The Effect of Nitrate Supplementation on Cycling Performance

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A thesis submitted to the Auckland University of Technology in fulfilment of the requirements for the degree of

Doctor of Philosophy

Auckland University of Technology

Faculty of Health and Environmental Science

ABSTRACT

Recently, nitrate (NO_3^-) supplementation by way of either beetroot juice or sodium nitrate ($NaNO_3^-$) has been shown to alter the physiology of, and enhance sport performance in, trained athletes. These improvements have been thought to occur via an oxygen independent NO_3^- - nitrite (NO_2^-) - nitric oxide (NO) pathway, with elevated levels of basal NO_3^- and NO_2^- leading to improvements in microvascular pressure, blood perfusion, protein handling and mitochondrial efficiency. As evidenced by Chapter 2, while numerous studies have shown performance improvements as a result of NO_3^-, historically the majority of such studies have been in longer duration (>8 min) trials, most often employing recreational participants or moderately-trained endurance populations. However, recent attention has focused on sporting events highly reliant on type II muscle, given that these fibres appear most likely to benefit from elevated NO_3^- intakes. Indeed, the ergogenic effects of NO_3^- appear to be mediated by the reduction of NO_2^- to NO. Interestingly, the transformation to NO appears to be catalysed during times of low oxygen availably suggesting that sporting events which are characterised by attainment or overshoot of VO_2max, with reliance on both anaerobic and aerobic energy systems may benefit from NO_3^- supplementation. As such, the mechanistic derived benefits of NO_3^- in combination with events that utilise type II fibres suggest a favourable environment for performance enhancements. However, as outlined in Chapter 2, to date, few studies have assessed the impact of NO_3^- on shorter duration performance, particularly in competitive well-trained populations. Therefore, the overall aim of this thesis was to determine the ergogenic effects of NO_3^- supplementation on supramaximal cycling performance in well-trained cyclists. To address this aim, 3 studies were conducted investigating: 1) the effect of NO_3^- on physiology and 4 km TT performance in well-trained cyclists; 2) the effect of dose and dose duration of NO_3^- supplementation on maximal-intensity cycling time-trial performances of various lengths; and 3) the influence of heat on the ergogenic effects of NO_3^- during maximal-intensity time-trial performance.

Study 1: The first study employed 8 well-trained cyclists to assess the effects of 6 to 8 d of NO_3^- supplementation on key physiological performance indicators and 4 km time-trial cycling performance. It was concluded that NO_3^- supplementation had no effect on

physiological measures or economy after 6 and 7 d of NO_3^- supplementation, respectively. However, 8 d of NO_3^- supplementation improved time-trial performance time (-0.7 s ± 0.9%), and power (2.4 W ± 2.5%), indicating a *likely beneficial* effect for well-trained (VO_2peak = 63 ml.kg^{-1}.min^{-1}) male endurance cyclists.

Study 2: Using a novel approach, the second study compared the effects of NO_3^- supplementation over a 3 and 6 d duration on economy and 4 km time-trial performance, and 4 and 7 d duration on 1 km time-trial performance in 9 highly-trained (VO_2peak = 68 ml.kg^{-1}.min^{-1}) male endurance cyclists. Relative to placebo, our findings indicated impairment to 1 km time-trial performance following 4 d of NO_3^- supplementation, but no effect, either positive or negative, after 7 d for 1 km, or 3 and 6 d for 4 km performance. There was no change in economy following consumption of NO_3^- over a 3 or 6 d period.

Study 3: Study 3 assessed the effect of NO_3^- supplementation on perception, thermoregulatory response and 4 km time-trial performance of 8 cyclists in hot conditions. It is possible that the whole-body vasodilation experienced as a consequence of NO_3^- consumption negatively alters the central vasoconstriction experienced in hot environments, thus impacting performance. Three d of NO_3^- supplementation resulted in *unclear* effects on rectal temperature (T_{re}) during low-intensity cycling, but did result in a small *very likely trivial* increase during moderate-intensity exercise. The ~0.10°C T_{re} increase during moderate intensity cycling did not appear to influence measures of perception or 4 km cycling time-trial performance. These outcomes suggests that in hot environments, elevated [NO_2^-] resulting from beetroot juice consumption, may elevate core-body temperature during steady state exercise, but not influence short-term, high-intensity performance of 6 min in well-trained (VO_2peak = 64 ml.kg^{-1}.min^{-1}) male endurance cyclists.

Collectively, the studies in this thesis demonstrate that the effectiveness of NO_3^- supplementation on physiological and performance measures appears to be sensitive to the physiological characteristics and training status of athletes, and the environmental conditions that the event is conducted in. The new findings resulting from this thesis include: 1) the effectiveness of NO_3^- supplementation may depend on the calibre of the cyclist, however 1 km time-trial performance may be impaired; 2) in the same

population, economy appears to be unaffected, when dosing strategies of 4 to 8 mmol NO_3^- are employed over a 3 to 7 d period; 3) similar to outcomes for economy, a range of key endurance markers appear to be uninfluenced as a result of NO_3^- supplementation; and 4) in hot environments, use of NO_3^- does not influence performance though may it lead to increased T_{re} during moderate intensity activity. As such, these findings suggest that effects of NO_3^- supplementation appear not to influence performance or a range of physiological measures to the same extent as lesser-trained populations.

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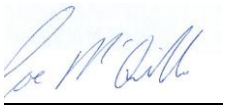
SYMBOLS AND ABBREVIATIONS

[ ]	concentration
Δ	difference
bpm	beats per minute
Ca^{2+}	calcium
CI	confidence interval
CL	confidence limits
cm	centimetres
CO_2	carbon dioxide
CV	co-efficient of variation
°C	degrees Celsius
d	day(s)
g	gravity
GET	gas exchange threshold
Ht	stature
HR	heart rate
$h.wk^{-1}$	hours per week
Incr.	incremental
kg	kilograms
kJ	kilojoules
km	kilometre
µL	microliters
µM	micromoles
m	metres
MBI	magnitude-based inference
min	minute(s)
mL	millilitres
$ml.kg^{-1}.min^{-1}$	millilitres per kilogram per minute
mm	millimetres
$mmol.L^{-1}$	millimoles per litre
MPO	mean power output
NIT	nitrate
nm	nanometres
NO	nitric oxide

NO_2^-	nitrite
NO_3^-	nitrate
PLA	placebo
PO_2mv	microvascular pressure of oxygen
PPO	peak power output
RH	relative humidity
RPE	rate of perceived exertion
RPM	revolutions per minute
s	second(s)
SD	standard deviation
SEM	standard error of measurement
SRM	Schoberer Rad Meßtechnik
TCA	trichloroacetic acid
TEM	typical error of measurement
T_{re}	rectal temperature
TT	time-trial
VO_2	pulmonary oxygen uptake
VO_2peak	peak oxygen uptake
VT_1	first ventilatory threshold
VT_2	second ventilatory threshold
W	watt
$W \cdot min^{-1}$	watts per minute

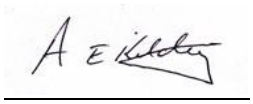
ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined). Similarly, it does not contain material, which to a substantial extent, has been submitted for the award of any other degree or diploma of university or institution of higher learning.



Joseph A. McQuillan
April 2017

Supervisor Signatures



Andrew Kilding
April 2017

Paul Laursen
April 2017

DEDICATION

I wish to dedicate this thesis to my Mum (Margaret) and Dad (Pat). Thank you for your love, support and encouragement in my formative years.

ACKNOWLEDGEMENTS

The fulfilment of this thesis is only possible through the contribution of many people. I consider myself to have been very fortunate to have the pleasure of working, and researching alongside these people.

Firstly, I would like to express my sincere thanks to my primary supervisor, Dr Andy Kilding, who has shown great patience, ongoing support, steadiness, and more often than once a sympathetic ear for me over the years. I have also been fortunate to have Dr Paul Laursen's support as my secondary supervisor. Your support with sourcing research funding made my life a lot easier, and super-responsive feedback on documents were much appreciated. Dr Deb Dulson (nee Fletcher) was instrumental and very patient in helping me get to grips with nitrite analytical procedures. As always Prof. Will Hopkins was ready with to advise on statistical issues as only Will can. Additionally, Drs Kyle Barnes, Jess Dent, Lisa McDonnell, Travis McMaster and Julia Casadio were great post-grad colleagues to spend time with.

While not part of my research team, Prof. John Cronin has been a teacher, advisor, mentor and friend going back 20 years, always with an open door, a positive outlook, and a bit of tough love when it was required.

I would like to thank High Performance Sport Performance New Zealand for provision of research funding. The funding would not have been possible with the support of Christel Dunshea-Mooij (Rowing NZ), Ien Hellemans (Triathon NZ), Craig Palmer, and Andy Reid (Cycling NZ) for their support in a successful funding application.

To find, let alone attract, well-trained athletes into a series of studies is difficult; therefore none of this research would have been possible without my extraordinary, selfless and talented cohort of participants, willing to give 100%, shed sweat and provide blood each and every time they were asked to.

Outside of the tight AUT post-grad niche, I have had fantastic on-going support from Dr Darrell Bonetti and Cliff Harvey. As colleagues and friends their advice and feedback was always gratefully received. To my other friends and my wider family, many thanks for your support and understanding during my PhD journey. I look forward to returning

to semi-normal hours of operation and catching up with you all. A thanks must go to Drs Sally Peters and Kirsten Petrie who have kindly allowed my time away from work to complete work and travel out of district as and when needed.

My partner Marika Avison, who is looking forward to the completion of the PhD possibly more than anyone else, has provided unrelenting support in the last 18 months. Looking forward to many (non-PhD) years together.

ETHICAL APPROVAL

Ethical approval for studies 1, 2 and 3 was obtained from Auckland University of Technology's Ethic Committee (AUTEC); Ethics Application Number 10/309.

SUBMISSIONS AND PUBLICATIONS

Chapters 3, 4 and 5 of this thesis represent 3 separate papers that have been accepted by peer-review journals for publication. These papers were prepared in collaboration with my supervisors Associate Professor Andrew Kilding, Professor Paul Laursen and Dr Deborah Dulson, while Chapter 5 was also assisted with collaboration with Julia Casadio. Additionally, Chapter 2 is currently in preparation for submission and has been prepared with assistance from Associate Professor Andrew Kilding. The percentage of work from each author is noted in brackets.

Co-authored works

Published peer-reviewed articles (Chapters 3 to 5)

McQuillan, J. A., Dulson, D. K., Laursen, P. B., and Kilding, A. E. (2016). The effect of dietary nitrate supplementation on physiology and performance in trained cyclists. *International Journal of Sports Physiology and Performance.* (Published Ahead of Print).

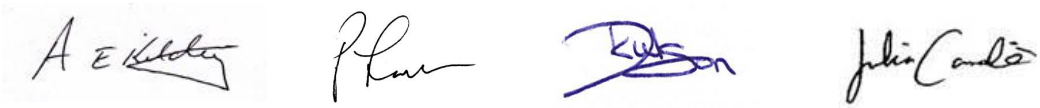
(Joe McQuillan 80%, Andrew Kilding 15%, Paul Laursen 2.5%, Deborah Dulson 2.5%)

McQuillan, J. A., Dulson, D. K., Laursen, P. B., and Kilding, A. E. (2016). Dietary nitrate fails to improve 1 and 4 km cycling performance in highly-trained cyclists. *International Journal of Sport Nutrition and Exercise Metabolism.* (Published Ahead of Print).

(Joe McQuillan 80%, Andrew Kilding 15%, Paul Laursen 2.5%, Deborah Dulson 2.5%)

McQuillan, J. A., Casadio, J. R., Dulson, D. K., Laursen, P. B., and Kilding, A. E. The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists. *International Journal of Sports Physiology and Performance.* (Accepted for Publication).

(Joe McQuillan 80%, Andrew Kilding 12.5%, Paul Laursen 2.5%, Deborah Dulson 2.5%, Julia Casadio 2.5%)



In preparation for submission

McQuillan, J. A., and Kilding, A. E. The effectiveness of nitrate supplementation as an ergogenic aid in competitive athletes.
(Joe McQuillan 85%, Andrew Kilding 15%)

Conference presentations

McQuillan, J. A., Casadio, J. R., Dulson, D. K., Laursen, P. B., and Kilding, A. E. (2016). Dietary nitrate fails to improve 1 and 4 km cycling performance in highly-trained cyclists. *Sport and Exercise Science New Zealand 2016, 29 to 30 October.*

CHAPTER ONE: INTRODUCTION

Background

The recent discovery that bolus doses of exogenous nitrate (NO_3^-), and more particularly nitrite (NO_2^-) and nitric oxide (NO), influence a range of therapeutic and performance outcomes has led to a plethora of research concerning the effectiveness of NO_3^- supplementation on health (Berry et al., 2015; Bode-Bodger et al., 1995; Jungersten, Ambring, Wall, & Wennmalm, 1997), physiology (Brown, 1995; Castello, David, McClure, Crook, & Poyton, 2006; Larsen, Weitzberg, Lundberg, & Ekblom, 2007), and exercise performance (Sandbakk et al., 2015) across a variety of populations (Bailey, Fulford, et al., 2010; Bryan et al., 2007; Kenjale et al., 2011; Peacock et al., 2012). NO_3^- is commonly found in high concentrations in beets and green leafy vegetables (Alexander et al., 2008; Hord, Tang, & Bryan, 1999) with diets high in these food types considered to be pivotal in the reduction of disease and longevity of life (Lundberg, Feelisch, Bjorne, Jansson, & Weitzberg, 2006; Lundberg, Weitzberg, Cole, & Benjamin, 2004). Primarily, endogenously derived NO is produced via oxygen dependent mechanisms via the L-arginine pathway and appears to facilitate a range of cellular level enhancements in neuronal, vascular and muscular tissue (Stamler & Meissner, 2001). More recently, research into the effects of exogenous NO_3^- on health and exercise related modalities suggest that in at-risk populations, consumption of NO_3^- elevates NO_2^- and NO bioavailability thereby reducing the risk of coronary heart disease (Lundberg, Weitzberg, & Gladwin, 2008), increases arterial compliance (Tordi et al., 2006), and improves indices of renal function and blood pressure (Kemmer et al., 2017). Furthermore, under exercising conditions, the same NO_2^--rich environment has been shown to improve the blood flow and exercise capacity in sufferers of peripheral arterial disease (Kenjale et al., 2011). Additionally, the conversion of NO_2^- to cell signalling NO is enhanced under lowered-oxygen and ischemic environments in which muscle pH is reduced (Cosby et al., 2003; Modin et al., 2001). Authors have therefore suggested that this secondary pathway compliments the L-arginine pathway for development of NO (Lundberg et al., 2008). The NO_2^- - NO mediated enhancements appear to be specific to type II muscle fibre (and in particular IIb and IId/x) (Ferguson et al., 2013; Ferguson, Hirai, et al., 2014; Ferguson, Holdsworth, et al., 2014), suggesting that for sporting applications, event intensity may play a part in the efficacy of NO_3^- on performance outcomes.

Dietary supplementation is employed by athletes with the primary aim of improving athletic performance (Stellingwerff, Maughan, & Burke, 2011). Supplementation of NO_3^- in athletic populations was inspired by a number of early studies demonstrating enhancements in economy, mitochondrial efficiency and exercise capacity via time to exhaustion (TTE) assessments in healthy, but untrained participants (Bailey, Winyard, et al., 2009; Bailey, Winyard, et al., 2010; Larsen et al., 2011). With a view to establishing the effects of NO_3^- on performance measures in trained athletes, several researchers have reported substantial improvements (1.2 to 2.8%) on all-out cycling performances of ~6 to 30 min duration (Cermak, Gibala, & van Loon, 2012; Lansley, Winyard, Bailey, et al., 2011). At the elite athletic level, the difference between success and failure in short- to moderate-term, endurance events completed within a 2 to 30 min timeframe can be as little as 0.3% (Bonetti & Hopkins, 2010; Paton & Hopkins, 2006; Smith & Hopkins, 2011); therefore improvements of such magnitudes are of significant interest to athletes, coaches and sport science practitioners.

Subsequent research into endurance-trained populations has examined the effects of NO_3^- within time-frames of 2 min to 2.15 hr (Peeling, Cox, Bullock, & Burke, 2015; Wilkerson et al., 2012), with the majority of research assessing the effects of NO_3^- in events of >8 min duration (Tables 3 and 4). Under hypoperfused conditions, increased [NO_2^-] appears to augment blood flow (Ferguson et al., 2013), elevate microvascular pressure (PO_2mv), and increase calcium turnover and contractile force (Hernandez et al., 2012). While the majority of these reactions are considered a standard physiological response under exercising conditions (Casey & Joyner, 2011), it appears that type II muscle is the primary benefactor of these mechanistic enhancements (Jones, Ferguson, Bailey, Vanhatalo, & Poole, 2016) under NO_2^--rich environments. These NO_3^- derived improvements would suggest that exercise of greater intensity would be most likely to benefit NO_3^- supplementation. Indeed, current applied research suggests that NO_3^- supplementation is unlikely to enhance performance events of >30 min (Cermak, Res, et al., 2012; Lane et al., 2014; Wilkerson et al., 2012). Despite some reliance on type II muscle in events >30 min, (Gollnick, Piehl, & Saltin, 1974), the mechanistic enhancements observed in type II muscle suggests that events shorter in duration, and higher in intensity, with greater reliance on type II muscle (Gollnick et al., 1974; Vøllestad & Blom, 1985) are likely to benefit from NO_3^- supplementation.

However, despite the substantial ground-breaking findings of Cermak, Gibala, et al. (2012) and Lansley, Winyard, Bailey, et al. (2011), the majority of subsequent investigations into the effects of NO_3^- in well-trained populations in events <30 min have largely failed to replicate the positive effects reported in these early studies (Boorsma, Whitfield, & Spriet, 2014; MacLeod et al., 2015; Nyakayiru et al., 2016; Peacock et al., 2012; Porcelli et al., 2015). As defined by Table 2, those that have shown performance improvements as a result of NO_3^- (Bond, Morton, & Braakhuis, 2012; Hoon, Jones, et al., 2014; Peeling et al., 2015) are typified by exercise of ~2 to 8 min duration (Bonetti & Hopkins, 2010; Smith & Hopkins, 2011). Coincidentally, this time frame is near identical to the 3 to 8 min duration that VO_2max is able to be held for (Billat, Dalmay, Antonini, & Chassain, 1994), thereby suggesting that sports in which attainment of VO_2max is associated would stand to benefit from exogenous NO_3^- supplementation. Interestingly, these studies have employed exercise activity that is considered either partially, or wholly dependent on upper body musculature (Bond et al., 2012; Hoon, Jones, et al., 2014; Peeling et al., 2015). To date, very few studies have investigated the effect of NO_3^- in short duration activity in lower body (cycling or running) activity. Those who have, demonstrate that, for events of 4 min, NO_3^- has at best, a neutral impact on running performance (Boorsma et al., 2014), and may have a negative effect in maximal-intensity cycling (Hoon, Hopkins, et al., 2014). Despite the mean group findings of Boorsma et al. (2014) reporting no influence on performance, several participants responded to NO_3^- supplementation, suggesting that as yet unidentified individual characteristics may play a part in the efficacy of NO_3^- on performance. Furthermore, the *possibly harmful* findings of Hoon, Hopkins, et al. (2014) were reported in the second of two 4 min cycling trials, with the first 4 min trial under NO_3^- conditions resulting in an *unclear* 1.3% improvement in power output (Hoon, Hopkins, et al., 2014). Therefore, with respect to the findings of Peeling et al. (2015), despite the limited effects of NO_3^- in shorter duration activity for lower body activity in endurance trained populations, further work is required to elucidate whether NO_3^- can enhance short-term, high-intensity activity in trained athlete populations.

NO_3^- Dosing Strategies

With regards to the length of dosing, both Bailey, Winyard, et al. (2009) and Larsen et al. (2011) reported that 3 to 6 d NO_3^- consumption by way of either $NaNO_3^-$ or NO_3^- improved mitochondrial efficiency, and led to improvements in economy, and exercise capacity (Bailey, Winyard, et al., 2009; Larsen et al., 2011). Although a number of studies have witnessed improvements in economy, exercise capacity, and endurance performance employing ≥3 d NO_3^- supplementation, previous findings suggest that, in untrained populations, a 1-off acute dose of NO_3^- is as effective as 5 or 15 d in improving exercise capacity and exercise physiology (Vanhatalo et al., 2010). Similarly, the same acute dose has demonstrated performance improvements in moderate- (Lansley, Winyard, Bailey, et al., 2011) and elite-level (Peeling et al., 2015) populations. Conversely, longer-term (>3 d) strategies have failed to improve a range of physiological and performance measures despite elevations in nitrite (NO_2^-) bioavailability (MacLeod et al., 2015; Porcelli et al., 2015). These findings suggest that while physiological and exercise related improvements resulting from NO_3^- might partially rely on longer term dosing strategies (Bailey, Winyard, et al., 2009; Larsen et al., 2011), shorter term dosing may positively enhance other mechanistic aspects. Therefore, in both trained and untrained populations, it would appear that the mechanism(s) by which NO_3^- exerts enhancements with regards to the duration of dosing period are yet to be elucidated.

