in your mouth help to activate the nitrate in the beetroot juice to do its job.

Our main interest is your performance of the 4 minute cycling test (MMP4), but we will also collect a few other pieces of information that will help us to understand what is going on. These tests will be:

- Collection of 5 ml of blood from your arm vein (maximum of 3-5 times per trial)
- Measurement of your blood pressure (4 times per trial)
- A finger prick blood sample at the completion of the race
An overview of the trial day is shown below.

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<td>Venous blood sample (5 ml)</td>
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<td>Supplement – Beet it beetroot juice shot</td>
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**Your rights**

Please note that this supplementation study is an additional activity within the 6 week training study. It is your prerogative to withdraw from the supplementation program at any time, and no explanation is required for such withdrawal. You will receive feedback about your individual response to buffering protocols as well as the group response.

If the data collected during the study is to be published in scientific and coaching journals and presented at conferences, your identity will not be disclosed unless prior written approval is provided. Results will be filed securely in a locked filing cabinet or secure electronic system for the following 5 years in accordance with the National Health and Medical Research Council Statement of Scientific Practice. After this period results will be destroyed. Access to results will only be made available to principle researchers of the study.

You are free to withdraw at any stage if you feel you cannot complete either of the tests or the supplementation protocols, or do not wish to complete the project.

*If you have any concerns with respect to the conduct of this Monitoring project, you may contact the Secretary of the AIS Ethics Committee (Ms. Helene Kaye) on 02 6214 1816.*
INFORMED CONSENT FORM

Project Title: The effect of beetroot juice supplementation on team pursuit performance in the London Olympics Games

Principal Researchers: Louise Burke, Matthew Hoon, Andrew Jones, Shona Halson, Dave Martin, Nathan Johnson

This is to certify that I, ________________________________ hereby agree to participate as a volunteer in a study of the effect of beetroot juice on cycling performance within the training study I am enrolled in at the Australian Institute of Sport

The investigation and my part in the investigation have been defined and fully explained to me by Louise Burke and Matt Hoon and I understand the explanation. A copy of the requirements of this monitoring process and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.

I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time.

I understand that any data or answers to questions will remain confidential with regard to my identity.

I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.

I am participating in this project of my own free will and I have not been coerced in any way to participate.

Signature of Subject: ________________________________  Date: ___/___/___

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: ________________________________  Date: ___/___/___
# 4 Minute Maximum Power Test

**Athlete Name and ID:**

**Date:**

**Instructions:**
1. Instruct subjects not to pedal
2. On watt bike open: Workout\Custom\1 00:04:00
3. Countdown everyone – 3,2,1 GO – start clock
4. After ensuring test has started, bike is working etc; cover bike screen with towel
5. After test, collect data below (avg power, energy) and then La approximately 1 minute after.

<table>
<thead>
<tr>
<th>4MMP No.1</th>
<th>Time:</th>
<th>Monitor:</th>
</tr>
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<tbody>
<tr>
<td>Average Power (W)</td>
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<td>Energy (KJ)</td>
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<td>La (mmol)</td>
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<tr>
<td>Notes</td>
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</table>

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<tr>
<th>4MMP No.2</th>
<th>Time:</th>
<th>Monitor:</th>
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<tr>
<td>Average Power (W)</td>
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<tr>
<td>Energy (KJ)</td>
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<tr>
<td>La (mmol)</td>
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<tr>
<td>Notes</td>
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</tbody>
</table>

Bike:_____________________

How much of yourself did you give (0-100%)?:________________

How do you feel physically (1 = terrible; 5 = fantastic)?:_____________
MINUTE

TO:       Prof. Louise Burke
FROM:     Ms Helene Rushby
SUBJECT:  Approval from AIS Ethics Committee   DATE:   25th March 2011

The AIS Ethics Committee gave consideration to your submission titled 'The effect of nutritional supplementation strategies on London 2012 team pursuit cycling performance' Out Of Session. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project is 20110213.

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

any proposed changes to the research design,
any adverse events that may occur,

Researchers are required to submit annual status reports to the secretary of the AIS Ethics Committee until completion of the project. Details of status report requirements are contained in the ‘Guidelines’ for ethics submissions.