Responders/Non-responders

The response of individuals to a treatment is considered to be one of the most important outcomes in intervention research (Hopkins, 2015). Indeed, a number of investigations have highlighted positive individual effects following NO_3^- supplementation, particularly when changes in basal [NO_2^-] and performance are taken into consideration (Hoon, Jones, et al., 2014; Wilkerson et al., 2012). For instance, under NO_3^- supplemented conditions, Wilkerson et al. (2012) reported a correlation between mean group reduction (-0.8%) in time taken to complete a 50 mile time-trial and increase in basal [NO_2^-] for 11 well-trained cyclists of r=0.83. Following NO_3^- supplementation the mean increase in plasma [NO_2^-] equated to 30%, however the authors subsequently removed 3 participants who had <30% increase in basal [NO_2^-] and demonstrated the remaining 'responder' mean group performance increased to 2.0%. A subsequent study by Hoon, Jones, et al. (2014) suggested that a 83% increase in basal [NO_2^-] following 8 mmol NO_3^- had a near

significant (P=0.055) relationship to improvements in 2000 m rowing performance in 11 trained rowers. Both of these examples highlight the positive effects of NO_3^- relative to athletic populations. However, when untrained and trained populations are compared, it would appear that in fact a negative correlation exists between increases in [NO_2^-] and changes in both 3 km running performance and VO_2peak (Porcelli et al., 2015), which highlights that athletic calibre, rather than changes in basal [NO_2^-], may well contribute to the effectiveness of NO_3^-. To date, it is not understood why the response within a homogenous cohort such as those in the Hoon, Jones, et al. (2014) or Wilkerson et al. (2012) studies are so variable, however, the change in [NO_2^-] as a result of NO_3^- may indicate the likelihood of positive changes (Wilkerson et al., 2012). As such, further research is warranted into the assessment of NO_3^- as an ergogenic aid for well-trained endurance athletes competing in short-duration, high-intensity endurance events.

Environmental Considerations

Olympic and World Championship events are often held in hot environments thus making it likely that warm temperatures may influence endurance performances (Peiser & Reilly, 2004). Interestingly, the whole-body vasodilating effects of NO_3^- (Bloomer, 2010) somewhat oppose those of the sympathetic mediated responses to exercise in the heat, i.e. central vasoconstriction in order to distribute blood to the periphery via vasodilation (Cheung & McLellan, 1998). To date, just two studies have reported on the effects of NO_3^- on physiology and performance in hot climates (Kuennen et al., 2015; Tyler, Coffey, & Hodges, 2016). Given the opposing mechanistic effects (whole body vasodilation via NO_3^- vs. central vasoconstriction via heat exposure), the likelihood that athletes are more likely to compete in a variety of environmental conditions (Peiser & Reilly, 2004), and the harmful effects reported for NO_3^- supplementation in recreationally trained participants during exercise in hyperthermic (41ºC; 20% relative humidity (RH)) conditions (Kuennen et al., 2015) it seems pertinent to assess the impact of NO_3^- on performance in thermally challenging environments.

Rationale, Thesis Aim and Research Questions

In addition to the aforementioned theoretical background, a strong applied need for the thesis existed and was a strong driver in the development of the thesis aim and research questions. Specifically, in the lead up to the 2012 Olympics, numerous New Zealand

high-performance sporting organisations were weighing up the pros and cons of using dietary NO_3^-, which at that stage was a relatively new, but very promising dietary supplement. Cycling, and in particular the 4 km time-trial event, was chosen due to my personal interest in cycling, the high reliability of cycling time-trials, and at that point in time the majority of NO_3^- related research had been carried out in cycling related measures, albeit adopting longer duration trials. Additionally, at the Olympic level, the time duration required to complete a 4 km time-trial was similar to that of 2000 m rowing, 1000 m flat-water sprint kayaking, 1500 m running, and 400 m swimming events, with reliance on both aerobic and anaerobic energy metabolism (Duffield, Dawson, & Goodman, 2005a, 2005b; Gastin, 2001). Notably, New Zealand had strong, medal capable athletes across all five of these sporting events, and as such, they were expected to contribute substantially to New Zealand's overall medal tally. Out of the five events, two (4 km track cycling team-pursuit and rowing) contributed a total of 6 of the countries 13 medals. It was considered a priority research area to assess the efficacy of NO_3^- for New Zealand athletes, and if ergogenic, a refinement of dosing approaches prior to the Olympic Games was needed.

Given its potential to impact performance and practice, the national sporting body responsible for funding for elite sport (High Performance Sport New Zealand) invested in specific research that assessed the effects of NO_3^- on events in which New Zealand was at least capable of achieving a final placing. Accordingly, the overarching aim of the thesis was to establish the effects of nitrate supplementation on short-distance, high-intensity cycling performance, in both temperate and hot climates, in competitive endurance cyclists. The thesis comprises a literature review and 3 experimental studies (Table 1) underpinned by the following specific research questions:

1. Does 6 to 8 days of NO_3^- supplementation influence VO_2peak, ventilatory thresholds, and economy in well-trained male endurance cyclists?
2. Does 8 days of NO_3^- supplementation influence 4 km cycling TT performance in well-trained male endurance cyclists?
3. Does the volume and/or duration of NO_3^- supplementation have any influence on a range of economy measures in well-trained male endurance cyclists?
4. Does the volume and/or duration of NO_3^- supplementation have any influence on 1 and 4 km time-trial performance in well-trained male endurance cyclists?

5. What is the impact of 3 days of NO_3^- supplementation on heart rate and rectal temperature in hot environments in well-trained male endurance cyclists?
6. What is the impact of 3 days of NO_3^- supplementation on short-term, high-intensity performance in hot environments in well-trained male endurance cyclists?

Table 1: Overview of the chapters as part of the thesis

Chapter	Title	Aim	Study Design	Rationale and Significance
Chapter 2	The effectiveness of nitrate supplementation as an ergogenic aid in competitive athletes	To establish a clear overview of the mechanistic basis for dietary supplements in which there is evidence of enhanced physiological measurement and/or performance outcomes during short-term high-intensity sprint endurance events.	Literature Review	Following NO_3^- supplementation, a range of positive physiological alterations in cardiovascular and muscular systems would appear to support its use to mediate performance enhancement (Jones et al., 2016). However, factors such as athletic calibre (Porcelli et al., 2015), duration and intensity of exercise, dose duration and environmental factors may have an influence on the effectiveness of NO_3^- as a performance enhancer. As such, it would be pertinent to be cognisant of these factors when administering NO_3^- in an attempt to aid performance in athletic populations. This review will analyse the effects of NO_3^- supplementation on physiology and performance of competitive athletic populations.
Chapter 3	Effect of nitrate supplementation on physiology and performance in well-trained cyclists	To evaluate the effectiveness of NO_3^- consumption on a range of physiology measures alongside 4 km cycling performance	Randomised, placebo-controlled, cross-over design	Several research studies have shown improvements in both physiological and short-term, high-intensity performance measures following NO_3^- supplementation. Outcomes such as these appear to be particularly evident in lesser-trained populations, whereby effects of NO_3^- has reduced economy (Cermak, Gibala, et al., 2012) and performance (Cermak, Res, et al., 2012; Lansley, Winyard, Bailey, et al., 2011) in competitive cyclists of 56 to 58 $ml.kg^{-1}.min^{-1}$. Despite such improvements, populations of greater aerobic calibre have predominantly demonstrated little to no alteration in shorter duration (4 to 16 min) performance following NO_3^- supplementation (Hoon, Hopkins, et al., 2014; MacLeod et al., 2015) potentially due to the training mediated physiological adaptations as a result of substantial training loads (Coyle, 1999; Jungersten et al., 1997). As such, it was of interest to assessing the influence of NO_3^- supplementation on a range of physiological measures and 4 km time-trial performance in well-trained cyclists. The information gained from this research would help to inform whether well-trained cyclists could improve performance, and if so, which physiological related measure(s) contribute(s) to these improvements.

Chapter 4	Dietary nitrate fails to improve 1 and 4 km cycling performance in highly-trained cyclists	To assess the effectiveness of short and longer duration NO_3^- supplementation on performance over two commonly used track cycling distances	Randomised, placebo-controlled, cross-over design	The mechanisms purported as being responsible for enhancements as a result of NO_3^- supplementation include upregulation of the hemodynamic, microvascular and protein handling properties of glycolytic, type II muscle (Jones et al., 2016). These physiological responses allow greater blood flow and increased vascularisation of type II muscle, which are several of the characteristics expressed by type I muscle. Potential benefits of these positive physiological enhancements would be a reduction in metabolite build up and increased force-generating capacity in exercising type II muscle during maximal intensity performance. To date, it is unknown whether such effects are dependent on event intensity, and if so, how the intensity of effort influences the effectiveness of NO_3^- in endurance-trained populations. The resulting knowledge would be of potential benefit to supra-maximal oxygen dependent performance.
Chapter 5	The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists	To investigate the impact of nitrate supplementation on cycling performance in hot conditions.	Randomised, placebo-controlled, cross-over design	It is well established that exercise of moderate to severe intensity induces intra-muscular ischemic and hypoxic conditions which, following NO_3^- consumption, catalyse the reduction of nitrite (NO_2^-) to nitric oxide (NO) (Cosby et al., 2003) leading to whole-body vasodilation (Bloomer, 2010). The reduction of NO_2^- to NO has resulted in positive physiological outcomes including reduced arterial blood pressure (Lee et al., 2015), and improved exercise capacity in populations with compromised peripheral blood flow (Kenjale et al., 2011). Whilst beneficial in some situations, the disregulation of the vagal response as a result of reduction of NO_2^- to NO, may, in some cases, counteract the sympathetic mediated responses to heat. For instance, during exercise in hot environments, NO_3^- has been associated with increased rectal temperature and an estimated decrease in exercise capacity, relative to placebo treatments (Kuennen et al., 2015). Given endurance athletes are likely to compete in hot environments (Garrett, Creasy, Rehrer, Patterson, & Cotter, 2012) it is pertinent to assess whether NO_3^- supplementation influences body temperature, and if so, what affect this has on performance.

Structure

The thesis consists of 6 Chapters including an overall introduction and rationalisation (preface) (Chapter 1) section, literature review (Chapter 2), 3 experimental studies (Chapters 3 to 5) and an overall discussion and conclusion (Chapter 6) to the thesis. A thesis structure is presented in **Figure 1**. Chapters 3, 4 and 5 are presented in their respective journal formats. The references for each chapter are collated as a whole at the end of the thesis in APA format. The appendices (Appendices 1 to 4) contain ethical approval, participant related information and instructions for the three experimental studies contained in the thesis.

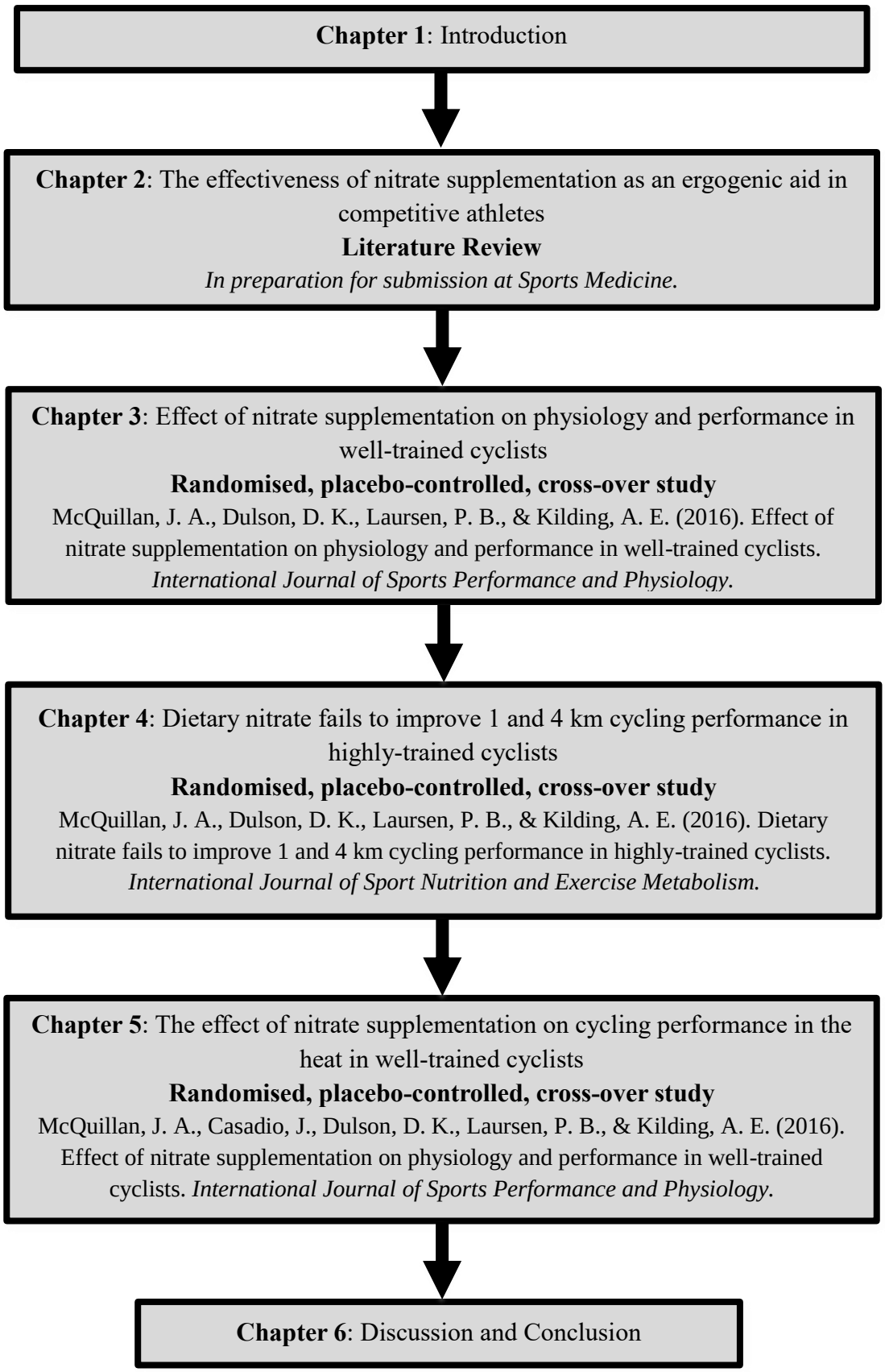


Figure 1: Overview of doctoral thesis structure and flow

Significance of the Thesis:

My personal interest in the effects of NO_3^- supplementation research into short-duration, high-intensity endurance performance was based on advising highly-trained athletes on the efficacy of supplementation of dietary NO_3^-. The outcomes that were of interest at the start of the series of investigations were largely focused on identifying:

- The efficacy of NO_3^- supplementation for well-trained cyclists in events of 1 and 4 km time-trial performance.
- The influence of NO_3^- supplementation on economy during moderate intensity cycling.
- The ability of NO_3^- supplementation to influence a range of key performance indicators in endurance trained athletes.
- The impact of NO_3^- supplementation in environments likely to be experienced in hot and humid conditions.
- The correlation between performance changes and basal [NO_2^-] as a result of NO_3^- supplementation.

Achieving these outcomes would help to inform coaches of endurance athletes of the potential for NO_3^- supplementation to influence a range of physiological outcomes. Additionally, establishing the efficacy of NO_3^- to augment high-intensity cycling performance in both ambient and thermally challenging environments would help inform athlete's choice on when, or even if, to use NO_3^- as an ergogenic aid.

CHAPTER 2: THE EFFECTIVENESS OF NITRATE SUPPLEMENTATION AS AN ERGOGENIC AID IN COMPETITIVE ATHLETES

Abstract

Dietary nitrate (NO_3^-) supplementation via either beetroot juice or sodium nitrate has been shown to improve a range of physiological and performance measures across a range of populations, exercise intensities and environmental conditions. Initially, this review presents an overview of proposed mechanisms for such improvements in addition to resulting effects of dietary NO_3^- supplementation on key endurance physiology markers. While improved mechanistic and/or physiological markers as a result of NO_3^- supplementation may infer an increase in performance ability, this is not always the case. Therefore, the second part of the review investigates the effectiveness of measures of endurance performance in athletic populations across a range of task durations and in various environments. From studies to date, it would appear that the physiological enhancements and performance improvements witnessed in moderately-trained populations (~57 $ml.kg^{-1}.min^{-1}$) as a result of NO_3^- supplementation are not as apparent in populations of greater aerobic ability. For instance, in these populations improvements in economy of 3.5 to 5.1% at low to moderate-intensity and 2 to 3% increase in time-trial performances of ~6.30 to 30 min duration have been previously reported following both acute and chronic supplementation strategies. However, in athletes of greater aerobic calibre (≥60 $ml.kg^{-1}.min^{-1}$), NO_3^- consumption appears less likely to improve either economy or time-trial performances of 4, 16, 60 or ~140 min. The reasons for the limited effects in athletes of ≥60 $ml.kg^{-1}.min^{-1}$, across a range of exercise intensities, may result from regular exposure to training stress, increased dietary intake (and therefore large NO_3^- intake) in an effort to match energy output, and a range of mechanistic alterations such as greater volume of type I muscle. However, while mean group outcomes may not always show clear benefit, there appears to be support for individual responders to NO_3^-, even within relatively homogenous populations.

Introduction

In athletic populations, the practice of nutritional supplementation is commonplace, with supplementation strategies recommended for athletes prior to (Jordan, Lukaszuk, Misic, & Umoren, 2010; Vandenbogaerde & Hopkins, 2010), during (Garth & Burke, 2013), and following (Saunders, Kane, & Todd, 2004) exercise. Indeed, numerous studies have

cited physiological improvements as a result of dietary supplementation (Bruce et al., 2000; Hill et al., 2007; van Loon et al., 2003). Supplementation derived performance benefits are reliant on underlying mechanistic alterations enhancing physiological response(s). For instance, consumption of either sodium bicarbonate or beta-alanine appears to benefit short-term, high intensity sport performances of 30 s to 8 min duration (Stellingwerff et al., 2011) in which elevations in muscular acidosis and hypoxia contribute to exercise fatigue typically represented as a loss in peak power output (Cairns, 2013). In contrast to shorter duration events, supplementation prior to, and during performance events in longer term exercise (≥30 min) tends to focus on mitigation of fatigue related factors such as glycogen depletion and dehydration and performance decrement via a number of strategies including use of multi-carbohydrate transporters, caffeine supplementation and regular hydration (Jeukendrup, 2011).

Recently, several studies have investigated the effects of NO_3^- on physiology (Larsen et al., 2014; Lee et al., 2015) and performance (Cermak, Gibala, et al., 2012). NO_3^- is commonly found in beets and green leafy vegetables with a total of 80% of NO_3^- coming from dietary sources (Hord et al., 1999). Endogenous NO_3^- production accounts for the remaining 20% with the human saliva a major source of NO_3^- (Lundberg & Govoni, 2004). Not only is NO_3^- antioxidant-rich (Wootton-Beard & Ryan, 2011), it is also, by way of nitric oxide synthase (NOS), thought to play a role in the protection of the gut from intestinal disorders (Benjamin et al., 1994) and is considered important for maintaining cardiovascular health (Hord et al., 1999). Although a number of therapeutic benefits have been found following NO_3^- consumption in clinical populations (Kapil et al., 2010; Kenjale et al., 2011) only more recently has its effect on exercise physiology and performance in athletic populations been considered more closely.

Research to date has predominantly focussed on nitrate ingestion via beetroot juice (McMahon, Leveritt, & Pavey, 2016). Following consumption of NO_3^- [~5 to 8 mmol], in this form, competitive endurance athletes have demonstrated enhanced time-trial performances in the order of 1.2 to 2.8% (Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011) via a range of potential mechanisms including increased mitochondrial efficiency (Larsen et al., 2011), and improved intra-muscular blood flow (Ferguson et al., 2013; Ferguson, Holdsworth, et al., 2014). However, the limited ecological validity of early studies in which foods high in NO_3^- were avoided and/or

assessments with high CVs were employed (Bailey, Winyard, et al., 2010; Larsen et al., 2011) have been superseded by investigations which ask participants to maintain dietary practices and assess interventions replicating realistic competition situations (Hoon, Hopkins, et al., 2014).

Therefore the primary purpose of this review is to examine the mechanistic basis of, physiological responses to, and impact of acute and chronic NO_3^- supplementation on endurance performance in endurance-trained populations. For purposes of structure, interpretation and meaningful comparison of effects, studies have been categorised and compared based on their performance trial duration: 1 to 8 min, 8 to 30 min and >30 min. When data relating to mechanistic or measurement outcomes is limited or not available in the specified population, references were made to untrained, recreationally trained populations or murine species in an attempt to better explain findings.

Mechanisms of Action of the NOS-independent (NO_3^--NO_2^--NO) Pathway

Until recently, the oxidisation of the amino acid L-arginine, via the oxygen dependent nitric oxide synthase (NOS) L-arginine - NOS - NO pathway was thought to be the only means by which the body was able to produce NO. As a signalling molecule, NO is heavily influential in the regulation of endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) responses essential for homeostasis, for instance in neuro-muscular function, and translational protein signalling (Stamler & Meissner, 2001). While NO is essential in the maintenance of homeostasis, ischemic conditions, such as those found during times of physiological stress, impairs the ability of NO as a mediator of homeostasis through the irreversible step-wise reduction of endogenously derived NO to NO_2^- and subsequently forms the relatively inert NO_3^-. Conversely, in the hours following the ingestion of ~4 mmol NO_3^-, the same low oxygen conditions enhance the reduction of bioactive NO^-_2 to NO (Webb et al., 2008). Therefore, this secondary pathway acts in a complementary fashion to the primary oxygen-dependent L-arginine - NOS pathway **(Figure 2).** The discovery has led to a multitude of studies highlighting the positive influence of exogenous NO_3^- in therapeutic (Berry et al., 2015; Carlstom et al., 2011; Kelly et al., 2012; Kenjale et al., 2011) and physiological (Kelly et al., 2012; Vanhatalo et al., 2010; Wylie et al., 2013) investigations. Particularly exciting for sporting populations, it would appear that, during times of high exogenous NO_3^- consumption,

ischemic (Bryan et al., 2007; Webb et al., 2008) and hypoxic (Masschelein et al., 2012) environments elevate the conversion of NO_2^- to NO (Cosby et al., 2003), which in turn appears to improve exercise tolerance. In a relatively short time period, the interest in the effects of NO_3^- on exercise physiology and measures of sport performance has generated several meta-analysis (Hoon, Hopkins, et al., 2014; McMahon et al., 2016) and literature reviews (Jones, 2014; Jones et al., 2016; Zafeirdis, 2014).