This Approval is valid until the 30th September 2012; re-approval will need to be sought should the project continue past this date.

Failure to comply with the above will render ethics approval null and void.

If you have any questions regarding this matter, please don’t hesitate to contact me on (02) 6214 1577.

Sincerely
Helene Rushby
Secretary, AIS EC
Plain Language statement:

The effect of beetroot juice supplementation on 2000m rowing performance. Researchers: Matthew Hoon, Louise Burke, Tony Rice, Liz Broad, Bronwen Lundy and Andy Jones

Background
We will be undertaking a concentrated series of investigations to allow members of the Australian Rowing Squad to fine tune their race day nutrition strategies, including the use of well-established sports supplements. Things that we would like better information about with supplement use include scenarios in which

- individually effective supplements are used in combination
- timing and dosage protocols are optimised for a specific athlete and race program

The current study involves an investigation of the potential benefits of taking beetroot juice drink to enhance rowing performance. This new idea comes from work of Professor Andy Jones, a British Sports Scientist, based on the knowledge that beetroot juice is a significant source of dietary nitrate. Professor Jones has recently conducted a series of studies showing that a single dose of the beetroot juice, taken 2.5 hours before exercise, can increase the efficiency (reduces the oxygen cost) of some exercise protocols. This is likely to allow an athlete to sustain optimal pace or work intensity for a longer time before fatiguing. Further work that needs to be undertaken includes:

- Confirmation that this effect occurs in well-trained individuals
- Establishing if this effect is apparent in rowing
- Experimentation to find the optimal dose to boost performance in athletes across a range of body sizes.

Why am I being invited to participate?
Although there has been promising results from cycling and running trials, the effect of beetroot juice on rowing has not been studied by scientists. Furthermore, there is some evidence to suggest the effect on a trained athlete is different to that of an unfit individual. Therefore we would like to determine if beetroot juice is suitable for use in competitive rowers and how this is best achieved.

You are invited to participate in a series of 3 supplement trials (separated by several days in between) to help us answer these questions regarding beetroot juice. Each testing day will focus on your performance of a 2000m time trial on a rowing ergometer (with a 30 minute warm up). This test is commonly used by coaches and physiologists at the Australian Institute of Sport to assess the condition of athletes. Involvement in this study will require you to consume a serve of beetroot juice 2.5hrs before your rowing trials which may aid in your performance. The duration of a testing day is expected to take roughly 3hrs. The information we gain from this study may provide you with a new tactic to use in your own racing, but will also provide a starting point for our development of the London Olympic Games nutrition program.

What will the study actually involve?
We will study a commercially produced vegetable juice drink from the UK (Beet it) which offers a range of useful nutrients in addition to its nitrate content. This drink is in the form of a 75 ml “shot” of concentrated beetroot juice which is equivalent to a normal commercial bottle of beetroot juice (250-300 ml). Beetroot juice can be an acquired taste for some individuals; therefore we opted to use a smaller serve with the same nutritional content. Given that this is a natural food product, we do not anticipate any side-effects occurring from the supplementation protocol although your urine may turn pink for a couple of hours and some persons may experience gastro symptoms. In some trials, we will also use an identical product in which the dietary nitrate has been removed. Both have the same texture, colour and taste so that you will be unaware of how much nitrate you have consumed. This will allow us to determine the optimal dose of nitrate to achieve the best outcome. There will be three protocols (separated by several days in between each) that will involve consumption of the following:

- 150ml of beetroot juice containing no nitrate
- 75ml of nitrate-rich beetroot juice (“Beet it”) and 75ml of nitrate-depleted beetroot juice
- 150ml of nitrate-rich beetroot

On trial days, we will ask you to consume your designated drink 2.5hrs before undertaking a 2,000m ergometer time trial. As an incentive, we are offering prizes of $300, $150 and $50 to the top three athletes who perform the best (relative to your PB) over all the trials. The order you will undertake each protocol is completely randomised and double blinded, meaning that both you and the researchers will
not know which trial you are completing until afterwards. The only preparation that you will need to undertake is to refrain from using anti-bacterial mouth wash and chewing gum in the 2 days leading into a trial, as the normal bacteria present in your mouth help to break down the nitrate in the beetroot juice. We also ask you keep a brief training and food diary for the two days prior to each trial (documents will be provided).