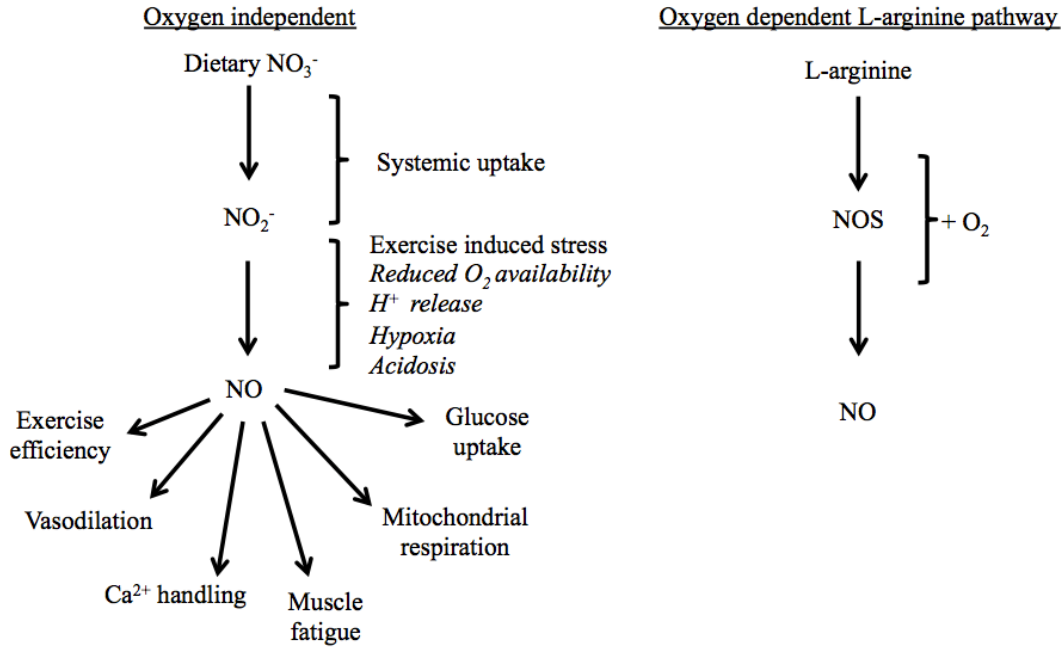


Figure 2: Pathways of nitric oxide (NO) generation in humans (modified from Bailey et al., 2012). The oxygen dependent pathway (right side) represents the primary pathway, while the oxygen independent pathway (left side) describes the more recently established method of NO production following oxidative processes under hypoxic conditions. Physiological drivers in italics lead to the oxidation of NO_2^-, with subsequent physiological enhancements occurring as a result of production of NO.

There have been various mechanisms explored and subsequently proposed as being responsible for facilitation of physiological and performance improvements following NO_3^- supplementation and consideration of these could help elucidate the reasons for the inconsistent findings with respect to sport performance enhancement. Intra-mitochondria mechanisms include a lowered cost of ATP turnover, reduced cost of PCr force production during maximal effort exercise (Bailey, Winyard, et al., 2010), and increased mitochondrial efficiency via a reduction in mitochondria H^+ leakage (Larsen et al., 2011). Additionally, improved extra-mitochondria effects resulting from NO_3^- supplementation have been reported in murine populations. For example, enhancements in microvascular pressure (PO_2mv) (Ferguson, Holdsworth, et al., 2014), rate of blood flow (Ferguson et al., 2013), calcium (Ca^{2+}) signalling and concentration [Ca^{2+}] (Hernandez et al., 2012).

Hernandez et al. (2012) also reported that as a result of elevated [Ca_2^+], force generation also increased, in agreement with previous research that reported increasing force generation proportional to increases in [Ca^{2+}] (Moss, Giulian, & Greaser, 1985). Notably, these findings appear exclusive to type IIb and d/x muscle and collectively suggest the potential for substantial mechanistic related enhancements in exercise of higher intensities, resulting from NO_3^- supplementation. As such, the benefits that appear solely representative in type IIb and d/x muscle may, in turn, explain the smaller effects witnessed in longer duration (≥60 min), lower intensity trials (Cermak, Res, et al., 2012; Wilkerson et al., 2012) relative to the larger improvements witnessed in events more reliant on energy production via type II muscle fibres.

Dietary Nitrate Supplementation Influence on Exercise Physiology

According to Joyner and Coyle (2008), the primary physiological determinants of aerobic based performance outcomes appear to be; 1) peak oxygen capacity (VO_2peak), 2) oxygen cost during sub-maximal steady-state exercise (economy), and, 3) second ventilatory (Lucia et al., 2004), anaerobic, or gas exchange threshold (GET) (Beaver, Wasserman, & Whipp, 1986). In elite endurance level athletes, a number of these characteristics are routinely reported to be some of the highest limits of human physiology (Bell, Furber, van Someren, Anton-Solanas, & Swart, 2016; Jones, 1997, 2006) and are often used as indicators of performance ability in endurance athletes (Beattie, Carson, Lyons, & Keny, 2017; Lucia et al., 2006; Lucia, Hoyos, & Chicharro, 2001). Additionally, depending on the mode of exercise, critical power (CP), or critical speed (CS) is thought to be essential to performance ability, as it defines the intensity at which energy supply is met by oxidative energy pathways without reliance on finite anaerobic energy sources (Jones, Vanhatalo, Burnley, Morton, & Poole, 2010). CP is typically an intensity that can be held for a duration of ~30 min (Poole, Burnely, Vanhatalo, Rossiter, & Jones, 2016). In healthy, but non-athletic populations, CP is ~80 to 85% VO_2peak (Poole, Ward, Gardner, & Whipp, 1998). In addition to those postulated by Joyner and Coyle (2008), in short-term high-intensity fast-start events categorised within a ‘heavy’ or ‘severe’ intensity domain, i.e. during which VO_2peak is attained, the responsiveness of oxygen uptake kinetics, and in particular the VO_2 slow component, is also considered a determinant of success (Jones & Carter, 2000).

While VO_2peak represents the ceiling of aerobic metabolism, the submaximal measures of economy, GET and oxygen uptake kinetics are heavily influenced by the severity of exercise and degree of involvement of muscle fibre type (Lucia, Hoyos, Margarita, & Chicharro, 2000; Pringle et al., 2003). Exercise that encompasses the heavy- and severe-intensity domains has negative consequences on the duration of activity due to a range of fatigue-mitigating factors (Jones & Burnley, 2009). In this section, the boundaries of aerobic exercise intensity will be defined by the demarcations of moderate (~60% VO_2peak, **Figure 3**), heavy (60 to 85% VO_2peak, **Figure 4**), and severe (85 to 100% VO_2peak) intensity exercise domains (Burnley & Jones, 2016; Jones & Burnley, 2009). For further reading, readers are directed to several focused reviews in the areas of domain intensities and the role of CP and oxygen kinetics (Burnley & Jones, 2016; Jones & Burnley, 2009).

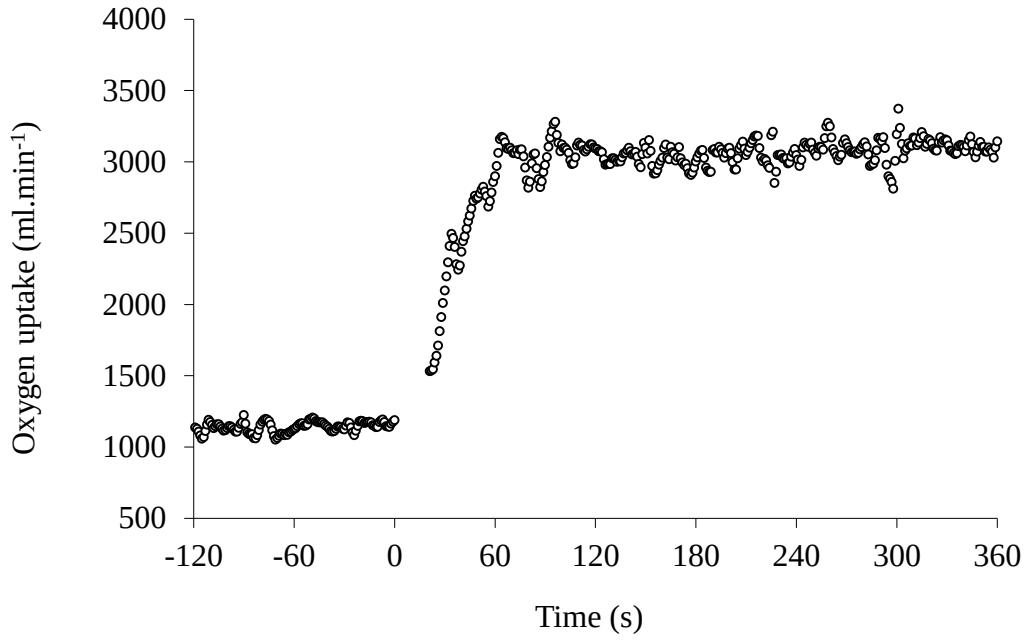


Figure 3: Example of oxygen kinetic response to moderate intensity exercise (unpublished observations).

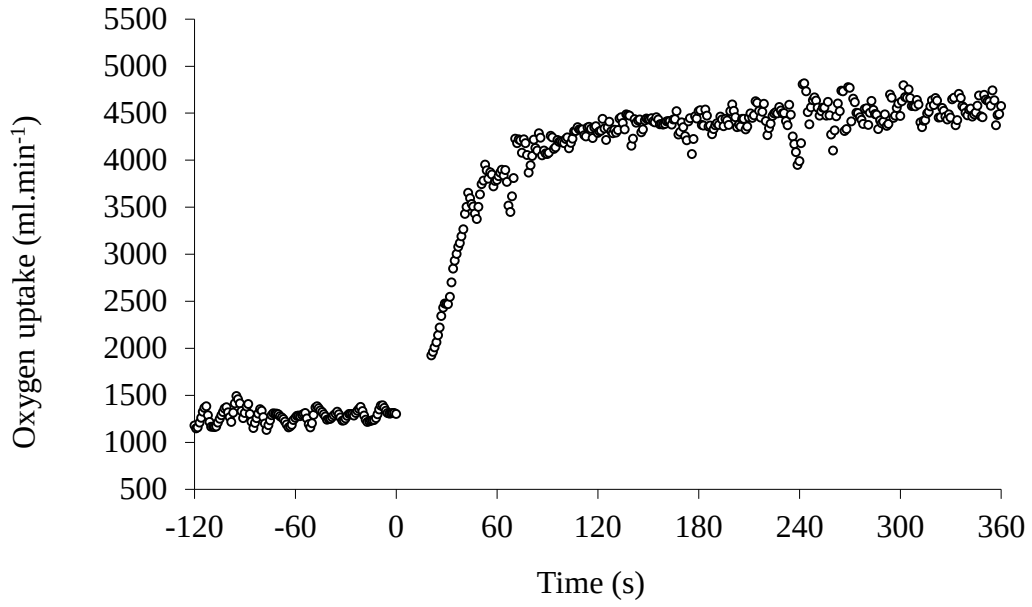


Figure 4: Example of oxygen kinetic response to heavy intensity exercise (unpublished observations).

VO_2peak and Oxygen Uptake Kinetics

In sedentary populations, VO_2peak has been shown to reduce following NO_3^- consumption alongside increased incremental peak power output (3.2%) in an incremental exercise assessment (Vanhatalo et al., 2010). Such findings are not surprising given that NO_3^-supplementation has been associated with a reduction in cost of steady-state, sub-maximal (<VO_2peak) exercise across a range of intensities in the identical populations (Bailey, Winyard, et al., 2009; Kelly, Vanhatalo, Wilkerson, Wylie, & Jones, 2013; Lansley, Winyard, Fulford, et al., 2011). These reductions are likely to be related to improved mitochondrial efficiency through increased oxidative phosphorylation ratio as a result of NO_3^- (Larsen et al., 2011). In athletic populations, VO_2peak, when measured using an incremental assessment protocol, appears to both decrease (Bescos et al., 2011), and be unaltered (Christensen, Nyberg, & Bangsbo, 2012) as a result of acute NO_3^- supplementation. Incremental assessments, specifically time to task failure, are considered a valid indicator of performance (Paton & Hopkins, 2001). Therefore, while a reduction in VO_2peak might suggest a negative result, the duration of the assessment was unchanged (Bescos et al., 2011), suggesting that NO_3^- does not influence exercise tolerance in incremental exercise. Previous findings suggest that a reduction in cost of ATP for muscle force production (Bailey, Fulford, et al., 2010), or enhanced oxidative phosphorylation (Larsen et al., 2011) may be solely, or jointly responsible for oxygen

sparing during maximal intensity aerobic exercise, without alterations to peak power output. However, Larsen et al. (2011) reported that improved mitochondrial outcomes were witnessed only after 3-days of NO_3^- supplementation, whereas Bescos et al. (2011) employed an acute (single dose) strategy. Given performance in incremental assessments can serve as a proxy for endurance performance (Hawley & Noakes, 1992), further research is warranted into the effects of NO_3^- on peak power, particularly in studies utilising more chronic (> 3 days) NO_3^- supplementation protocols.

Oxygen Uptake Kinetics

An enhanced oxygen kinetic response enables the sparing of anaerobic energy, thereby allowing for a greater contribution to an all-out effort from aerobic energy systems and is typically enhanced chronically through training (Bailey, Wilkerson, DiMenna, & Jones, 2009; Carter et al., 2000), and acutely via pre-event priming (Palmer, Jones, Kennedy, & Cotter, 2009), and initial pacing of performance (Jones, Wilkerson, Vanhatalo, & Burnley, 2008). Oxygen kinetic response is partially regulated by the speed of the VO_2 slow component, and as such, may be detrimental to exercise performance within the heavy and severe intensity domains (Burnley & Jones, 2016; Jones & Burnley, 2009), i.e. 'fast-start' endurance events (Palmer et al., 2009). Given that 85% of VO_2 slow component is derived from the initiation of type II muscle (Poole et al., 1991), muscular related benefits mediated by NO_3^- would appear to be beneficial during moderate and heavy domain intensity exercise. Despite NO_3^- consumption leading to improved oxygen kinetic response in recreational level populations (Bailey, Winyard, et al., 2009), these effects are yet to be realised in trained athletes (Christensen et al., 2012; Porcelli et al., 2015). For instance, 6-days of NO_3^- (~5 $mmol.day^{-1}$) supplementation failed to improve oxygen kinetics in highly-trained runners in bouts of moderate- (Porcelli et al., 2015), or cyclists in bouts of heavy- (Christensen et al., 2012) intensity exercise. The findings suggest that effects of NO_3^- on oxygen uptake kinetic responses in athletic populations ≥65 $ml.kg.^{-1}min^{-1}$ appears to be reduced, potentially due to chronic training adaptions (Bailey, Wilkerson, et al., 2009; Carter et al., 2000), and/or the higher basal [NO_2^-] that endurance athletes appear to possess (Jungersten et al., 1997).

Economy

In cycling, for which the majority of NO_3^- related studies have been completed, it is well-known that the oxygen 'cost' per unit of power (W) at moderate intensity, steady-state exercise is equivalent to ~10 $ml.min^{-1}$ and appears not to be influenced by variables such as age and training status (Jones & Poole, 2005). However, initial investigations into the effects of NO_3^- on economy of recreational level populations suggested that the oxygen cost of cycling is reduced (Bailey, Winyard, et al., 2009; Bailey, Winyard, et al., 2010; Larsen et al., 2011; Vanhatalo et al., 2011). In a number of these early studies (Bailey, Winyard, et al., 2010; Larsen et al., 2011) participants were required to avoid or substantially reduce dietary intake of NO_3^- rich foods during the supplementation phase. While this controlled for baseline dietary NO_3^- intake, the ecological validity of this approach could be questioned. However, in subsequent research, participants who maintained their regular dietary intake during NO_3^- supplementation experienced similar improvements in economy (Cermak, Gibala, et al., 2012) suggesting that effects of NO_3^- are not sensitive to acute reductions in dietary NO_3^- intake.

In order to assess the effects of NO_3^- on economy in trained endurance athletes, Cermak, Gibala, et al. (2012) administered NO_3^- to endurance-trained (58 $ml.kg^{-1}.min^{-1}$) cyclists, leading to a reduction in oxygen utilisation of 3.5 and 5.1% during moderate and heavy intensity cycling. Subsequently, the economy of well-trained (63 $ml.kg^{-1}.min^{-1}$) cyclists was assessed by Wilkerson et al. (2012) during a 50-mile cycling time-trial. Authors reported a 1.8% (P=0.06) improvement in economy during heavy intensity (~75% VO_2peak) cycling following an acute dose of NO_3^-. The authors noted the smaller, and non-significant improvement of oxygen cost was potentially due to the smaller elevations in basal [NO_2^-] (~33%) of participants, which, in previous research on lesser-trained populations, was elevated to a greater (138%) degree following NO_3^- consumption. Wilkerson et al. (2012) also suggested the possibility of responders and non-responders to NO_3^- supplementation, due to 3 of the 8 participants not increasing [NO_2^-] by more than 30%. However, despite the removal of the 3 participants from economy calculations, outcomes remained non-significant (Wilkerson et al., 2012). A notable contrast between the Cermak, Gibala, et al. (2012) and Wilkerson et al. (2012) studies were the alternate supplementation strategies employed. While Wilkerson et al. (2012) provided a one-off, acute 6.2 mmol NO_3^- dose, Cermak, Gibala, et al. (2012) provided participants with ~8 mmol NO_3^- daily over a 6-day period. Larsen et al. (2011) reported that mitochondrial

alterations occurred only after a 3-day loading period. Therefore, the smaller improvements reported by Wilkerson et al. (2012) may have resulted from the dosing period being too brief. Comparatively, it would appear that in endurance-trained athletes >65 $ml.kg^{-1}.min^{-1}$, 6 d of NO_3^- supplementation does not improve economy. However, the same duration of supplementation improves sedentary (~36 $ml.kg^{-1}.min^{-1}$), and lower-trained (~51 $ml.kg^{-1}.min^{-1}$) populations, by 10 and 7% in respectively (Porcelli et al., 2015). The lack of effects in economy in trained endurance athletes following NO_3^- consumption may result from increases in the size and number of mitochondria in muscle as a result of endurance training exposure (Holloszy & Coyle, 1984). As such, this would likely reduce the improved mitochondrial biogenesis witnessed in recreation level populations following NO_3^- supplementation (Larsen et al., 2011).

Blood Lactate Response

Increasing exercise intensity leads to a greater demand on type II muscle, which is high in glycogen storage, but has poor oxidative metabolism (Stallknecht, Vissing, & Galbo, 1998). These characteristics, which contrast that of type I muscle, appear at least partially responsible for the production of, and inability to clear lactate, relative to type I muscle (Aunola & Rusko, 1986). Following NO_3^- consumption in rats, type II muscle appears to reduce dependency on glycogen (Ashmore et al., 2015), increase PO_2mv (Ferguson, Holdsworth, et al., 2014), and improve vascularisation (Ferguson et al., 2013), which suggests that both the production and clearance of lactate during high-intensity exercise may be improved under NO_3^- conditions. Given that production of lactate appears to be driven predominantly by utilisation of type II muscle (Aunola & Rusko, 1986), and the numerous benefits associated with NO_3^- in type II muscle (Hernandez et al., 2012; Jones et al., 2016), the supplementation of NO_3^- in athletic populations may influence both the rate of production and rate of clearance. In humans, for the same workload, a reduced blood lactate response indicates improved exercise capacity (Lucia et al., 2000), and/or enhancements in mitochondrial enzymatic function, leading to, among other enhancements, a sparing of glycogen during exercise (Holloszy & Booth, 1976; Holloszy & Coyle, 1984), therefore mechanistic improvements to type II muscle is of interest for athletes competing in events or sports in which bouts of high-intensity exist.