Our main interest is your performance of the 2000m time trial, but we will also collect a few other pieces of information that will help us to understand what is going on. These tests will be
- Collection of 20 ml of blood from your arm vein (twice per trial)
- Measurement of your blood pressure (twice per trial)

An overview of the trial day is shown below:

Your rights
Please note your participation in the study is completely voluntary. It is your prerogative to withdraw from the supplementation program at any time, and no explanation is required for such withdrawal. If the data collected during the study is to be published in scientific and coaching journals and presented at conferences, your identity will not be disclosed unless prior written approval is provided. Results will be filed securely in a locked filing cabinet or secure electronic system for the following 5 years in accordance with the National Health and Medical Research Council Statement of Scientific Practice. After this period results will be destroyed. Access to results will only be made available to principle researchers of the study.

You are free to withdraw at any stage if you feel you cannot complete either of the tests or the supplementation protocols, or do not wish to complete the project. If you have any further questions concerning the study, please feel free to consult any of the researchers. If you have any concerns with respect to the conduct of this Monitoring project, you may contact the Secretary of the AIS Ethics Committee (Ms. Helene Kaye) on 02 6214 1816.
‘INFORMED CONSENT’ FORM

Project Title: Can nitrate supplementation aid rowing performance?

Principal Researchers: Louise Burke, Matthew Hoon, Tony Rice, Liz Broad, Bronwen Lundy, Nathan Johnson

This is to certify that I, ___________ hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Louise Burke.

The investigation and my part in the investigation have been defined and fully explained to me by ___________ and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

Signature of Subject: _______________________________ Date: ___/___/___

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: _____________________________ Date: ___/___/___
Dietary standardisation pre-ergo trial
Beetroot juice study

To tease out the effects of the beetroot juice on your performance, we need to standardise the other factors that can influence the outcome as well as possible.

Your recent nutrition in one such factor.

Our approach is to allow you to organise your own version of an ideal pre-event fuelling and hydration plan, then get you to repeat this on each occasion. We will concentrate on the period from lunch time the day before up to the ergo trial.

PLEASE COMPLETE THIS RECORD FROM LUNCH THE DAY BEFORE YOUR TRIAL RIGHT THROUGH TO THE ERGO TEST

Note that we have given you some guidelines for the type of menu plan that would promote good preparation

Please fill in the following table with as much detail as possible about the food and fluid you consume in the critical period leading into the ergo trial.

✓ Carry the diary with you at all times and write everything down as it happens. Don’t rely on memory at the end of the day…. a messy diary is a well kept diary.
✓ Stick to a version of this plan that reflects your usual habits and preferences. You will be required to replicate this next week as well, so choose a plan that can be repeated easily and enjoyably.
✓ Remember to include all the things that you add to food when eating (eg. margarine, oil, sugar, dressings).
✓ Please remember to describe foods in as much detail as possible (eg. white/wholemeal, fat left on/trimmed off, sweetened/unsweetened, full cream/reduced fat).
✓ List the ingredients and special features of mixed dishes such as pizza, pasta sauce, stir fries, casseroles, ingredients in salad.
✓ Record the quantity of foods and fluids as accurately as possible. Number of scoops, package size, dimensions (e.g. for meat / chicken etc 12cm x 20cm), tablespoons or teaspoons, number of slices etc.
✓ Include all water, cordial, sports drink and milk.