Despite positive alterations in mitochondrial efficiency (Larsen, Weitzberg, Lundberg, & Ekbolm, 2010), and type II fibres (Ferguson, Hirai, et al., 2014; Ferguson, Holdsworth, et al., 2014; Hernandez et al., 2012) following NO_3^-, relative to placebo NO_3^- supplementation appears to have no effect on lactate during exercise. For example, no change in post-exercise blood lactate levels were observed at the conclusion of 6 x 500 m repeat maximal-intensity rowing ergometer intervals following 6-days of NO_3^- supplementation (Bond et al., 2012). Likewise, mean blood lactate response between the 5 and 10 km distance of a 20 km time-trial did not different between placebo and NO_3^- trials (Glaister, Pattison, Muniz-Pumares, Patterson, & Foley, 2015). Similarly, following supplementation of sodium nitrate, Bescos et al. (2012) reported no change in blood lactate response during a 40 min time-trial. In longer-duration (~135 min) cycling performance, blood lactate was unchanged over the duration of the event (Wilkerson et al., 2012). Despite recent findings for NO_3^- reducing glycogen dependency in mice (Ashmore et al., 2015), to date it appears that there is an apparent inability of NO_3^- to alter substrate utilisation in humans (Cermak, Gibala, et al., 2012). An inability to influence substrate utilisation in humans following NO_3^- supplementation is somewhat surprising given that Ca^{2+} activity within the sarcoplasmic reticulum, which is enhanced following NO_3^- supplementation (Hernandez et al., 2012), is intricately involved in glycogen release during exercising conditions (Stallknecht et al., 1998). The lack of effects witnessed in humans, relative to rats, may be as a result of differing dietary patterns, and/or muscle physiologies. While low pH values are associated with a reduced Ca^{2+} sensitivity and binding affinity (Hernandez et al., 2012), rodents appear to have lower pH threshold which may be partially related to the greater volume of type II muscle, relative to human populations (Allen, Lamb, & Westerblad, 2008). Furthermore, NOS activity in rat muscles appears to correlate with the volume of type II muscle, whereas, in humans, NOS activity is more consistent regardless of fibre type (Stamler & Meissner, 2001). Finally, baseline [NO_2^-] appears to be elevated, and is strongly correlated to lactate anaerobic threshold, in athletic populations (Totzeck et al., 2014). The relationship between [NO_2^-] and this quasi-performance related measure would suggest that as athletic ability increases, the effects of NO_3^-, and bioavailability of NO_2^- to improve moderate to severe exercise intensity exercise diminishes.

Heart Rate

During exercise, heart rate monitoring provides an easy means to detect information relating to exercise intensity (Achten & Jeukendrup, 2003), and in highly intensive aerobic training sessions at intensities ≤VO_2peak heart rate may provide an objective assessment of athlete fatigue (Craig & Norton, 2001). Using heart rate based zones, coaches and sports scientists are able to prescribe training intensities at key thresholds across the duration of the season despite changes in power output (Lucia et al., 2000) and manage training loads over the duration of a season (Jeukendrup & van Dieman, 1998). In healthy populations the administration of NO_3^- has been shown to improve haemodynamic response at rest and during exercise, leading to improved oxygen delivery and a reduction in stroke volume and cardiac output at rest and low workloads, leading to an increase in time to fatigue (Lee et al., 2015). In pathological conditions NO_3^- improves a range of vascular related events such as ischemic-reperfusion injury (Carlstom et al., 2011; Lundberg et al., 2006) and has been shown to reduce recovery heart rates following exercise in sufferers of peripheral arterial disease (Kenjale et al., 2011). These cardiovascular-related enhancements may confer positive benefits in athletic populations, either during exercise or following exercise. Given that heart rate is a relatively easy physiological response to measure, it would make the identification of responders and non-responders relatively simple.

In untrained populations (Vanhatalo et al., 2010) both mean and peak heart rate were unchanged following NO_3^- supplementation during moderate intensity exercise and at the conclusion of an incremental peak power assessment. Mean heart rate response during a 20 km time-trial performance was unchanged in 14 competitive female cyclists following NO_3^- (Glaister et al., 2015) following an acute supplementation strategy. Similarly, the findings of Lane et al. 2014, suggest that mean heart rate was unchanged under NO_3^- supplemented conditions in well-trained male and female cyclists in time-trial simulations of 44 and 29 km, respectively. In trained athlete populations (60 to 63 ml.kg.min) heart rate was unchanged following supplementation of NO_3^- (Wilkerson et al., 2012) low-, moderate- (Cermak, Gibala, et al., 2012) and heavy- (Bescos et al., 2012) intensity exercise. NO_3^- supplementation appears to have no effect on heart rate response, regardless of athletic ability or intensity of effort (Bescos et al., 2012; Cermak, Gibala, et al., 2012; Vanhatalo et al., 2010). To date, therefore, NO_3^- supplementation would appear

to have no effect on heart rate response, regardless of athletic ability or intensity of effort (Bescos et al., 2012; Cermak, Gibala, et al., 2012; Vanhatalo et al., 2010).

Respiratory Exchange Ratio

Moderate- to long-term, sub-maximal endurance performance is heavily reliant on a ready supply of carbohydrate and glycogen (Jeukendrup, 2011). Therefore, strategies that spare this limited resource are of interest. In this regard, in rats, it has been shown that NO_3^- consumption, by way of sodium nitrate, elevated fatty acid oxidation in the presence of enhanced mitochondrial biogenesis (Ashmore et al., 2015). Similarly, in healthy humans, a statistically significant reduction in RER (P=0.02), and enhanced mitochondrial efficiency (P=0.02) were reported following 3 days of sodium nitrate supplementation (Larsen et al., 2011) suggesting substrate utilisation can be altered acutely. However, untrained populations may be more sensitive to interventions to reduce reliance on glycogen, as regular endurance training appears to improve fat oxidation during exercise, thus sparing glycogen sources (Coyle, 1999). Indeed, it would appear that in most cases NO_3^- supplementation does not influence substrate utilisation during moderate- (Betteridege et al., 2016; Vanhatalo et al., 2010) or severe-intensity (Bailey, Winyard, et al., 2009) exercise in recreational populations, or in endurance-trained populations (Christensen et al., 2012; Glaister et al., 2015). For instance, while economy was improved during low- to moderate-intensity cycling, RER was unchanged following 6-days of NO_3^- supplementation (Cermak, Gibala, et al., 2012). Similarly, RER was unchanged when cyclists exercised at intensities equating to 70% incremental peak power following 6 d NO_3^- (Christensen et al., 2012) and at 85% VO_2peak after an acute dose of NO_3^- (Bescos et al., 2012) or during a 20 km time-trial performance following NO_3^- supplementation in female cyclists (Glaister et al., 2015).

In summary, it is apparent that a number of physiological enhancements reported in murine species, sedentary or recreationally trained participants as a result of NO_3^- supplementation are not as readily observed in endurance-trained populations. However, even within these higher trained cohorts, NO_3^- supplementation appears to have a limited effect on key physiological parameters, for example, several studies have shown improved economy in competitive endurance populations of <60 $ml.kg^{-1}.min^{-1}$ (Cermak, Gibala, et al., 2012), yet these appear to dissipate for athletes of ≥65 $ml.kg^{-1}.min^{-1}$

(Porcelli et al., 2015). These limited effects are likely to be due to a combination of physiological adaptations mediated through frequent exposure to high training loads and/or high-intensity interval training (Coyle, 1995; Coyle, Sidosis, Horowitz, & Beltz, 1992; Joyner & Coyle, 2008). Indeed, the endurance prescribed adaptations which may limit the effectiveness the NO_3^- derived mechanistic adaptations include, but are not limited to: elevated basal [NO_3^-] (Jungersten et al., 1997), improved oxidative capacity via increased capillary and mitochondrial density (Costill, Fink, & Pollock, 1976), improved calcium signalling and mitochondrial biogenesis (Coffey & Hawley, 2007), and greater expression of oxidative dependent, type I muscle (Coyle et al., 1992). While research into murine populations using in vivo and isolated muscle techniques appears to support supplementation NO_3^-, the location and metabolism of mitochondria and muscle architecture of such species differ to that of human populations (Allen et al., 2008; Holloszy & Coyle, 1984; Stamler & Meissner, 2001). Additionally, given that an achievable daily intake of 400 g NO_3^--rich food delivers ~2.5 mmol NO_3^- (Wink & Paolocci, 2008), it would appear that the typical human diet may offer a small to moderate delivery of NO_3^- relative to the standard chow fed to rodent populations (Ashmore et al., 2015). Indeed, a number of previous studies have shown performance improvements following whole-beetroot consumption (Bond et al., 2012; Murphy, Eliot, Heuretz, & Weiss, 2012). Finally, to meet their large energy expenditures as a result of substantial training loads, athletes typically consume a larger dietary intake than their sedentary counterparts, which is inadvertently likely to mean a greater intake of exogenous NO_3^- (Porcelli et al., 2015). Therefore, while there appears to be a range of physiological mechanisms supporting the use of NO_3^- as an ergogenic aid for athletic populations, there are numerous factors that may limit the efficacy of NO_3^- as a performance aid.

The Effect of Nitrate Supplementation on Performance

A number of early studies investigating the effect on NO_3^- supplementation on performance employed fixed-rate, time-to-exhaustion (TTE) assessments to ascertain its effectiveness in recreational activity level populations (Bailey, Winyard, et al., 2009; Kelly et al., 2013; Lansley, Winyard, Fulford, et al., 2011). While the NO_3^- supplemented groups reported improvements of 15 to 20% in TTE, these trials, which are measures of exercise capacity (Currell & Jeukendrup, 2008) have lower ecological validity, and a high coefficient of variation (CV) of >10%, relative to direct measures of performance, such as time-trial assessments (Hopkins, Hawley, & Burke, 1999). However, for untrained

populations, TTE assessments are likely to be easier to manage, given that time-trials require a learnt pacing strategy and therefore, may be more suited to athletes who regularly compete in such events (Currell & Jeukendrup, 2008; Jeukendrup, Saris, Brouns, & Kester, 1996; Stone, Thomas, Wilkinson, St Clair Gibson, & Thompson, 2011). Indeed, investigations employing TTE trials to assess the ergogenicity of NO_3^- would appear to support it's use in non-athletic populations (Hoon, Johnson, Chapman, & Burke, 2013). For purposes of this review on the effects of NO_3^- on endurance performance, demarcations were made by way of time with the initial 1 to 8 min range selected due to the a number of short-term, high-intensity endurance events that require a notable contribution from both aerobic and anaerobic energy pathways (Craig & Norton, 2001; Duffield et al., 2005a, 2005b; Jeukendrup, Craig, & Hawley, 2000).

Performance Events of 1 to 8 mins

Energy contribution during maximal-intensity events of 1 to 8 min duration has drawn substantial interest over recent years, with particular focus on either running (Duffield et al., 2005a, 2005b) or cycling energetics (Corbett, Barwood, & Parkhouse, 2008; Hettinga, De Koning, Meijer, Teunissen, & Foster, 2007; Stone et al., 2011). Specific interest has been targeted at the role of pacing strategies on event energetics (Watkins, Platt, & McGawley, 2017), EMG activity (Stone et al., 2011), or identification of differences in energy contribution between male and female athletes (Duffield et al., 2005b). While the contribution of aerobic and anaerobic metabolism appears to be similarly matched for events of 1 to 2 min (Gastin, 2001; Hettinga et al., 2007), longer duration activity relies more heavily on aerobic energy. For example, aerobic metabolism contributes 75 and 85% of total energy in events of 3 (Corbett et al., 2008), and 4 (Watkins et al., 2017) min duration, respectively. Despite the rapid reduction in anaerobic energy for events between 2 and 4 min, events closer to 8 min duration still demand ~15% contribution from anaerobic metabolism (Duffield et al., 2005b). Indeed, observations of pacing strategies during the final 10% of a 6 min all-out effort suggests that anaerobic energy may contribute as much as 25% of overall energy metabolism in order to support a final 'kick' (Stone et al., 2011). As such, given the reliance on the aerobic pathway within these durations, research into the effects of NO_3^- on events of this duration is warranted given the higher anaerobic contributions observed at the back end of a trial, relative to mid-distance, and may partially, or wholly explain observed NO_3^--mediated benefits in events

of similar duration (Lansley, Winyard, Bailey, et al., 2011). Indeed, at least two studies have reported that NO_3^- supplementation appears to enhance the second half of maximal intensity exercise (Bond et al., 2012; Lowings, Shannon, Deighton, Matu, & Barlow, 2017). It is well known that events of higher-intensity demand a greater involvement of type II muscle relative to longer duration events which are more reliant on type I muscle. Given the intense nature of such events within this time-frame, the production of numerous fatigue mitigating factors is high (Cairns, 2013). It is thought that in such environments, NO_3^- supplementation has potential to aid performance due to a raft of mechanistic benefits specific to high-intensity exercise and type II fibre (see sections 2 and 3).

To date, potentially as a result of the mechanistic support for improved outcomes, numerous investigations have been undertaken in trials of 1 to 8 min to assess the ergogenic actions of NO_3^- (Table 2). In the studies that have, the outcomes are somewhat conflicting (Hoon, Hopkins, et al., 2014; Peeling et al., 2015). For instance, in order to inform practitioners how dosing NO_3^- strategy would affect repeat cycling performance Hoon, Hopkins, et al. (2014) invited 26 competitive cyclists to complete two 4 min bouts of all-out cycling. The 4 min duration is representative of the time to complete the 4 km track-cycling pursuit event at an elite level, while the time between performances was sequenced based on the pursuit event format in realistic track cycling competition environment. Relative to placebo, however, the initial acute NO_3^- dose resulted in no change to 4 min mean power output while a second acute dose of NO_3^- reduced the mean power output achieved in the second and final 4 min attempt, resulting in a *possibly harmful* outcome on performance (Hoon, Hopkins, et al., 2014). Conversely, in 6 elite kayakers, an acute dose of 70 ml (4.8 mmol) NO_3^- led to a 0.7% or *possibly beneficial* increase in distance covered in 4 min all-out kayaking, relative to placebo (Peeling et al., 2015). Subsequently, 500 m kayaking performance in 5 elite female kayakers was also improved following a 140 ml (9.6 mmol) dose of NO_3^-. Notably, the trial was conducted on-water, with NO_3^- consumption leading to a 1.7% reduction (114.6 vs 116.7 s) in completion time for the 500 m distance and equated to a *very likely beneficial* improvement in performance (Peeling et al., 2015). Simulated rowing over

Table 2: Effects of NO_3^- on physiology, and performance events <8 min						
Study	*Participants (VO_2peak) ml.kg^{-1}.min^{-1})*	*NO_3^- dosing protocol and procedures*	*Physiological Measures*	*Physiological Outcomes*	*Performance Measures*	*Performance Outcomes*
Bond et al. (2012)	14 well-trained junior M rowers	6 d BR 5.5 mmol NO_3^-			6 x 500 m rowing ergometer intervals	↓ time (-1.0%) int 1 to 3 ↑ time (1.7%) int 4 to 6 ↑ time (0.4%) int 1 to 6
Boorsma et al. (2014)	8 elite M middle-distance runners (80)	Acute BR 19.5 mmol NO_3^-	Econ at 50, 65, 80% VO_2peak	↔ in all measures	1500 m running	↔ Time (0.1%)
Boorsma et al. (2014)	8 elite M middle-distance runners (80)	8 d BR 19.5 mmol NO_3^-	Econ at 50, 65, 80% VO_2peak	↔ in all measures	1500 m running	↔ Time (-0.4%)
Christensen et al. (2012)	10 elite M cyclists (72)	6 d BR 4.0 mmol NO_3^-	O_2 kinetics, Econ, HR, RER, RPE	↔ in all measures	Sprint peak power	↔ PPO (0.1%)
Hoon, Hopkins, et al. (2014)	26 elite M cyclists	Acute BR 4.2 mmol NO_3^- 150 min pre TT 1			4 min cycling distance trial (W)	↔ MPO (1.2%) TT 1 ↓ MPO (-0.4%) TT 2
Hoon, Hopkins, et al. (2014)	26 elite M cyclists	Acute BR 4.2 mmol NO_3^- 75 min pre TT 1			4 min cycling distance trial (W)	↔ MPO (1.7%) TT 1 ↓ MPO (-0.5%) TT 2
Hoon, Hopkins, et al. (2014)	26 elite M cyclists	Acute BR 4.2 mmol NO_3^- 75 min pre TT & 2.4 mmol NO_3^- pre TT 2			4 min cycling distance trial (W)	↔ MPO (1.1%) TT 1 ↔ MPO (-0.1%) TT 2
Hoon, Jones, et al. (2014)	10 highly trained M rowers	Acute BR 4.2 mmol NO_3^-			2000 m rowing ergometer time	↔ (0.1%)
Hoon, Jones, et al. (2014)	10 highly trained M rowers	Acute BR 8.4 mmol NO_3^-			2000 m rowing ergometer time	↑ Time (-0.4%)
Lansley, Winyard, Bailey, et al. (2011)	9 competitive M cyclists (58)	Acute BR 6.2 mmol NO_3^-	PO/VO_2	↑ PO/VO_2 (11%)	4 km TT cycling	↑ Time (-2.8%) ↑ MPO (5.0%)

(Lowings et al., 2017)	10 (5 M, 5 F) trained swimmers	Acute BR 12.5 mmol NO_3^-			168 m backstroke	↔ Time (0.9%)
Peeling et al. (2015)	6 elite M canoeists (57)	Acute BR 4.8 mmol NO_3^-	VO_2 during trial	↑ Econ (-2.2%)	4 min kayak ergometer distance trial	↑ Distance (0.7%)
Peeling et al. (2015)	5 elite F canoeists (48)	Acute BR 9.6 mmol NO_3^-			500 m on-water kayak trial	↑ Time (-1.7%)
Shannon et al. (2017)	8 trained M runners and triathletes (62)	Acute BR 12.5 mmol NO_3^-	VO_2 during trial post exercise BL	↔ VO_2 ↑ BL (-7.5%)	1500 m treadmill run	↑ Time (-1.9%)

↑= improvement, ↓= reduction, ↔= no change, (x%) = % change, - = decrease, BL = blood lactate, BM = body mass (kg), BR = beetroot juice, d = day, Econ = economy, F = female, HR = heart rate, int = intervals, M = male, m = metre, MPO = mean power output, NO_3^- = nitrate, PO = power output, PPO = peak power output, RER = respiratory exchange ratio, RPE = rate of perceived exertion, TT = time-trial, VO_2 = oxygen consumption, VO_2peak = peak oxygen uptake, W = watt(s)

a 2000 m distance is reported to require athletes to maintain 90% VO_2peak for the duration of the event (Gillies & Bell, 2000). Given that hypoxic and acidic environments act as a catalyst for the conversion of NO_2^- to NO, events of this intensity appears to be ideal for such supplementation regimes. Indeed, Hoon, Jones, et al. (2014) reported that while a 70 ml dose of NO_3^- had a *likely trivial* effect on 2000 m rowing performance, a 140 ml dose had a *possibly beneficial* effect across the same distance. Notably, the participants were well-trained rowers, who on average took 6.22 min to complete the 2000 m distance, suggesting that events with smaller anaerobic contributions may still benefit from NO_3^- exposure. Bond et al. (2012) reported that 6 days of NO_3^- supplementation had a *likely beneficial* improvement on repeat 500 m ergometer based intervals in well-trained rowers. Overall, the 0.8 s decrease in mean completion time for the 6 repeats, equated to a 0.4% improvement, relative to placebo. In swimming, for which to date only one NO_3^- study has been performed, an acute dose of 140 ml NO_3^- had a *likely trivial* influence on overall swim time for an event of ~130 s (Lowings et al., 2017). However, while overall performance was unchanged, relative to placebo, NO_3^- supplementation resulted in a 64% chance that effects on the back-half (final 4 laps) of the 168 m distance were *likely beneficial*. Second half performances were slower by ~10% relative to the first 4 laps, indicating the onset of fatigue as a result of over-pacing which may have assisted the creation of an acidic and hypoxic environment within the exercising muscle. These findings support the notion that NO_3^- may have beneficial effects in the later half of a short-term, high-intensity activity allowing enhanced NO signalling within working muscles.

The notably different outcomes reported by Peeling et al. (2015) and Bond et al. (2012) to that of Hoon, Hopkins, et al. (2014) may relate to participants of the two former studies being short-duration specialists, in which events are between ~115 s for 500 m kayaking (Peeling et al., 2015), and 6 to 7 min for 2000 m rowing (Smith & Hopkins, 2011). In contrast, the participants employed by Hoon, Hopkins, et al. (2014) were potentially training for longer duration, mixed-intensity, road-race cycling events, in which development of type I fibre and up-regulation of oxidative pathways is observed (Coyle, 1999; Coyle et al., 1992). Kayak training has been shown to increase type II muscle in upper body musculature (Baker & Hardy, 1989), which might explain the tendency for outcomes to be positive in studies utilising upper-body muscle in the high-intensity domain (Bond et al., 2012; Peeling et al., 2015). Moreover, the consumption

of NO_3^- has been shown to systematically enhance vasodilation, arterial compliance, blood flow dynamics (Ferguson et al., 2013; Ferguson, Holdsworth, et al., 2014) and rate of force development (Hernandez et al., 2012) in non-oxidative, type II muscle fibres. These mechanistic alterations would suggest potential benefit for high power output sporting events that are heavily reliant on glycolytic, type II muscle fibres (Stellingwerff et al., 2011). Furthermore, exercise at such high intensity induces an acidic and hypoxic environment (Cairns, 2013) which ameliorates the effects of exogenous NO_3^-, thereby facilitating the reduction of nitrite (NO_2^-) to nitric oxide (NO) (Modin et al., 2001). Furthermore, while endurance athletes train substantial distances, competitive demands for kayakers (Bonetti & Hopkins, 2010) and rowers (Smith & Hopkins, 2011) and are substantially different to cyclists competing in long, single day races or multi-day tours (Rehrer, Hellemans, Rolleston, Rush, & Miller, 2010).