For example;

2 slices of toast with butter and vegemite, 1 glass of orange juice
4 weet bix with Tone milk and 2 tablespoons of sugar and 2 glasses of water
2 scoops of rice with 2 scoops of chicken & cashew stir fry and 1 glass of cordial plus 1 hot chocolate
<table>
<thead>
<tr>
<th>Meal Type</th>
<th>Time</th>
<th>Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lunch</strong></td>
<td></td>
<td><em>(Goals: include carbohydrate-rich foods such as bread, or leftovers which include rice or pasta)</em></td>
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<tr>
<td></td>
<td></td>
<td>Time</td>
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<tr>
<td></td>
<td></td>
<td>Intake</td>
</tr>
<tr>
<td><strong>Afternoon Training</strong></td>
<td></td>
<td><em>(Goals: stay well hydrated and fuelled)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Type and duration of training:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Food &amp; liquid intake during training:</td>
</tr>
<tr>
<td><strong>Recovery snack</strong></td>
<td></td>
<td><em>(Goals: hydrate well after the session)</em></td>
</tr>
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<td></td>
<td>Time:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intake:</td>
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<tr>
<td><strong>Dinner and dessert/supper</strong></td>
<td></td>
<td><em>(Goals: include carbohydrate-rich foods such as bread, rice or pasta; Dessert may include sweetened yoghurts/custard/icecream and fruit. Drink to promote good hydration)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time:</td>
</tr>
<tr>
<td><strong>Breakfast</strong></td>
<td></td>
<td><em>(Day of trial)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>(Goals: follow your standard pre-ergo breakfast to provide fuel and fluid but keep gut comfortable to perform hard)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Time:</td>
</tr>
</tbody>
</table>
Recording sheet

Date:

Athlete:________________________

D.O.B:

Body Weight: Base BP:

Drink time: 60min BP:

Test time: Lab conditions:

2000m Ergometer

Erg #

Completion time:

[La] 1 min post: [La] 4 min post:

Splits (Taken from Concept after test):

<table>
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<tr>
<th></th>
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<th>1,000m</th>
<th>1,500m</th>
<th>2,000m</th>
<th>Average</th>
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<tr>
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<td></td>
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<tr>
<td>Power</td>
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<tr>
<td>Heart rate</td>
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<tr>
<td>Stroke</td>
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</tbody>
</table>

Post-trial questions

Did you experience any gastric symptoms (1= none; 5= very unpleasant)?:________

How much effort did you give (0-100%)?:______________

How do you feel physically (1 = terrible; 5 = fantastic)?

Before the erg:_________________

After the erg:_______________

How would you rate your performance today (1 = terrible; 5 = fantastic)?:_______

Notes:
A.I.S Beetroot Supplementation Study
End of Study Questionnaire

Name:______________________          Age:

No. yrs rowing experience:____________

How many hours a week do you train?:__________

1. Did you think beetroot juice worked for you?  Y   /   N
2. Can you guess which order you performed the following trials:
   i.  150ml nitrate rich beetroot juice: Trial_____  
   ii. 75ml nitrate beetroot juice (and 75ml placebo): Trial _____ 
   iii. 150ml of placebo beetroot: Trial ______

3. After ingesting beetroot juice, how did you feel physically compared to normal ergo sessions?:
   a) During the warm up and time trials:

   |   |   |   |   |   |
   | 1 | 2 | 3 | 4 | 5 |
   | felt really bad | the same | felt really good |

   b) Following the tests:

   |   |   |   |   |   |
   | 1 | 2 | 3 | 4 | 5 |
   | felt really bad | the same | felt really good |

4. Did you experience any discomfort or side effects from the beetroot juice? If yes please elaborate:
____________________________________________________________________________________________________________________________________________________
____________________________________________________________________________________________________________________________________________________
____________________________________________________________________________________________________________________________________________________

5. Following your experience with the study, would you use beetroot juice before racing/training in the future?
____________________________________________________________________________________________________________________________________________________
____________________________________________________________________________________________________________________________________________________

6. Are there any other comments you would like to make?
____________________________________________________________________________________________________________________________________________________
____________________________________________________________________________________________________________________________________________________
____________________________________________________________________________________________________________________________________________________
Rowers Wanted For Supplement Study

A joint project between the Australian Institute of Sport and the University of Sydney is seeking competitive rowers to participate in a study investigating the effect of nitrate supplementation (via beetroot juice) on rowing performance. We are looking for:

- Males OR females aged 18-35 yrs
- Have been training 4+ times a week for the last 2yrs
- Males who can perform a 2,000m erg in under $6:45$
- Females who can perform a 2,000m erg in under $7:15$
  (i.e. roughly state level competitors)

Specifically, the project will:

- be conducted at the A.I.S. in Canberra
- require 3 trials (each ~2hrs) - each trial will involve drinking 150ml of beetroot juice and a 2,000m erg
- provide participants with training and recovery food
- require two blood samples during each trial
- Have a **prize pool of $500** for the best performing participants (relative to your PB)

**For more information on the study, please contact Matthew Hoon:**

Email (preferred): Mhoo7389@uni.sydney.edu.au

Phone: 0431173648
MINUTE

TO: Mr Matthew Hoon
FROM: Ms Laura Juliff
SUBJECT: Approval from AIS Ethics Committee
DATE: 16th December 2011

At the last meeting of the AIS Ethics Committee, held on the 13th of December 2011, the Committee gave consideration to your submission titled "Can nitrate supplementation aid rowing performance?" The Committee saw no ethical reason why the study should not proceed subject to the following amendments:

- Report gastric disturbances as a potential side effect of the beetroot juice in the "information to participant form".
- Clearly state the prize pool for the participants and how this is to be assessed in the "information to participant form".
- Clarify the subject number to the ethics committee.

The approval number for this project is 20111208.

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

- any proposed changes to the research design,
- any adverse events that may occur,

Researchers are required to submit annual status reports to the secretary of the AIS Ethics Committee until completion of the project. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

This Approval is valid until the 30th of July 2012; re-approval will need to be sought should the project continue past this date.

Failure to comply with the above will render ethics approval null and void.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577.

Sincerely,
Laura Juliff
Secretary, AIS EC
The Effect of Beetroot Juice Supplementation on Muscle Contraction

PARTICIPANT INFORMATION STATEMENT

(1) What is the study about?

You are invited to participate in a study investigating the effect of beetroot juice supplementation on muscle function. After several days of beetroot juice supplementation we expect that there will be an increase in the force production capability of muscle.

(2) Who is carrying out the study?

The study is being conducted by Mr. Matthew Hoon and will form the basis for the degree of Doctor of Philosophy at The University of Sydney under the supervision of Dr. Nathan Johnson.

(3) What does the study involve?

The total study comprises of 3 visits to the Exercise and Sports Science Laboratories at the University of Sydney, Cumberland in Lidcombe. During these visits, the contraction force of the quadricep muscles will be tested via electrical stimulation. This will involve two electrode pads being placed on the muscle and an electrical current will be sent to cause your muscle to contract. The resulting force will be recorded by a force transducer which the leg is secured to. Although the sensation of the electrical stimulation may be initially surprising, the maximum current is set at your own tolerance. Part of the testing protocol also involves muscle stimulation while leg blood flow is partially occluded (much alike having your blood pressure measured). Additionally on the first visit, you will be asked to perform a 30s Wingate cycle test.
Between visits

Each testing session will be separated by at least 7 days. On one occasion, you will be asked to consume one bottle of beetroot juice each day (which will be provided by researchers) for 5 days preceding a visit. We also ask on this occasion that you refrain from using mouthwash and chewing gum for the 5 days. On another occasion you will be asked to consume one bottle of beetroot juice each day (provided) for 5 days – on this occasion the active nitrate ingredient will have been removed.

(4) How much time will the study take?

The overall time commitment for the study is estimated to be between 4.5-5 hrs (broken into three separate testing days), with visit 1 lasting approximately 90 min and visits 2 & 3 approximately 60 min each.

(5) Is there any risk associated with the study?

The electrical stimulation of the muscle may cause some transient discomfort, however we do not expect it to be painful. The maximum intensity of the stimulations will be set so it is tolerable for you. After consumption of beetroot juice, some people report a slight discolouration of their urine and stools, however this effect is harmless.

(6) Can I withdraw from the study?

Being in this study is completely voluntary - you are not under any obligation to consent and - if you do consent - you can withdraw at any time without affecting your relationship with The University of Sydney or the researchers.