Performance Events of 8 to 30 mins

Similar to events of 1 to 8 min, performances of 8 to 30 min time-frame appear to be enhanced (1.2 to 2.7%) following NO_3^- supplementation (Table 3). The first study to report positive outcomes for athletic populations following NO_3^- supplementation examined its effect on 16.1 km time-trial performance in cyclists (56 $ml.kg^{-1}.min^{-1}$) (Lansley, Winyard, Bailey, et al., 2011). Using stationary cycling ergometers, the participants improved their 16.1 km time-trial performance by 2.7% (Lansley, Winyard, Bailey, et al., 2011). The findings of Lansley, Winyard, Bailey, et al. (2011) were subsequently supported by those of Cermak, Gibala, et al. (2012) who reported 8 mmol NO_3^- supplementation over a 6-day period reduced 10 km time-trial cycling performance by 12 s. While not as substantial as the findings of Lansley, Winyard, Bailey, et al. (2011), the 1.2% overall improvement provides additional evidence for the potential of NO_3^- as an ergogenic aid.

The ground-breaking findings of Lansley, Winyard, Bailey, et al. (2011) and Cermak, Gibala, et al. (2012) would appear to support the use of NO_3^- in athletic populations competing in events of 16 to 30 min duration. However, despite these initial positive findings, subsequent research utilising endurance-trained populations has routinely demonstrated limited effects on performance within this time-frame (Boorsma et al., 2014; Nyakayiru et al., 2016; Porcelli et al., 2015). For instance, in endurance trained

athletes, possessing VO_2peak of ≥65 ml.kg^{-1}.min^{-1}, neither running (Boorsma et al., 2014; Porcelli et al., 2015; Sandbakk et al., 2015), nor cycling (MacLeod et al., 2015; Nyakayiru et al., 2016) appear to be improved as a result of NO_3^- supplementation. Relative to the populations employed by Lansley, Winyard, Bailey, et al. (2011) and Cermak, Gibala, et al. (2012), the volunteers for these more recent studies are of greater aerobic fitness. Indeed, a recent study by Porcelli et al. (2015) eloquently highlighted the possibility of an inverse relationship between aerobic fitness and enhancements in performance following NO_3^- supplementation. Porcelli et al. (2015) reported 6-days of NO_3^- consumption enhanced 3 km running performance in participants with low (28 to 44 ml.kg^{-1}.min^{-1}) and moderate (46 to 57 ml.kg^{-1}.min^{-1}) aerobic fitness, but not in participants noted as being of high (64 to 81 ml.kg^{-1}.min^{-1}) aerobic fitness. More recently, several authors (Boorsma et al., 2014; Nyakayiru et al., 2016) have used a novel approach to investigate whether short or long exposure to NO_3^- might benefit performances of 8 to 30 min. However, it appears that neither acute nor multiple (6 to 8) days of NO_3^- supplementation influences performance in well-trained endurance athletes (Boorsma et al., 2014; Nyakayiru et al., 2016). Finally, in trained athletes, the concentration of 8.7 to 19.5 mmol NO_3^- does not appear to influence performance outcomes (Boorsma et al., 2014).

Table 3: Effects of NO_3^- on physiology, and performance events 8 to 30 min

Study	*Participants (VO_2peak) ml.kg^{-1}.min^{-1}*	*NO_3^- dosing protocol and procedures*	*Physiological Measures*	*Physiological Outcomes*	*Performance Measures*	*Performance Outcomes*
Cermak, Gibala, et al. (2012)	12 trained M cyclists (58)	6 d BR 8 mmol NO_3^-	VO_2peak, Econ (45 and 65% PPO), BL, RER, substrate, energy, HR	Econ: ↑ 45% (3.1%) ↑ 65% (5.5%), ↔ all measures/workloads	10 km TT cycling	↑ Time (-1.2%) ↑ MPO (2.1%)
Lansley, Winyard, Bailey, et al. (2011)	9 competitive M cyclists (56)	Acute BR 6.2 mmol NO_3^-	PO/VO_2	↑ PO/VO_2 (7%)	16 km TT cycling	↑ Time (-2.7%) ↑ MPO (6.0%)
Nyakayiru et al. (2016)	17 trained M cyclists (65)	Acute $NaNO_3^-$ 12.9 mmol NO_3^-	Cycling Econ (45 and 65% PPO), RPE	↔ all measures at both workloads	10 km cycling TT	↔ Time (0.5%)
Nyakayiru et al. (2016)	17 trained M cyclists (65)	6 d $NaNO_3^-$ 12.9 mmol NO_3^-			10 km cycling TT	↔ Time (-1.3%)
Peacock et al. (2012)	10 trained M skiers (65)	Acute KNO_3^- 7.7 mmol NO_3^-	Econ (10 and 14 km.hr^{-1})	↔ at either speed	5 km track run	↔ Time (0.5%)
Porcelli et al. (2015)	6 highly-trained M runners (72)	6 d $NaNO_3^-$ 5.5 mmol NO_3^-	Econ (80% GET), VO_2peak	↔ at either measure	3 km track run	↔ Time (-0.3%)
Shannon et al. (2017)	8 trained M runners and triathletes (62)	Acute BR 12.5 mmol NO_3^-	VO_2 during trial and post exercise BL	↔ VO_2 and BL	10000 m treadmill run	↔ Time (-0.3%)

↑ = improvement, ↓ = reduction, ↔ = no change, (x%) = indicates % change, - = decrease, BL = blood lactate response, BM = body mass (kg), BR = beetroot juice, Econ = economy, GET = gas exchange threshold, HR = heart rate, KNO_3^- = potassium nitrate, M = male, m = metre, MPO = mean power output, $NaNO_3^-$ = sodium nitrate, NO_3^- = nitrate, PO = power output, PPO = peak power output, RER = respiratory exchange ratio, RPE = rate of perceived exertion, TT = time-trial, VO_2 = oxygen consumption, VO_2peak = peak oxygen uptake, W = watt(s)

Performance Events of >30 mins

To date, only a handful of studies have adopted performance trials of between 40 (Bescos et al., 2012) and >2 hr (Wilkerson et al., 2012) to assess the efficacy of NO_3^- supplementation on endurance performance (Table 4). Using a time-trial distance of 50 miles, Wilkerson et al. (2012) investigated the effectiveness of an acute dose of 500 ml NO_3^- on a group of 8 well-trained (63 ml.kg.$^{-1}$min^{-1}) male cyclists. While the 0.8% reduction in time to complete the 50-mile distance was not statistically significant, removing 3 non-responders (those who's basal [NO_2^-] had risen by <30% following NO_3^-) resulted in a mean 2% reduction in time for the remaining 5 participants. In cycling, changes as small as 1% are considered worthwhile (Paton & Hopkins, 2006), therefore, the identification that responders to NO_3^- supplementation could improve performance by at least 2.0% is of note. Subsequently, Cermak, Res, et al. (2012) assessed the effects of an acute dose of NO_3^- on time to complete a 1000 kJ workload trial (equating to a mean group duration of ~60 min), in the shortest possible time in trained (60 ml.kg.$^{-1}$min^{-1}) cyclists. However, trial duration was unchanged following NO_3^- suggesting that in trials of ~1 hr, NO_3^- has little to no effect on performance. Likewise, the distance covered in a 40 min cycling distance-trial by 13 trained cyclists (60 ml.kg.$^{-1}$min^{-1}) was unchanged following 3 d of either NO_3^--rich or NO_3^--depleted supplementation (Bescos et al., 2012). Together, these findings suggest that irrespective of fitness levels, supplementation of NO_3^- has little to no effect on performance, however, it is important to investigate for an individual response to NO_3^- supplementation (Wilkerson et al., 2012).

Table 4: Effects of NO_3^- on physiology, and performance events >30min						
Study	*Participants (VO_2peak) ml.kg^{-1}.min^{-1}*	*NO_3^- dosing protocol and procedures*	*Physiological Measures*	*Physiological Outcomes*	*Performance Measures*	*Performance Outcomes*
Bescos et al. (2012)	13 well-trained, M cyclists and triathletes (60)	3 d 10 mg.kg BM Na NO_3^-			40 min distance trial	↔ MPO (0.4%)
Cermak, Res, et al. (2012)	20 competitive M cyclists and triathletes (60)	Acute BR 8.7 mmol NO_3^-	BL, HR, FFA, Glu, and Ins	↔ all measures	~1 hr TT cycling	↔ MPO (1.1%)
Christensen et al. (2012)	10 elite M cyclists (72)	6 d BR 4.0 mmol NO_3^-	O_2 kinetics, Econ, HR, RER, and RPE	↔ all measures	400 kcal cycling TT (~ 1 hr)	↔ MPO (1.75%)
Wilkerson et al. (2012)	8 well-trained M cyclists (63)	Acute BR 6.2 mmol NO_3^-	Econ	↑ PO/VO_2 (3.2%)	50 mile cycling TT	↔ MPO (0.8%)

↑ = improvement, ↓ = reduction, ↔ no change, (x%) = % change, - = decrease, BL = blood lactate response, BM = body mass (kg), BR = beetroot juice, d = day, Econ = economy, HR = heart rate, M = male, MPO = mean power output, $NaNO_3^-$ = sodium nitrate, NO_3^- = nitrate, PO = power output, RER = respiratory exchange ratio, RPE = rate of perceived exertion, TT = time-trial, VO_2 = oxygen consumption

In summary, it is evident, that in lesser-trained populations (56 to 58 $ml.kg^{-1}.min^{-1}$), dietary NO_3^- supplementation appears to be a promising ergogenic aid, particularly in cycling specific events of ~6 to 27 min. While effects of NO_3^- appear to be limited in populations of greater athletic status, it's use prior to shorter duration events, maybe warranted (Peeling et al., 2015). Specifically, it is possible that events of ~115 s duration may benefit from acute NO_3^- due to a range of mechanistic enhancements in type II muscle (Jones et al., 2016), however, it is yet to be determined if longer duration dosing has a greater impact on these short-term events. Despite previous research suggesting that a 3-d NO_3^- dosing protocol was central in improving economy via elevated mitochondrial biogenesis (Larsen et al., 2011), it appears that the acute NO_3^- supplementation strategies improve events between 115 s (Peeling et al., 2015) and 27 min (Lansley, Winyard, Bailey, et al., 2011) duration. As such, it would be of interest to extend NO_3^- dosing strategies to ≥3-d in order to assess the impact of longer duration NO_3^- consumption has on performance, especially those events with greater aerobic demand.

Despite evidence to the contrary, the majority of NO_3^- related studies report no meaningful positive change in performance (McMahon et al., 2016) leaving questions about the efficacy of NO_3^- supplementation in endurance-trained athletes. An attractive and plausible explanation for the lack of effects witnessed in higher trained populations is that in an attempt to meet energy expenditure, athletes will typically consume a greater volume of food, and by default increase the daily amount of nitrate-source foods (Jonvik et al., 2016). Furthermore, frequent endurance training has been shown to elevate basal NO_2^- (Jungersten et al., 1997). Indeed, higher resting levels of both NO_3^- and NO_2^- have been reported in well-trained athletes, relative to lesser-trained populations (Porcelli et al., 2015). However, recent evidence appears to suggest that higher basal plasma [NO_2^-] may not be a moderator in the responsiveness of NO_3^- on performance in athletes of 64 $ml.kg^{-1}.min^{-1}$ (Christensen, Petersen, Signe, Weitzberg, & Nybo, 2017). Additionally, a range of long-term physiological enhancements mediated through frequent exposure to endurance training may also reduce the impact of NO_3^- supplementation on endurance performance (Coffey & Hawley, 2007). These enhancements include transformation of type II to type I muscle (Coyle et al., 1992), improved mitochondrial functioning (Befroy et al., 2008) and exercise-induced Ca^{2+} perturbation (Summermatter et al., 2012). Together with dietary intake, these training-specific alterations may negate, or at least

reduce the effectiveness of proposed mechanistic alterations following NO_3^- supplementation (Jones et al., 2016; Larsen et al., 2011). Typically, endurance athletes training takes place in outdoor environments, than more sedentary populations. Recently, the combination of NO_3^- supplementation and ultra-violet (UV)-A exposure has been shown to enhance the bioavailability of NO_2^- above that of NO_3^- supplementation alone (Muggeridge et al., 2015). Enhanced bioavailability of NO_2^- subsequently led to improved time-trial performance in the NO_3^- + UV-A cohort, relative to control (Muggeridge et al., 2015).

Therefore, not only is further research required to elucidate if longer duration supplementation is effective in improving short-term, high-intensity performance of 1 to 8 min, but there appears to be a number of dietary and environmental related exposure related factors that warrant consideration in future research when assessing the efficacy of NO_3^- to enhance performance in athletes.

Dual Dietary Supplementation Strategies Involving Nitrate

In an attempt to improve sporting performance, athletes will often consume several dietary supplements before competition. Parallel or dual dietary supplement consumption is an attempt to maximise the individual mechanism/s of each supplement, thus leading to a larger magnitude of enhancement. However, while these supplements may have shown positive effects individually, there is no guarantee that both supplements will provide a beneficial effect when combined. To date, several authors have attempted to assess the effects of NO_3^- supplementation, in parallel with a second dietary supplement, on performance outcomes.

In the lead up to the 2012 London Olympic Games, Lane et al. (2014) investigated the combined, and single effects of NO_3^- (8.4 mmol NO_3^-) and caffeine (3 $mg.kg^{-1}$ body mass) supplementation on competitive cyclists. Cycling performance was assessed using a computer-simulated version of the road cycling time-trial course at the 2012 Olympics, whereby 12 female (60 $ml.kg^{-1}.min^{-1}$), and 12 males (72 $ml.kg^{-1}.min^{-1}$) completed distances of 29 and 44 km respectively, following acute consumption of either, or a combination of both supplements. Relative to placebo, mean power output (MPO) was enhanced following supplementation of caffeine (3.9%), and in combination with NO_3^-

(3.0%). However, there was no improvement for NO_3^- (-0.4%) when used on its own. The authors surmised that the longer duration of the event and the acute loading period might have contributed to the lack of effects under NO_3^- conditions.

A subsequent investigation carried out by Glaister et al. (2015) on 14 competitive female cyclists and triathletes (52 $ml.kg^{-1}.min^{-1}$) employed the same combination of supplements as Lane et al. (2014). Similarly to the findings of Lane et al. (2014), acute supplementation of caffeine (5 $mg.kg^{-1}$ body mass) for both individual, and combined effects with NO_3^- (~7.3 mmol NO_3^-) revealed no change in mean power output under NO_3^- conditions, over a 20 km cycling time-trial distance but improved effects for both caffeine (3%) and combined caffeine and NO_3^- (3%) trials. Glaister et al. (2015) suggested that a potential reason for lack of effects under NO_3^- may have been the high intensity of the 20 km time-trial, which was completed at ~83% of VO_2peak suggesting that the intensity was too high for economical benefits to be conferred. Conversely, several of the mechanisms by which NO_3^- is thought to enhance performance (Modin et al., 2001), are elicited during events of shorter duration, namely, low-oxygen, highly acidic conditions (Cairns, 2013), with benefits particularly evident in type II muscle (Jones et al., 2016). While both Lane et al. (2014) and Glaister et al. (2015) reported benefits of caffeine in isolation, and with NO_3^-, the trial distance may have been too long, and as such, intensity too low to fully invoke the mechanistic actions of NO_3^- within type II muscle. As such further research is warranted into the effects of NO_3^- supplementation in parallel with caffeine and other dietary supplements with evidenced-based support for use in events of shorter duration (Stellingwerff et al., 2011).

A recent study compared the combined effects of L-arginine and NO_3^- on 5 km run performance of 9 junior elite (69 $ml.kg^{-1}.min^{-1}$) cross-country skiers (Sandbakk et al., 2015). The benefit of combined supplementation of both L-arginine and NO_3^- is the targeting of both endogenous (NOS) and exogenous (NO_3^- - NO_2^- - NO) pathways in the production of NO. Elevated NO levels are associated with improvements in economy, vascular conductance and mitochondrial efficiency and as such findings indicate that individually both L-arginine (Bailey, Winyard, et al., 2010) and NO_3^- (Vanhatalo et al., 2010) have enhanced physiological measures in recreational populations. However, no performance increases were witnessed following either combined or individual treatments.

Environmental Considerations

The majority of NO_3^- related studies have been undertaken in normoxic, sea-level environments within contemporary and standardised laboratory (18 to 21°C; 20 to50% RH) settings (Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011; Porcelli et al., 2015) with few studies conducted either 'in the field' (Peeling et al., 2015) or, in simulated environmental conditions replicating potential field conditions (Kuennen et al., 2015). While standardised conditions increase research trial reliability via controlled indoor environmental conditions, athletes compete across a variety of terrains and environmental conditions (Peiser & Reilly, 2004), often influencing athlete's physiological stress and performance (Altareki, Drust, Atkinson, Cable, & Gregson, 2009), regardless of ability (Bell et al., 2016). The subsequent research findings should be more attractive to practitioners working with athletes particularly when the reduction of NO_2^- to NO appears to be facilitated under hypoxic and/or thermally challenging environments, potentially making their findings more valid for specific environmental conditions. Therefore, the following section discusses the effect of hypoxic and thermally challenging environments on the effectiveness of NO_3^- supplementation.

Altitude Simulated Environments (Normoxia vs. Hypoxia)

Given the catalysing effect on the conversion of NO_2^- to NO observed under normoxic conditions (Modin et al., 2001), the potential to improve physiology and performance following NO_3^- in hypoxic environments is attractive. Certainly, it appears that in populations possessing no greater than moderate fitness levels, the efficacy of NO_3^- in combatting the effects of hypoxic environments is well-founded. For instance, in order to establish the mechanistic basis for the effects of NO_3^- during hypoxic exercise and subsequent recovery, Vanhatalo et al. (2011) investigated the effects of 5-days NO_3^- on the recovery kinetics of [PCr] following maximal single-leg knee-extension activity over two (10 min and 24 s) time-periods. The authors reported enhanced muscle energetics and better oxygen matching during hypoxia, with faster recovery of [PCr] in both normoxia and hypoxia following NO_3^- supplementation. In applying the effects of NO_3^- to a competitive simulation, Muggeridge et al. (2014) assessed NO_3^- on competitive male cyclists (52 ml.kg. $^{-1}$min^{-1}) during which participants completed cycling based exercise trials at a simulated altitude of 2500 m. Under acute (~5 mmol)

NO_3^- supplemented conditions, both economy (7%), and 16.1 km time-trial (~28 min) performance improved (2.5%), relative to placebo in hypoxia. Conversely, at the same altitude, Arnold, Oliver, Lewis-Jones, Wylie, and MacDonald (2015) reported that 10 km run performance was unchanged following an acute bout of ~7 mmol NO_3^-. The authors also reported no change in incremental running assessment at 4000 m, suggesting that NO_3^- had no effect on maximal intensity performance at either 2500 or 4000 m simulated altitude. The outcomes reported by Arnold et al. (2015) are surprising, particularly when hypoxic environments are known to enhance the bioavailability of NO, thereby thought to improve physiological outcomes. However, the results reported may be indicative of the cohorts' higher endurance ability (66 $m.kg^{-1}.min^{-1}$), relative to the lesser-trained populations employed in previous research (Vanhatalo et al., 2011), indicating a limiting effect of NO_3^- in hypoxic conditions. If a ceiling effect does exist, by using a cohort of trained male cyclists possessing VO_2peaks of 60 $m.kg^{-1}.min^{-1}$, MacLeod et al. (2015) have helped to identify the minimal level at which acute NO_3^- supplementation strategies appears to be ineffective. Following ~6 mmol NO_3^- neither cycling economy nor 10 km time-trial performance improved in hypoxic conditions, relative to placebo conditions. Notably, the authors report identical outcomes when these interventions were assessed under normoxic conditions, which further supports the suggestion that aerobic calibre, by way of training adaptions, influences the effectiveness of dietary nitrate supplementation on physiology and performance.

Exercise in the Heat

While beneficial in some therapeutic environments (Kenjale et al., 2011), the disregulation of the vagal response from NO, may, in some cases, counteract homeostatic responses during times of either hot (Tripathi, Mack, & Nadel, 1990), or cold (Gagge, Stolwijk, & Hardy, 1967) exposure. Typically, in hot environments, blood flow is distributed to the periphery of the body in an attempt to maintain core temperature equilibrium (Tripathi et al., 1990). However, as a result of whole body vasodilation (Bloomer, 2010), a range of physiological responses such as increased blood flow (Ferguson et al., 2013), and sympathetic derived responses (Tripathi et al., 1990) may be muted under NO_3^- conditions.

Notably, the effects of NO_3^- supplementation on exercise in hot environments have only recently been assessed (Kuennen et al., 2015; Tyler et al., 2016). The studies employed healthy, but non-athletic male participants who took part in either marching (Kuennen et al., 2015) or moderate-intensity cycling (Tyler et al., 2016). Specifically, Kuennen et al. (2015) assessed the effects of beetroot juice over a 6-day period on military style marching (~5 $km.hr^{-1}$). While the authors observed a reduction in oxygen consumption, an increase in core body temperature in the final third of a 45 min march in a 41ºC; 20% RH environment was observed (Kuennen et al., 2015). As a result, the authors projected that critical core body temperature (40ºC) would be reached earlier and lead to a 10% reduction in distance marched due to hyperthermic stress imposed by consumption of NO_3^- (Kuennen et al., 2015). Conversely, Tyler et al. (2016) reported that an acute 10 g dose of L-arginine had no effect on oxygen consumption or rectal temperature (T_{re}) during moderate intensity exercise in 35ºC; 40% RH environments.

While the outcomes of Kuennen et al. (2015) are of interest, there is limited application to endurance-trained populations, given the various training related enhancements, such as higher resting level of NOS (Jungersten et al., 1997) that athletes appear to possess as a result of substantial training loads. Moreover, training stimulus enables a greater buffering to the exhaustive effects of exercise in hot environments, relative to untrained populations (Cheung & McLellan, 1998). Additionally, the seasonal timing of studies should be taken into account particularly as UV-A exposure elevates NO_2^- bioavailability (Muggeridge et al., 2015), while melatonin levels appear to reduce ischemic-hypoxic damage (Blanco, Hernandez, Franchelli, Ramos-Alverez, & Peinado, 2017). Notably, while it is unknown over which season(s) the Tyler et al. (2016) study was undertaken, the Kuennen et al. (2015) study was conducted in winter months, whereas greater sun exposure (leading to increased UV-A exposure) is more likely in summer months (Muggeridge et al., 2015). Clearly, further studies are required to ascertain the effects of NO_3^- consumption on perceptual responses, thermal regulation and performance, especially in athletes who may be exposed to hot environments during competition.

Conclusions and Future Directions

A wide variety of assessment protocols, in various sporting disciplines, have been employed to determine the effectiveness of NO_3^- supplementation on the physiology

and performance of trained endurance athletes. To date, in moderately trained (~58 $ml.kg^{-1}.min^{-1}$) populations, both exercise economy and performances of 2 to 30 min duration appear to benefit from the supplementation of NO_3^-. Despite these sizable effects, populations of greater aerobic ability ($\geq$65 $ml.kg^{-1}.min^{-1}$) appear not to benefit from NO_3^- supplementation. However, more research is needed to ascertain if a 'ceiling' effect exists with regards to NO_3^- and aerobic ability, and if it does, what are the key determinants. Research appears to support the use of NO_3^- supplementation in events of 1 to 8 min duration for well-trained athletes, particularly in kayaking (Peeling et al., 2015), rowing (Bond et al., 2012), and potentially swimming (Lowings et al., 2017), notably sporting events that are reliant on upper body musculature that is predominantly type II (Baker & Hardy, 1989). Effects for cycling are less clear, with their being potential for performance reduction. To date, there appears to be no benefit in NO_3^- supplementation for events >40 min duration in trained endurance athletes. Although relatively limited in number, research investigating the effects of NO_3^- supplementation of higher-intensity in athletic populations appears to favour events of between ~2 (Peeling et al., 2015) and 6.30 min (Lansley, Winyard, Bailey, et al., 2011) potentially due to the apparent enhancements of both aerobic (Larsen et al., 2011) and anaerobic (Jones et al., 2016) related factors. These enhancements may potentially be due to improvement in expression of type II muscle (Ferguson, Hirai, et al., 2014; Ferguson, Holdsworth, et al., 2014), the enhancement of oxygen kinetic response in these fast-start events (Bailey, Winyard, et al., 2009), and/or the increased bioavailability of NO_2^- during exercise in which oxygen availability is low and acidity levels are elevated (Cairns, 2013; Cosby et al., 2003). Notably, in longer duration, sub-maximal VO_2peak events of ~28 min, large performance effects (2.7%) have been reported as a result of NO_3^- supplementation (Lansley, Winyard, Bailey, et al., 2011). Given the potential mechanistic enhancements, and relatively promising to findings to date (Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011; Peeling et al., 2015), it was interest to explore the potential benefits of performance within this time duration. Finally, improved mitochondrial efficiency (Larsen et al., 2011) and enhanced measures of economy (Bailey, Winyard, et al., 2009; Kelly et al., 2013) as a result of NO_3^- supplementation, supported inclusion of literature concerning endurance events >30 min. Researchers and practitioners should be on the lookout for responders and non-responders to NO_3^- supplementation, and as such, further work is required to identify what mechanism/s mediate such responses. Finally, the mechanistic actions of NO_3^-

appear to support its use in hypoxic exposure, however these same actions may counteract sympathetic mediated responses and thus impair performance during exercise in hot environments. From a practical standpoint, it is clear that NO_3^- supplementation may benefit shorter-term, high-intensity endurance events, in trained endurance athletes. Further research is required to investigate the effectiveness of NO_3^- supplementation in endurance-trained athletes with specific focus on physiological and performance alterations, individual responses, dosing strategies and event duration in a variety of environmental conditions.

CHAPTER 3: THE EFFECT OF DIETARY NITRATE SUPPLEMENTATION ON PHYSIOLOGY AND PERFORMANCE IN TRAINED CYCLISTS

Abstract

Purpose: To determine the effect of dietary nitrate (NO_3^-) supplementation on physiology and performance in well-trained cyclists following six to eight d of NO_3^- supplementation. **Methods:** Eight competitive male cyclists (mean ± SD; age = 26 ± 8 y; body mass = 76.7 ± 6.9 kg; VO_2peak = 63 ± 4 ml.kg^{-1}.min^{-1}) participated in a double-blind, placebo-controlled, crossover-design study in which participants ingested 70 ml beetroot juice containing ~4 mmol NO_3^- (NIT) or a NO_3^- depleted placebo (PLA), each for 8 d. Replicating pre-treatment measures, participants undertook an incremental ramp assessment to determine VO_2peak, first (VT_1), and second (VT_2) ventilatory thresholds on day 6 (NIT6 and PLA6), moderate-intensity cycling economy on day 7 (NIT7 and PLA7), and a 4 km time-trial on day 8 (NIT8 and PLA8). **Results:** Relative to PLA, 6 d of NIT supplementation produced *unclear* effects for VO_2peak (mean ±95%CL: 1.8 ±5.5%) and VT_1 (3.7 ±12.3%) and *trivial* effects for both VT_2 (-1.0 ±3.0%) and exercise economy on day 7 (-1.0 ±1.6%). However, effects for time-trial performance time (-0.7 ±0.9%), and mean power (2.4 ±2.5%), on day 8 were *likely beneficial*. **Conclusions:** Despite mostly *unclear* outcomes for standard physiological determinants of performance, 8 d of NO_3^- supplementation resulted in *likely beneficial* improvements to 4 km time-trial performance in well-trained male endurance cyclists.

Key Words: Beetroot juice, time-trial, economy, cycling

Introduction

Dietary nitrate (NO_3^-) supplementation, either through ingestion of pharmacological sodium NO_3^- (Bescos et al., 2012; Larsen et al., 2010) or naturally occurring beetroot juice (Bailey, Winyard, et al., 2009) has generated significant interest amongst researchers, coaches and sport science practitioners, due to its marked physiological effects (Larsen et al., 2010). Specifically, NO_3^- ingestion, both acutely, and chronically for up to 15 days has been shown to reduce oxygen consumption during submaximal exercise (Bailey, Winyard, et al., 2009; Cermak, Gibala, et al., 2012), improve ventilatory threshold (Vanhatalo et al., 2010) and reduce the VO_2 slow component and mean response time (Bailey, Winyard, et al., 2009). Individually and collectively, such enhancements are of interest in athletic populations given their relevance to sport performance in endurance events (Jones & Carter, 2000).

The performance enhancing effects of NO_3^- supplementation in endurance events are well documented (Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011), however, ergogenic effects appear to reduce as performance trial duration increases (Bescos et al., 2012; Wilkerson et al., 2012), and/or as athlete calibre increases (Porcelli et al., 2015). The range of performance outcomes highlights the need to consider trial duration, athletic calibre and athlete type (sprint vs endurance) when interpreting the effect of NO_3^- supplementation. For example, in cycling, following a single acute dose of NO_3^-, moderately-trained (VO_2peak 56 to 63 $ml.kg.^{-1}min^{-1}$) cyclists improved performance over shorter (~6.45 to 27.30 min) (Lansley, Winyard, Bailey, et al., 2011) but not longer (40 to 137 min) duration time-trials (Bescos et al., 2012; Wilkerson et al., 2012). In contrast to the findings of Lansley, Winyard, Bailey, et al. (2011) national level cyclists produced *unclear* and *possibly harmful* effects for 4 min time-trial MPO following acute doses of NO_3^- (Hoon, Hopkins, et al., 2014). Such findings suggest that acute NO_3^- supplementation strategies enhance the performance of moderately trained cyclists within events of shorter - and therefore greater relative intensity - than longer duration trials. Short-duration (1 to 12 min), maximal intensity exercise leads to a rapid increase in blood acidosis (Cairns, 2013) and although this may not directly infer a reduction in performance (Allen et al., 2008) the stepwise reduction of nitrite (NO_2^-) to nitric oxide (NO) is catalysed in more acidic and hypoxic environments (Cosby et al., 2003), indicating that higher intensity exercise may enhance the effectiveness of NO_3^- supplementation on performance.

Improvements in mitochondrial respiration (Larsen et al., 2011) and a reduction in ATP cost during muscle force production (Bailey, Fulford, et al., 2010) are several mechanisms postulated to positively enhance measures of performance and physiology as a result of longer term (≥3 d) NO_3^- supplementation (Bailey, Fulford, et al., 2010; Larsen et al., 2011). Based on these physiological enhancements, and given the 1.2% mean improvement over longer (10 km) distance following a 6 d supplementation period (Cermak, Gibala, et al., 2012), it is of interest to explore whether a similar dose duration augments improvements over and above that of the 2.7% enhancement for 4 km reported by Lansley, Winyard, Bailey, et al. (2011) following an acute dose in a similarly trained population. Moreover, this shorter 4 km distance may serve to produce an ideal environment for the reduction of NO_2^- to NO (Cosby et al., 2003). Therefore, the aim of this study was to determine the effects of six to eight d of beetroot juice supplementation on 4 km time-trial performance and economy in trained cyclists.

Methods

Subjects

Nine well-trained endurance male cyclists (mean ± SD; age 26 ± 8 y; body mass 76.7 ± 6.9 kg; sum of 8 skinfold 52 ± 10 mm; VO_2peak 63 ± 4 $ml.kg^{-1}.min^{-1}$; weekly training duration 9 ± 3 $h.wk^{-1}$) acted as their own controls in this double-blind, placebo-controlled, crossover-design study. With ethical approval from the Auckland University of Technology research ethics committee, participants were fully informed about the study prior to providing consent. The information provided also explained that the aim of study was to investigate the effects of beetroot supplementation on cycling physiology and performance. Participants were asked to maintain their usual diet but to abstain from caffeine and alcohol, 24 and 48 h prior to testing, respectively. Participants were requested to refrain from exercise the day of the test and limit exercise the day prior to no more than 2 h at moderate intensity. All experimental trials occurred at the same time of day (± 2 h) to control for biological variation.

Design

Over a 5 wk period participants attended 15 separate sessions to perform a range of physiological and performance assessments on an electromagnetically braked cycle

ergometer (Velotron, Racermate, Seattle, USA) in a sea-level altitude, temperature-controlled laboratory (19°C; 60% RH). The cycle ergometer was fitted with the participant's own pedals with replication of seat and handle bar position from the participant's own bike to the ergometer. On the first visit participants anthropometric characteristics were measured along with VO_2peak and peak power output (PPO) from an incremental cycling assessment. Within the week participants completed two 4 km time-trial familiarisation sessions. Based on MPO from the second 4 km time-trial familiarisation, participants were then randomly assigned according to one of two 12 day experimental periods in which nitrate (NIT) or placebo (PLA) supplements were administered with several physiological and performance measures carried out prior to and following each treatment phase. A 7 d washout period followed the first treatment before participants undertook the second, alternate treatment.

Procedures and Assessments

Incremental Ramp Assessment: Participants carried out the incremental ramp assessment to determine ventilatory thresholds, VO_2peak, and PPO. The protocol started with 3 min at 50 W, followed by a 20 $W \cdot min^{-1}$ increase until volitional exhaustion on the cycle ergometer. Participants were asked to maintain a cadence of between 70 and 90 $rev \cdot min^{-1}$ throughout the test, and this pedal rate was recorded and repeated during subsequent assessments. The test was terminated when participants' cadence fell below 70 $rev \cdot min^{-1}$. During this, and subsequent economy trials, gas-exchange and ventilatory measures were assessed using a breath-by-breath metabolic system (Metamax 3B, Cortex Biophysik, Leipzig, Germany). Calibration of the system took place prior to each test using alpha standard gases (BOC Gases, Auckland, NZ), while a turbine volume sensor was calibrated using a 3 L syringe (Hans Ruldolph, Shawnee, USA). VO_2peak was established from peak 30 s mean data using raw breath-by-breath measures for oxygen consumption. Ventilatory thresholds were established following the removal of artefacts from raw breath-by-breath data. First (VT_1) and second (VT_2) ventilatory thresholds were established using methods of Whipp, Davis, Torres, and Wasserman (1981) and (Beaver, Wasserman, & Whipp, 1985), respectively.

Economy: Following the incremental assessment and within a 48 to 72 h period, participants completed two x 6 min constant-load square-wave bouts of exercise,

separated by a 6 min bout of 'unloaded' cycling, at a prescribed power equivalent to 80% of each individual's VT_1 power during which VO_2 was recorded. The mean VO_2 during the last 60 s of each 6 min bout was averaged and used to reflect the underlying oxygen cost.

Performance Trials: Following the economy assessment and within a 48 to 72 h period, participants returned to the laboratory for the first of two 4 km familiarisation time-trials; the second following another period of 2 to 3 d. A standardised warm-up protocol was employed consisting of 5 min at 100 W, 5 min at 150 W, 3 min at the individual's heavy intensity domain (~90% of PPO group mean) and finally 5 min at 100 W. Participants were instructed to complete the 4 km distance in the shortest time possible. Subsequent trials were performed if the individual variance in performance time was >1% until this was achieved. The time-trial mode of the cycle ergometer allowed for the use of self-selected gearing and cadence to best reflect actual individual competition performance. During each trial, the lead researcher provided consistent encouragement to participants while feedback - including power output, gear selection and distance completed - was provided visually using the Velotron software. Duration, MPO, average heart-rate and cadence were recorded during all time-trials. The 4 km time-trial assessment has previously been used before in NO_3^- research (Lansley, Winyard, Bailey, et al., 2011).

Beetroot Juice Supplementation

At the conclusion of the two pre-supplement testing periods, participants were provided with 8 x 70 ml supplements of either unlabelled NO_3^- rich beet-juice [NO_3^- ~4.0 mmol.L^{-1}]; (James White Drinks, Ipswich, UK) or a NO_3^- depleted [NO_3^- ~0.003 mmol.L^{-1}] placebo of identical taste, smell and appearance, supplied by the same manufacturer, consistent with previous research (Cermak, Gibala, et al., 2012). Participants were asked to consume the juice at a rate of 1 x 70 ml daily and, on the day of a trial, to ingest the juice 2 h before their scheduled lab appointment. The tester administering the beverage was blinded to the supplement condition. Participants were instructed to avoid spitting, chewing gum or using antibacterial mouthwash during the supplementation interventions, as these actions are associated with a lowering of plasma/serum NO_2^- levels (Govoni, Jansson, Weitzberg, & Lundberg, 2008; Webb et al., 2008). Participants were asked to record their dietary intake in the 24 h prior to the first time-trial and repeat this for all subsequent visits.

Habitual Training

Quantification of the cyclist's habitual training load (mean for duration, distance, and power) were determined during both experimental and washout phases using a calibrated wireless crank-based power meter (SRM, Julich, Germany), which was installed on each participant's bike.

Blood collection

Participants were asked to sit motionless in a reclined position for 15 min on their arrival at the laboratory. Thereafter, a venous blood sample from an antecubital vein was collected via an evacuated SST monovette tube (Becton Dickinson Biosciences). The 10 ml of extracted blood was then left to clot for 20 min before being centrifuged at 1218 x g for 10 min at 4°C **(**Heraeus Megafuge 16R, Thermo Scientific) as per the manufacturer's instructions. Serum was then aliquoted into micro containers and stored at -80°C for later analysis.

Serum NO_2^- Analysis

NO_2^- analysis was carried out using the Griess Method (Chae, Lee, Kim, & Bac, 2003) with a commercially available kit (Promega, Wisconsin, USA). Following thawing samples were deproteinized by adding 400 µL trichloroacetic acid (TCA) to 400 µL serum, vortexed and centrifuged at 14,500 x rpm for 5 min **(**Espresso, Thermo Scientific) (Ghasemi et al 2007). Thereafter, 150 µL of supernatant was added to 130 µL of deionized water followed by 20 µL of Griess reagent, prior to an incubation period of 30 min, as per manufacturers instructions. Absorbance was measured at 548 nm. The resulting [NO_2^-] levels were reported in µM, consistent with previous research using the Griess analysis method (Tsikas, 2007).

Statistical Analysis

Data are presented as mean ± SD unless otherwise reported. Comparisons were made between each of the four NIT and PLA time-points for 4 km time-trial performance, economy and heart-rate using a customised analysis spread-sheet (Hopkins, 2006). Performance data were log-transformed for analysis to reduce bias arising from non-uniformity of error, and subsequently back-transformed to obtain changes in mean and variation as percentages. To make inferences about true (population) values of the effect

of NO_3^- supplementation on cycling performance and incremental PPO, the uncertainty in the effect was expressed as 95% confidence limits and as likelihoods that the true value of the effect represents substantial change (harm or benefit). We present these probabilities in quantitative values and qualitative terms in preference to a statistical inference based on a null hypothesis test. An effect was deemed *unclear* if the chance of benefit was sufficiently high to warrant use of the treatment but the associated risk was unacceptable. Such *unclear* effects were identified as those with an odds ratio of benefit-harm <57, a ratio corresponding to an effect that is borderline *possibly beneficial* (12.5% chance of benefit) and borderline *most unlikely harmful* (0.25% chance of harm) (Hopkins, Marshall, Batterham, & Hanin, 2009). We assumed 1% and 0.4% as smallest worthwhile change for power and time, respectively for 4 km time-trial performance and 1% for incremental PPO (Paton & Hopkins, 2006). The default values and qualitative terms were set at: <0.5%, *most unlikely*; 0.5-5%, *very unlikely*; 5% - 25%, *unlikely*; 25 to 75%, *possibly*; 75 – 95%, *likely*; 95 – 99.5%, *very likely*; >99.5%, *most likely*. Smallest worthwhile change for remaining (non-clinical) measures were calculated as 0.3 of the coefficient of variation of measurement error arising from their respective reliability trials in agreement with previous studies (Bonetti, Hopkins, & Kilding, 2006), with effect sizes reported as 0.2 of the between-subject SD (Hopkins et al., 2009). Effect sizes (ES) were calculated using Cohens *d*, with an ES of <0.2 considered *trivial*, >0.2 *small*, >0.6 *moderate*, >1.2 *large* and >2.0 *very large*.

Results

One participant withdrew due to illness during the study. Remaining participants' (n=8) self-reported adherence to supplementation was 100% for both treatment periods. All participants reported experiencing beeturia during the supplementation phases, however no other side effects were reported. Four of the 8 participants received NIT as their initial intervention. Participants reported similar daily training loads across the two supplement phases of the study (mean ± SD) for duration (PLA: 1.4 ± 1.1 $h.d^{-1}$; NIT: 1.5 ± 0.9 $h.d^{-1}$) or intensity (222 ± 18 W; 202 ± 14 W). No significance was detected between trials ($P<0.05$) for either condition, for training time (0.34) or intensity (0.14). Measurement error (CV) for time-trial to time-trial was 0.7 and 2.1% for 4 km time and power, respectively and 1.7, 4.1, 3.5, 6.5 and 11.8% for incremental peak power, VO_2peak, economy, VT_1 and VT_2, respectively.

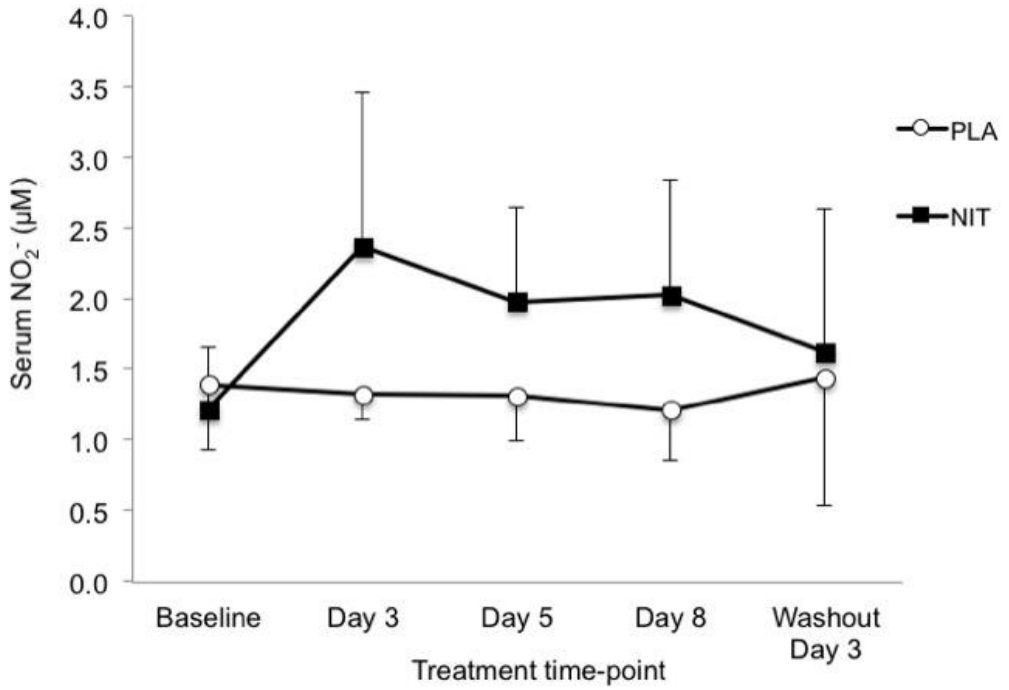


Figure 5: Serum (NO_2^-) levels at baseline, during placebo (PLA; mean - SD), and nitrate (NIT; mean + SD) treatments.