(7) Will anyone else know the results?

All aspects of the study, including your contact details and results, will be strictly confidential and only the researchers will have access to information on participants.

A report of the study may be submitted for publication, but individual participants will not be identifiable in such a report.

(8) Will the study benefit me?
The study will provide you with several days worth of beetroot juice to drink. Studies have demonstrated the positive effects it has on cardiovascular health as well as exercise capacity. This trial will allow you to try for yourself without having to front the cost of the juice. The results from this study may help inform your own nutritional practices.

(9) Can I tell other people about the study?

Yes and the researchers do encourage you to pass on information to those you believe are suitable for this project. The chief investigator’s contact details are available at the beginning of this document.

(10) What if I require further information about the study or my involvement in it?

When you have read this information, Matthew Hoon will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact:

Matthew Hoon, Chief Investigator:  
matthew.hoon@sydney.edu.au  
+61 2 9036 7364

Nathan Johnson, Research Supervisor:  
nathan.johnson@sydney.edu.au  
+61 2 9351 9328

(11) What if I have a complaint or any concerns?

Any person with concerns or complaints about the conduct of a research study can contact The Manager, Human Ethics Administration, University of Sydney on +61 2 8627 8176 (Telephone); +61 2 8627 8177 (Facsimile) or ro.humanethics@sydney.edu.au (Email).

This information sheet is for you to keep
PARTICIPANT CONSENT FORM

I, ..........................................................[PRINT NAME], give consent to my participation in the research project:

TITLE: THE EFFECT OF BEETROOT JUICE SUPPLEMENTATION ON MUSCLE CONTRACTION

In giving my consent I acknowledge that:

1. The procedures required for the project and the time involved have been explained to me, including any inconvenience, risk, discomfort or side effect, and their implications, and any questions I have about the project have been answered to my satisfaction.

2. I have read the Participant Information Statement and have been given the opportunity to discuss the information and my involvement in the project with the researcher/s.

3. I understand that being in this study is completely voluntary – I am not under any obligation to consent.

4. I understand that my involvement is strictly confidential. I understand that any research data gathered from the results of the study may be published however no information about me will be used in any way that is identifiable.

5. I understand that I can withdraw from the study at any time, without affecting my relationship with the researcher(s) or the University of Sydney now or in the future.
6. I consent to:

- Receiving Feedback  YES ☐  NO ☐

   If you answered YES to the “Receiving Feedback” question, please provide your details i.e. mailing address, email address.

   **Feedback Option**

   **Address:** __________________________________________________________

   **Email:** ____________________________________________________________
AHA/ACSM Health/Fitness Facility Preparticipation Screening Questionnaire

Assess your health needs by marking all true statements.

History
You have had:

___ A heart attack
___ Heart surgery
___ Cardiac catheterization
___ Coronary angioplasty (PTCA)
___ Pacemaker/implantable cardiac defibrillator/rhythm disturbance
___ Heart valve disease
___ Heart failure
___ Heart transplantation
___ Congenital heart disease

If you marked any of the statements in this section, consult your physician or other appropriate healthcare provider before engaging in exercise. You may need to use a facility with a medically qualified staff.

Other health issues

___ You have diabetes
___ You have or asthma other lung disease.
___ You have burning or cramping in your lower legs when walking short distances.
___ You have musculoskeletal problems that limit your physical activity.
___ You have concerns about the safety of exercise.
___ You take prescription medication(s).
___ You are pregnant.

Symptoms

___ You experience chest discomfort with exertion.
___ You experience unreasonable breathlessness.
___ You experience dizziness, fainting, blackouts.
___ You take heart medications.

Cardiovascular risk factors

___ You are a man older than 45 years.
___ You are a woman older than 55 years, you have had a hysterectomy, or you are postmenopausal.
___ You smoke, or quit within the previous 6 mo.
___ Your BP is greater than 140/90.
___ You don't know your BP.
___ You take BP medication.
___ Your blood cholesterol level is >200 mg/dL.
___ You don't know your cholesterol level.
___ You have a close blood relative who had a heart attack before age 55 (father or brother) or age 65 (mother or sister).
___ You are physically inactive (i.e., you get less than 30 min. of physical activity on at least 3 days per week).
___ You are more than 20 pounds overweight.

If you marked two or more of the statements in this section, you should consult your physician or other appropriate healthcare provider before engaging in exercise. You might benefit by using a facility with a professionally qualified exercise staff to guide your exercise program.

___ None of the above is true.

You should be able to exercise safely without consulting your physician or other healthcare provider in a self-guided program or almost any facility that meets your exercise program needs.


www.acsm-msse.org/GlobalCoreTemplateJournal/msse/media/6688c.htm

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PARTICIPANTS NEEDED!

For a study on

MUSCLE STRENGTH AND BEETROOT JUICE SUPPLEMENTATION

We are currently seeking healthy individuals to participate in a University trial investigating changes to muscle force following a diet supplemented with beetroot juice.

WHAT IS INVOLVED?

- Attend 3 separate testing sessions, lasting 60 – 90min each at the University of Sydney, Cumberland.
- On two occasions, for a period of 5 days, drink one bottle of beetroot juice (provided) daily
- Undergo a series of Functional Electrical Stimulation leg tests

WHAT DO YOU GET?

- The study will provide you with several bottles of beetroot juice to try, which has been found to be an effective sports supplement as well as highly beneficial for cardiovascular health. The results of this study may inform your own nutritional practices.

IF YOU ARE INTERESTED IN PARTICIPATING OR WOULD LIKE MORE INFO, PLEASE CONTACT:

Mr. Matthew Hoon: matthew.hoon@sydney.edu.au (preferred);
(02) 9036 7364; Room H111 Cumberland Campus
Research Integrity
Human Research Ethics Committee

Wednesday, 23 April 2014

Mr Matthew Hoon
Exercise Health and Performance; Faculty of Health Sciences
Email: matthew.hoon@sydney.edu.au

Dear Matthew

I am pleased to inform you that the University of Sydney Human Research Ethics Committee (HREC) has approved your project entitled “Nitrate supplementation and muscle contractile force”.

Details of the approval are as follows:

Project No.: 2014/252
Approval Date: 23 April 2014
First Annual Report Due: 23 April 2015
Authorised Personnel: Hoon Matthew; Johnson Nathan; Chapman Phillip;

Documents Approved:

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</tr>
<tr>
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<td>Participant Consent Form</td>
<td>Informed consent</td>
</tr>
</tbody>
</table>

HREC approval is valid for four (4) years from the approval date stated in this letter and is granted pending the following conditions being met:

**Condition/s of Approval**

- Continuing compliance with the National Statement on Ethical Conduct in Research Involving Humans.
- Provision of an annual report on this research to the Human Research Ethics Committee from the approval date and at the completion of the study. Failure to submit reports will result in withdrawal of ethics approval for the project.
- All serious and unexpected adverse events should be reported to the HREC within 72 hours.
- All unforeseen events that might affect continued ethical acceptability of the project should be reported to the HREC as soon as possible.
- Any changes to the project including changes to research personnel must be approved by the HREC before the research project can proceed.
- Note that for student research projects, a copy of this letter must be included in the candidate’s thesis.
Chief Investigator / Supervisor’s responsibilities:

1. You must retain copies of all signed Consent Forms (if applicable) and provide these to the HREC on request.

2. It is your responsibility to provide a copy of this letter to any internal/external granting agencies if requested.

Please do not hesitate to contact Research Integrity (Human Ethics) should you require further information or clarification.

Yours sincerely

Dr Stephen Assinder
Chair
Human Research Ethics Committee

This HREC is constituted and operates in accordance with the National Health and Medical Research Council’s (NHMRC) National Statement on Ethical Conduct in Human Research (2007), NHMRC and Universities Australia Australian Code for the Responsible Conduct of Research (2007) and the CPMP/ICH Note for Guidance on Good Clinical Practice.
Supplementary Figures

Supplementary Figure 1. Setup of participants during muscle function testing.