Relative to PLA, there were small to moderate *unclear* increases in mean [NO_2^-] in NIT following NO_3^- supplementation over d 3, 5 and 8 [(factor ×/÷95%CL) 1.19 ×/÷1.37, 1.35 ×/÷1.65 and 1.10 ×/÷1.15, respectively (**Figure 5**)].

Pre- and post-intervention mean group outcomes are summarised in Table 5, whilst between group differences and their subsequent magnitude-based inference (MBI) are reported in Table 6. Relative to PLA, effects of 6 d of NIT supplementation were *unclear* for VO_2peak (mean ±95%CL: 1.8 ±5.5%), and VT_1 (3.7 ±12.3%), whilst VT_2 (-1.0 ±3.0%) was *trivial*. Effects for economy (-1.0 ±1.6%) were *trivial* after 7 d of NIT supplementation. At d 8, an 88% chance of benefit lead to a *likely beneficial* decrease in 4 km time-trial time (-0.7 ±0.9%; Table 6) under NIT supplemented conditions.

Table 5: Pre- and post-measures for performance and physiological measures for placebo (PLA) and nitrate (NIT) treatments. Data are mean ± SD.

	Treatment outcomes			
	PLA Pre	**PLA Post**	**NIT Pre**	**NIT Post**
4-km TT time (s)	344.2 ± 11.3	344.8 ± 14.0	345.4 ± 13.2	343.6 ± 14.3
4-km TT MPO (W)	377 ± 32	375 ± 40	374 ± 40	380 ± 41
VO_2peak ($L.min^{-1}$)	4.73 ± 0.29	4.64 ± 0.34	4.70 ± 0.32	4.70 ± 0.55
Incr. PPO (W)	422 ± 33	429 ± 31	423 ± 31	423 ± 31
Economy ($L.min^{-1}$)	2.73 ± 0.26	2.75 ± 0.26	2.74 ± 0.25	2.73 ± 0.25
VT_1 ($L.min^{-1}$)	3.82 ± 0.32	3.77 ± 0.34	3.60 ± 0.46	3.71 ± 0.46
VT_2 ($L.min^{-1}$)	4.39 ± 0.43	4.41 ± 0.43	4.38 ± 0.43	4.36 ± 0.48
4-km TT HR (bpm)	170 ± 8	171 ± 9	172 ± 6	169 ± 8

TT = time-trial; MPO = mean power output; Incr. PPO = incremental peak power output; VT_1 = first ventilatory threshold; VT_2 = second ventilatory threshold; HR = heart rate

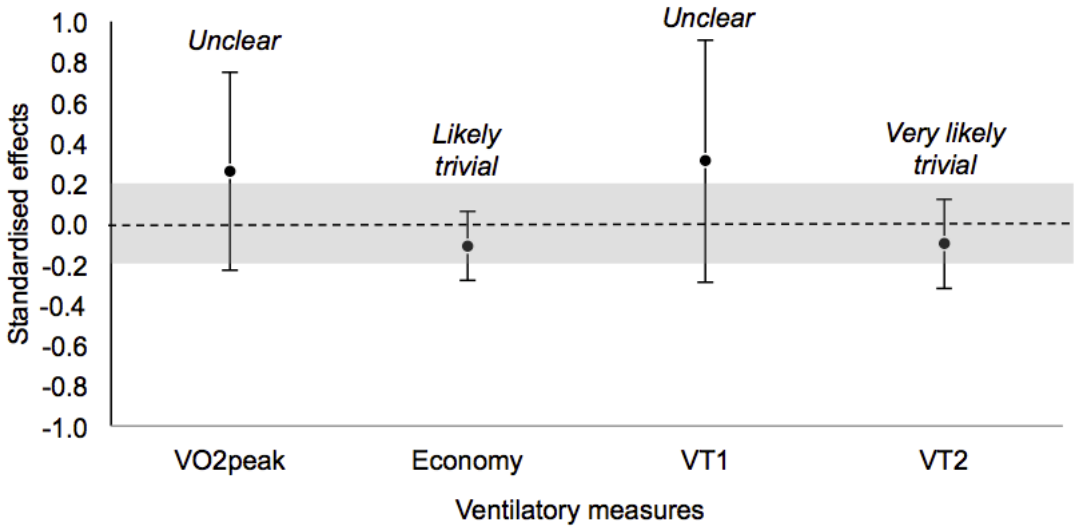


Figure 6: Standardised effects for ventilatory measures. Error bars indicate uncertainty in the true mean with 95% confidence intervals; if error bars overlap both the opposing positive and negative trivial (shaded area representing ± 0.2 SD) value, changes are deemed *unclear*.

Discussion

The current study aimed to determine whether NO_3^- supplementation over a 6 to 8 d period would improve high-intensity cycling performance and economy in well-trained endurance cyclists. Employing identical statistical methods and terminology as previous investigations (Hoon, Hopkins, et al., 2014; Lansley, Winyard, Bailey, et al., 2011) the major outcomes of this study indicated that relative to PLA, consuming NO_3^- had a *likely beneficial* (-0.7 ±0.9%) effect on 4 km time-trial performance and *unclear* (3.7 ±12.3%) and *trivial* (-1.0 ±3.0 and -1.0 ±1.6%) effects for VT_1, VT_2 and economy respectively, in well-trained cyclists.

Whereas previous findings employing a MBI approach indicated a 98.9% or *very likely* chance of benefit for 4 km performance, (Lansley, Winyard, Bailey, et al., 2011) we report a smaller (75%) *likely beneficial* outcome, alongside chances of *triviality* and *harm* of 24% and 1%, respectively (Table 6). The smallest worthwhile enhancement for cyclists competing in road time-trials has been reported to be 0.6% (Paton & Hopkins, 2006), therefore our 0.7% improvement indicates a small but meaningful performance improvement. However, the magnitude of performance enhancement is substantially lower than the 2.7% performance increase reported by Lansley, Winyard, Bailey, et al. (2011) over an identical distance. A potential explanation for such less pronounced effects reported by Lansley, Winyard, Bailey, et al. (2011) may relate to differences in athletic ability (Lansley, Winyard, Bailey, et al., 2011). Under placebo conditions, the 4 km performance time of 405 s reported by Lansley, Winyard, Bailey, et al. (2011) relative to our current investigation of 345 s highlights the difference in athletic calibre of the respective cohorts. The only other cycling related study of comparable duration reported *unclear* and *possibly harmful* outcomes for two x 4 min all-out trials in national level cyclists (Hoon, Hopkins, et al., 2014). Notably, despite our 4 km time-trial equating to a 43% longer time duration than the 4 min trial employed by Hoon, Hopkins, et al. (2014), the MPO for our study is only 6% lower than that of Hoon, Hopkins, et al. (2014). The relative closeness of MPO between the current study and that of Hoon, Hopkins, et al. (2014) suggests that our participants were highly-trained. Collectively, the recent findings of Hoon, Hopkins, et al. (2014) and the current study would indicate a reduced effectiveness of NO_3^- supplementation as cycling ability increases (Jones, 2014). Relative to lesser-trained populations, well-trained athletes appear to have significantly elevated

resting levels of circulating NO_3^- (Porcelli et al., 2015) which may partially explain the attenuated individual effects in highly trained populations (Hoon, Hopkins, et al., 2014). As a result, it may be necessary to elevate resting NO_2^- levels via the provision of larger doses (>4 mmol) of NO_3^-, given that increased availability of NO_3^- will subsequently promote NO_2^- levels and therefore NO availability (Wylie et al., 2016). Indeed several studies have reported improvements in time to exhaustion in untrained (Wylie et al., 2013), and time-trial performance in trained (Hoon, Jones, et al., 2014) populations following doses of ~8 but not 4 mmol NO_3^-. Moreover, the significant (Wylie et al., 2013) and near significant relationship (Hoon, Jones, et al., 2014) between changes in performance and resting basal [NO_2^-/NO_3^-] following NO_3^- supplementation in the aforementioned studies highlights the influence of basal [NO_2^-/NO_3^-] on performance. Whilst further work is required to elucidate the impact of higher doses of NO_3^- on performance in athletes of varying ability the present findings appear to be consistent with published outcomes in athletic outcomes utilising a 4 mmol dose of NO_3^- as they relate to athletic calibre (Hoon, Hopkins, et al., 2014; Lansley, Winyard, Bailey, et al., 2011).

In contrast to gains in performance, we report minimal differences in several physiological and performance measures, many of which have been shown to underpin performance in endurance athletes (Jones & Carter, 2000). Firstly, there were *unclear* (mean ±95%CL: 1.8 ±5.5%) improvements in VO_2peak following NO_3^- supplementation whereas previous studies have reported unaltered (Porcelli et al., 2015) or reduced (Bescos et al., 2011) VO_2peak in endurance trained athletes. Secondly, there was a *trivial* (-1.0 ±3.0%) tendency for VT_2 to decrease, which is comparable to Bescos et al. (2012) who reported no change in VT_2, respiratory exchange ratio (NIT 0.96; PLA 0.96) or blood lactate (NIT 7.5; PLA 7.4 mmol) following sodium NO_3^- supplementation in a trained cycling population (Bescos et al., 2012). Thirdly, similar trends were observed for measures of submaximal exercise economy (*trivial* and *unclear*) following NO_3^- supplementation. Our findings are surprising since 3 d of NO_3^- supplementation has previously been shown to positively alter mitochondrial efficiency (Larsen et al., 2011) by reducing the cost of ATP force production leading to improved economy (Bailey, Winyard, et al., 2009; Cermak, Gibala, et al., 2012; Vanhatalo et al., 2010). In a similarly trained cycling population to that of our current study, Cermak, Gibala, et al. (2012) noted improvements of 3.5% and 5.1% in economy at 45% and 65% of PPO, respectively

following 6 d of NO_3^- supplementation (Cermak, Gibala, et al., 2012). However, it should be noted that Cermak, Gibala, et al. (2012) employed a greater NO_3^- concentration (~8 mmol NO_3^-) compared to the current study. Other studies using identical dosages to that of Cermak, Gibala, et al. (2012) have also demonstrated improvements in economy for population's of low to moderate, but not high fitness levels (Porcelli et al., 2015), again suggesting economy related effects of NO_3^- are constrained to sub-elite athletes. Fourthly, incremental peak power (or speed) is often used as a surrogate marker of endurance performance (Hopkins et al., 1999). A change in PPO would predict that measures of performance requiring substantial aerobic contribution would also change. However, a *possibly harmful* (-1.8 ±2.8%) effect of NO_3^- on PPO opposed the *likely beneficial* effect on 4 km time-trial performance (Table 6). Similarly to our findings, Porcelli et al. (2015) reported a divergence of effects whereby peak run speed reduced, yet 3 km run performance improved in both low- and moderately-trained cohorts following NO_3^- ingestion (Porcelli et al., 2015). Collectively, therefore, the findings of the current and aforementioned studies (Bescos et al., 2012; Porcelli et al., 2015), suggest that despite improved time-trial performance, NO_3^- supplementation has limited effect on several physiological or performance indices synonymous with endurance performance in well-trained athletes (>60 $ml.kg^{-1}.min^{-1}$).

To date the majority of NO_3^- related studies have analysed blood plasma using the chemiluminescence technique (Hoon, Jones, et al., 2014; Wilkerson et al., 2012; Wylie et al., 2013), with significant increases in [NO_2^-] observed in sedentary (Bailey, Winyard, et al., 2009) (96%) and moderately-trained (Lansley, Winyard, Bailey, et al., 2011) (138%) cohorts. However, like others (Porcelli et al., 2015) we employed the Griess technique to measure serum [NO_2^-] and while an initial ~85% increase in basal serum [NO_2^-] was observed on day one of supplementation, we report small-to-moderate *unclear* effects of NO_3^- supplementation on serum [NO_2^-] (**Figure 5**). In support, increases in [NO_2^-] appear to be much lower (Hoon, Hopkins, et al., 2014) in well-trained cohorts potentially due to high resting levels of [NO_3^-] (Porcelli et al., 2015). Furthermore, despite substantial increases in [NO_2^-] in some studies following NO_3^- supplementation, performance effects have been inconsistent (Bailey, Fulford, et al., 2010; Hoon, Hopkins, et al., 2014; Lansley, Winyard, Bailey, et al., 2011) suggesting that factors other than alterations in NO_2^- determine the ergogenic potential of NO_3^- supplementation. Further research is needed to better define the relationship of [NO_2^-

/NO_3^-] response relative to any performance changes in well-trained endurance athletes following NO_3^- supplementation. There appears to be large individual variation on day 3 of the washout phase for both NIT and PLA [NO_2^-]. Under NIT conditions, the influence of additional exogenous NO_3^- may have, in some participants, limited endogenous NO_3^- production, however this would not explain the variation witnessed under PLA conditions. Although speculative, the variation witnessed may have occurred in part due to changes in participants training load and/or diet following the conclusion of that particular phase of the study. Given the heavy testing schedule that bookended each 10 d phase, the limitations of minimal training and somewhat repetitive dietary intake that accompanied it, it is likely that participants took full advantage of the washout period to train and, or eat, without the 'in-study' limitation. Exercise and dietary intake influence basal [NO_2^-], therefore alterations to either of these variables may impact basal [NO_2^-].

Practical Applications

- An 8 d period of beetroot juice (~4.0 mmol NO_3^-) supplementation is a beneficial ergogenic strategy for well-trained male cyclists competing within high-intensity short duration (5 to 6 min) time-trial events
- Athletes wishing to use NO_3^- supplementation should assess its effectiveness using time-trial simulations over the specific distance(s) of interest in order to gauge individual responses

Conclusions

The results of this current study add to the growing body of knowledge regarding the ergogenic effects of nitrate supplementation on high-intensity endurance performance. Relative to placebo, daily supplementation of 70 ml beetroot juice (~4.0 mmol NO_3^-) over an 8 d period had a *likely beneficial* enhancement on 4 km time-trial performance with *non-clear* outcomes for a range of physiological measures in well-trained competitive cyclists. Future studies should explore the ergogenic effects of NO_3^- on shorter duration trials given performance improvements appear not to be reliant on enhancements in economy in well-trained cyclists.

Acknowledgements

We thank the participants for their time, enthusiasm and effort over the course of this research project. We also wish to thank Professor Will Hopkins and Joe Chang for their invaluable guidance with statistical and nitrite analysis, respectively.

CHAPTER 4: DIETARY NITRATE FAILS TO IMPROVE 1 AND 4 KM CYCLING PERFORMANCE IN HIGHLY-TRAINED CYCLISTS

Abstract

We aimed to compare the effects of two different dosing durations of dietary nitrate (NO_3^-) supplementation on 1 and 4 km cycling time-trial performance in highly-trained cyclists. In a double-blind crossover-design, nine highly-trained cyclists ingested 140 ml of NO_3^--rich beetroot juice containing ~8.0 mmol [NO_3^-], or placebo, for seven d. Participants completed a range of laboratory-based trials to quantify physiological and perceptual responses and cycling performance: time-trials on d 3 and 6 (4 km) and on d 4 and 7 (1 km) of the supplementation period. Relative to placebo, effects following 3 and 4 d of NO_3^- supplementation were *unclear* for 4 (-0.8;95%CL, ±2.8%, *P*=0.54) and *likely harmful* for 1 km (-1.9;±2.5%CL, *P*=0.17) time-trial mean power. Effects following 6 and 7 d of NO_3^- supplementation resulted in *unclear* effects for 4 (0.1;±2.2%CL, *P*=0.93) and 1 km (-0.9;±2.6%CL, *P*=0.51) time-trial mean power. Relative to placebo, effects for 40, 50 and 60% peak power output (PPO) were *unclear* for economy at d 3 and 6 of NO_3^- supplementation (P>0.05). Dietary NO_3^- supplementation appears to be detrimental to 1 km time-trial performance in highly-trained cyclists after 4 d. Whilst, extending NO_3^- dosing to ≥6 d reduced the magnitude of harm in both distances, overall performance in short duration cycling time-trials did not improve relative to placebo.

Introduction

In athletic populations, the practice of nutritional supplementation is common place, even if it is largely due to athletes relying on the assumption that a specific supplement has ergogenic properties (Burke, 2009). In sports events which require sustained supramaximal (>VO_2peak) effort with reliance on both anaerobic and aerobic energy systems (Gastin, 2001), such as rowing and a number of track cycling events (Stellingwerff et al., 2011), the use of nutritional supplements may be most successful. For example, supplements such as sodium-bicarbonate and beta-alanine have been shown to buffer extra-cellular and intra-cellular fatigue inducing factors (Stellingwerff et al., 2011), respectively, subsequently resulting in meaningful performance enhancements (Hobson et al., 2013).

Recently, dietary nitrate (NO_3^-) supplementation, either by way of beetroot juice or sodium nitrate ($NaNO_3^-$), has become a popular and well-researched sporting ergogenic aid (Jones, 2014). Indeed, the potential ergogenic effects of NO_3^- in recent years has resulted in more than 50 publications and two meta-analyses (Hoon et al., 2013; McMahon et al., 2016) both of which have reported favourable effects on exercise performance following NO_3^-supplementation, particularly via beetroot juice consumption (McMahon et al., 2016).

The consumption of NO_3^- has been shown to systematically enhance vasodilation, arterial compliance, blood flow dynamics (Ferguson et al., 2013; Ferguson, Holdsworth, et al., 2014) and rate of force development (Hernandez et al., 2012) in non-oxidative, type II muscle fibres. These mechanistic alterations would suggest potential benefit for high power output sporting events that are heavily reliant on glycolytic, type II muscle fibres (Stellingwerff et al., 2011). Furthermore, exercise at such high intensity induces an acidic and hypoxic environment (Cairns, 2013) which ameliorates the effects of exogenous NO_3^- , thereby facilitating the reduction of nitrite (NO_2^-) to nitric oxide (NO) (Modin et al., 2001).

In an attempt to deduce the most effective NO_3^- supplementation strategy, recent investigations have assessed the influence of dose duration on events of ~4 (Boorsma et al., 2014) and ~16 (Nyakayiru et al., 2016) min duration in trained athletes. In these studies, neither acute (Boorsma et al., 2014; Nyakayiru et al., 2016), 6 (Nyakayiru et al.,

2016), or 8 (Boorsma et al., 2014) d of NO_3^- supplementation improved performance. Notably, the studies reporting positive outcomes as a result of NO_3^- supplementation assessed performance over ~90 s durations (Bond et al., 2012; Peeling et al., 2015), suggesting that in highly-trained athletes, events of ~90 s duration may be more sensitive to enhancement than those of 4 to 16 min. These reported improvements may be due, in part, to the greater reliance on type II muscle, which are sensitive to the positive mechanistic actions of NO_3^- (Ferguson, Holdsworth, et al., 2014; Hernandez et al., 2012). To our knowledge, no study to date has reported the effects of NO_3^- supplementation in relation to both intensity of the performance trial and dose duration in highly-trained athletes. Therefore, the aim of this study was to compare the effects of two different dosing durations of dietary NO_3^- supplementation on 1 and 4 km cycling time-trial performance in highly-trained cyclists.

Methods

Participants

Nine male endurance trained cyclists (mean ± SD; age: 27 ± 9 y; body mass: 74.0 ± 6.8 kg; VO_2peak: 68 ± 3 ml.kg^{-1}.min^{-1}) participated in this study, which was approved by the ethics committee at AUT University (10/309). All athletes provided informed consent, were injury free and had been regularly training (11.4 ± 2.6 h.wk^{-1} and 310 ± 27 km.wk^{-1}) and competing in fortnightly cycle races in the 3 mo preceding the study. Participants were informed that the aim of study was to investigate the effects of beetroot supplementation on cycling physiology and performance.

Experimental Design

A double-blind, crossover-design study was adopted during which participants were randomly assigned to a nitrate (NIT) or placebo (PLA) group and acted as their own controls. Participants visited the laboratory on 8 separate occasions, at the same time of d (±2 h) to perform several 4 km (d 3; NIT3, PLA3 and 6; NIT6, PLA6), and 1 km (d 4; NIT4, PLA4 and 7; NIT7, PLA7) time-trials and physiological and perceptual assessments. Each seven d treatment period was separated by a 10 d washout. Participants were required to abstain from caffeine and alcohol in the 48 and 24 h prior to testing, respectively, and to perform only moderate intensity exercise the day prior to any assessments.

All physiological assessments and performance trials took place in a well-ventilated, temperature-controlled, sea-level altitude laboratory (18 to 20°C; 55 to 70% RH) on a calibrated, reliable and accurate electromagnetically-braked cycle ergometer (Velotron, Racermate, Seattle, USA) (Stone et al., 2011). The ergometer was fitted with the participant's own pedals and adjusted to replicate the participants own bicycle for all assessments.

Incremental Test

Participants first performed an incremental cycle test consisting of 3min at 50 W followed by increases of 25 $W \cdot min^{-1}$ until volitional exhaustion. During the test, pulmonary gas exchange was measured breath-by-breath using a calibrated metabolic cart (Oxycon Pro; Jaeger; Hoechberg; Germany). The VO_2 data was averaged on a 30 s basis and VO_2peak was determined as the highest 30 s mean VO_2 prior to termination of the test. Participants were excluded if VO_2peak was <65 $ml.kg^{-1}.min^{-1}$. PPO was defined as the highest power output obtained during the test and was used to determine individualised power output for subsequent submaximal economy measures.

Blood collection

Participants were asked to sit motionless in a reclined position for 15min at the start of each visit to the laboratory. Thereafter, a venous blood sample from an antecubital vein was collected via an evacuated SST monovette tube (Becton Dickinson Biosciences). The 10 ml of extracted blood was then left to clot for 20 min before being centrifuged at 1218 x g for 10min at 4°C **(**Heraeus Megafuge 16R, Thermo Scientific) as per the manufacturer's instructions. Serum was then aliquoted into micro containers and stored at -80°C for later analysis.

Economy

On d 3 (PLA3, NIT3) and 6 (PLA6, NIT6) participants completed 3 x 10 min constant-load bouts of cycling at 40, 50 and 60% of PPO at 95 $rev \cdot min^{-1}$ to determine economy. Pulmonary gas exchange was recorded throughout each 10 min bout with the mean VO_2 during the final 5 min of each bout used for subsequent analysis. Economy was expressed

as the P:O ratio, i.e. the ratio between power output and absolute VO_2 ($W.L^{-1}.min^{-1}$) for each workload.

Performance Trials

Following a 30 min incremental warm-up and 3 min passive recovery, participants undertook either a 1 km (d 4; NIT4, PLA4 and 7; NIT7, PLA7) or 4 km (d 3; NIT3, PLA3 and 6; NIT6, PLA6) time-trial on the ergometer. Participants were able to self-select gearing and cadence to best reflect actual individual competition performance. During each trial, the lead researcher provided consistent encouragement to participants while feedback - including power output, gear selection and distance completed - was provided visually using the Velotron software. Completion time (s) and mean power output (MPO) (W) were recorded during each time-trial. Two familiarisation time-trials preceded the experimental trials.

Perceptual Responses

Perceptual ratings of gastric discomfort were obtained during each trial at the conclusion of each 30 min economy measure. Participants were asked to verbally rate on a scale of 1 (significant gut discomfort) to 5 (no gut discomfort) how they felt using language terms they were familiar with.

Serum [NO_2^-]

NO_2^- analysis was carried out using the Griess Method (Chae et al., 2003) with a commercially available kit (Promega, Wisconsin, USA). Samples were deproteinized by adding 400 µL trichloroacetic acid (TCA) to 400 µL serum, vortexed and centrifuged at 14,500 x rpm for 5 min **(**Espresso, Thermo Scientific) (Ghasemi et al 2007). Thereafter, 150 µL of supernatant was added to 130 µL of deionized water followed by 20 µL of Griess reagent, prior to an incubation period of 30 min, as per manufacturers instructions. Absorbance was measured at 548 nm. The resulting [NO_2^-] was reported in µM, consistent with previous research (Tsikas, 2007).

Beetroot Juice Supplementation

We adopted the supplement brand and loading volume employed in previous research (Cermak, Gibala, et al., 2012) in which daily ingestion of either 140 ml of nitrate rich (~8.0 mmol NO_3^-) beetroot juice (James White Drinks, Ipswich, UK) or an identical

looking and tasting non-nitrate placebo juice (~0.003 mmol NO_3^-) took place over a 7 d period. Five of the nine participants supplemented with NIT as their first treatment. On d 3, 4, 6 and 7 participants were instructed to consume the 140 ml dose 2.5 h prior to the start of that day's scheduled time-trial, whereas on non-trial days they consumed at a time of their own choosing. During data collection, all researchers were blinded as to which conditions were NIT or PLA. A 10 d washout period separated the cross-over trial supplement conditions. During supplementation, particularly over the time of assessment procedures, participants were instructed to avoid spitting, chewing gum or using antibacterial mouthwash, as these actions are associated with a lowering of [NO_2^-] (Govoni et al., 2008; Webb et al., 2008). Participants were asked to record their dietary intake in the 24 h prior to the first time-trial and repeat this for all subsequent visits.

Statistical Analysis

Data are presented as mean ± SD unless otherwise reported. Comparisons were made between each of the four NIT and PLA time-points for 4 and 1 km time-trial performance, economy and perceptual outcomes using a customised analysis spread-sheet (Hopkins, 2006). Performance data were log-transformed for analysis to reduce bias arising from non-uniformity of error, and subsequently back-transformed to obtain changes in means and variations as percentages. To make inferences about true (population) values of the effect of NO_3^- supplementation on cycling performance, the uncertainty in the effect size (ES) was expressed as 95% confidence limits (CL) and as likelihoods that the true value of the effect was *beneficial*, *trivial* or *harmful* in relation to the threshold value for benefit and harm of ±1% (Paton & Hopkins, 2006). An effect was deemed *unclear* when the chance of benefit was sufficiently high to warrant use but the risk of harm was unacceptable. Such *unclear* effects were identified as those with an odds ratio of benefit-harm of >57, a ratio that corresponds to an effect that is borderline *possibly beneficial* (12.5% chance of benefit) and borderline most *unlikely harmful* (0.25% harm) (Hopkins, 2004). All other effects were deemed clinically *clear* and expressed as the chance of the true effect being *trivial*, *beneficial* or *harmful* with the following default values and qualitative terms were set at: <0.5%, *most unlikely*; 0.5-5%, *very unlikely*; 5-25%, *unlikely*; 25-75%, *possibly*; 75-95%, *likely*; 95-99.5%, *very likely*; >99.5%, *most likely*. Non-performance (mechanistic) measures were calculated as 0.2 of the between-subject SD. If the confidence interval overlapped the thresholds for substantial positive and negative values (±0.2 of the between-subject SD in the PLA condition), the effect was

declared *trivial* and reported as *unclear;* all other effects were deemed *clear* and were evaluated probabilistically as described previously and expressed qualitatively as follows; <0.2 *trivial,* >0.2 *small,* >0.6 *moderate,* >1.2 *large* and >2.0 *very large.* Measurement reliability was assessed by determining the percent TE and expressed as coefficient of variation (CV) (Hopkins, 2003). Serum [NO_2^-] is shown as factor ×/÷confidence limits. Performance and physiological data were also analysed using a two-tailed paired *t*-test with statistical significance set at $P<0.05$.

Results

Participants' self-reported adherence to supplementation was 100% for both treatment periods, with 5 of the 9 participants receiving NIT as their initial intervention. All participants experienced beeturia during the supplementation phases, however no other side effects were reported. CV for time-trial time and mean power was 1.1% and 2.5% (1 km) and 1.2% and 3.1% (4 km), respectively, and 2.7% for economy.

Serum [NO_2^-]

Relative to pre-supplementation, there was a tendency for mean [NO_2^-] to increase following NO_3^- supplementation (factor ×/÷confidence limits: 1.36 ×/÷3.04, 1.60 ×/÷2.15, 1.64 ×/÷2.40 and 1.51 ×/÷1.98 greater for NIT3, NIT4, NIT6 and NIT7, respectively, **Figure 5**), though differences in [NO_2^-] between NIT and PLA were statistically *unclear* (P>0.05).

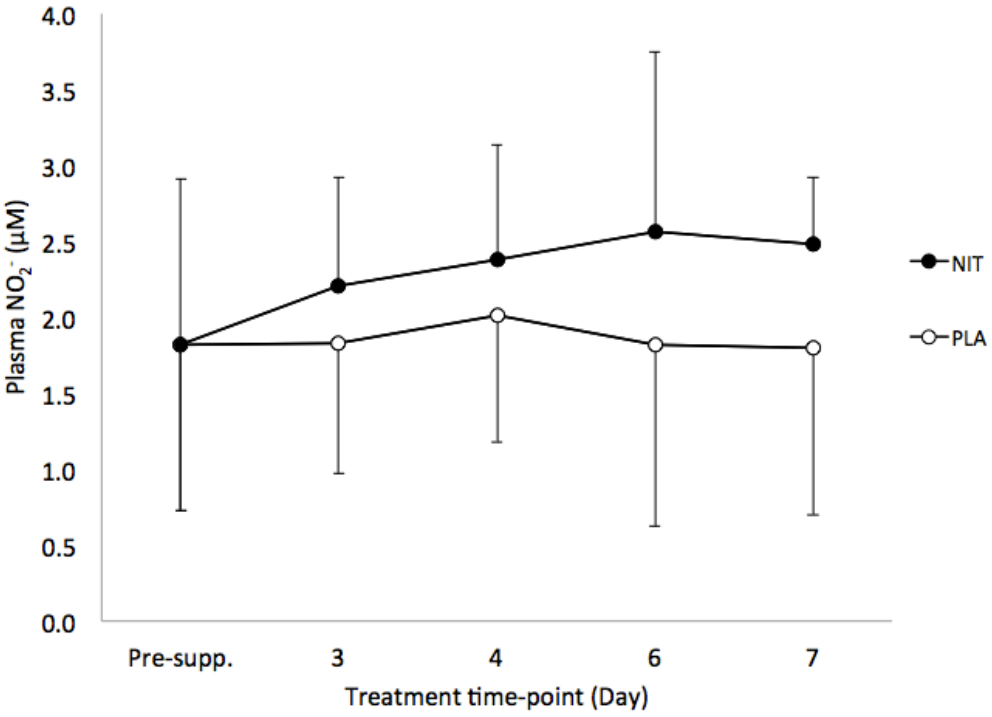


Figure 7: Mean (± SD) serum [NO_2^-] for each supplementation condition over the dosing duration.

Economy

Relative to PLA, effects for NIT conditions were either *unclear* or *trivial* for all measures of economy (P>0.05) following NO_3^- supplementation (**Figures 8 and 9**).

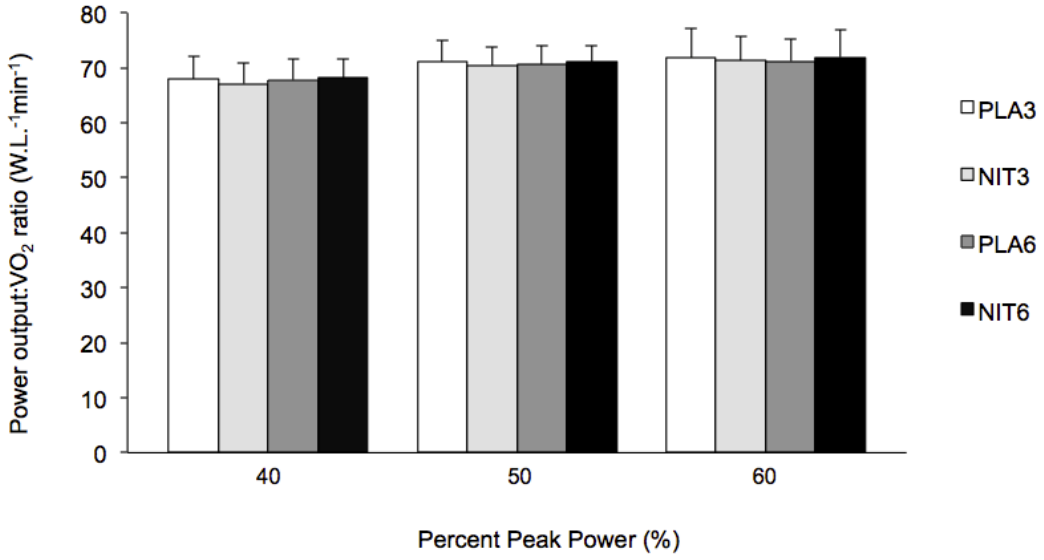


Figure 8: Mean economy (± SD) at 40, 50 and 60% of incremental peak power output following nitrate supplementation for 3 and 6 d (NIT3 and NIT6, respectively), and placebo (PLA3 and PLA6, respectively) treatments.

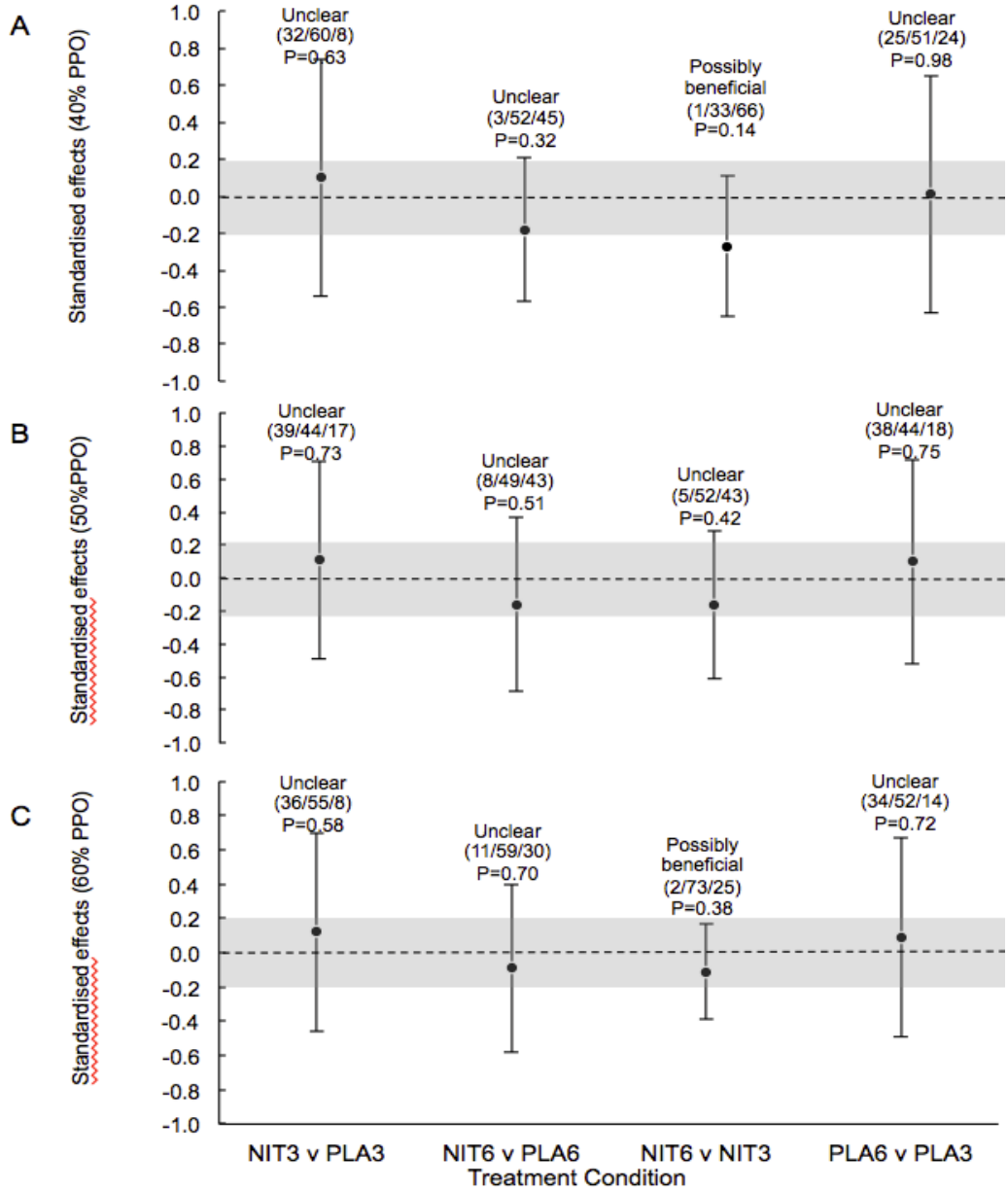


Figure 9: Standardised effects ± 95% confidence limit, qualitative inference and chances (beneficial/trivial/harmful) and significance (P<0.05) for intra- and inter-treatment comparisons for economy measures for nitrate (NIT); d 3 and 6 (NIT3 and NIT6), and placebo (PLA); d 4 and 7 (PLA4 and PLA7) treatments at [A] 40, [B] 50 and [C] 60% of incremental peak power output (PPO). Error bars indicate uncertainty in the true mean with 95% confidence intervals; if error bars overlap the opposing positive or negative *trivial* (shaded area representing ± 0.2 SD) value, changes are deemed *unclear*.

Performance Outcomes

Group mean performance data are presented in Table 7, with group and individual responses for 1 and 4 km time-trials shown in **Figure 10**. Following 3 and 4 d of NO_3^- supplementation, effects were *unclear* for 4 km (-0.8;95%CL, ±2.8%, *P*=0.54) and *likely*

harmful for 1 km (-1.9;±2.5%CL, *P*=0.17) MPO, relative to PLA. Continued supplementation at 6 d resulted in *unclear* effects for 4 (0.1;±2.2%CL, *P*=0.93) km MPO. Despite a *possibly beneficial* (1.2;±1.8%CL, *P*=0.14) effect of extending NO_3^- supplementation from 4 to 7 d, outcomes for 1 km MPO on d 7 were *unclear* (-0.9;±2.6%CL, *P*=0.51), relative to placebo at the same time-point (**Figure 11**). Remaining outcomes were *unclear* and not significantly different (*P*>0.05). We observed no relationship between changes in performance and serum [NO_2^-] for either 1 km (r=0.40, *unclear*) or 4 km (r=0.44, *unclear*) time-trial performance despite the tendency for [NO_2^-] to be increased (albeit *unclear*) following NO_3^- supplementation (**Figure 7**).

Table 6: Performance data for 1 and 4 km time-trials following various nitrate (NIT) or placebo (PLA) supplementation durations. Data are mean ± SD.

Measure	Condition			
	PLA4	NIT4	PLA7	NIT7
1 km TT time (s)	79.2 ± 2.9	79.6 ± 3.5	79.0 ± 3.0	79.3 ± 3.3
1 km TT MPO (W)	503 ± 51	495 ± 61	505 ± 52	501 ± 59
	PLA3	NIT3	PLA6	NIT6
4 km TT time (s)	340 ± 10	341 ± 12	340 ± 11	340 ± 10
4 km TT MPO (W)	393 ± 37	390 ± 45	393 ± 37	394 ± 38

NIT4, NIT7 = day 4 or 7 of nitrate supplementation, MPO = mean power output; PLA4, PLA7 = day 4 or 7 of placebo; TT = time-trial.

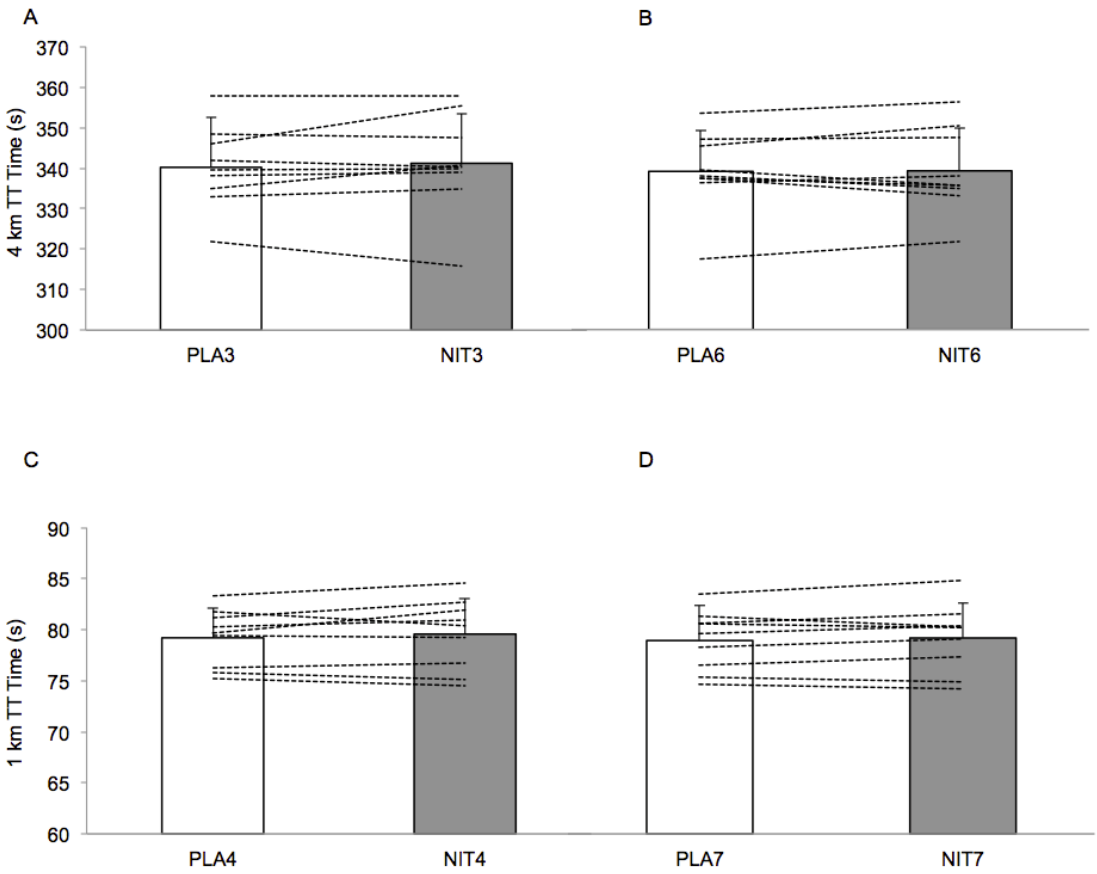


Figure 10: Mean 1 and 4 km time-trial performance time (± SD) and individual responses (dashed lines) for 4 km time-trial performance for nitrate (NIT) and placebo (PLA) conditions. Figures A and B represent effects for 4 km time-trial time for 3 d of nitrate (NIT3) and placebo (PLA3), and 6 d of nitrate (NIT6) and placebo (PLA6) respectively. Figures C and D represent effects for 1 km time-trial time for 4 d of nitrate (NIT4) and placebo (PLA4), and 7 d of nitrate (NIT7) and placebo (PLA7) respectively.

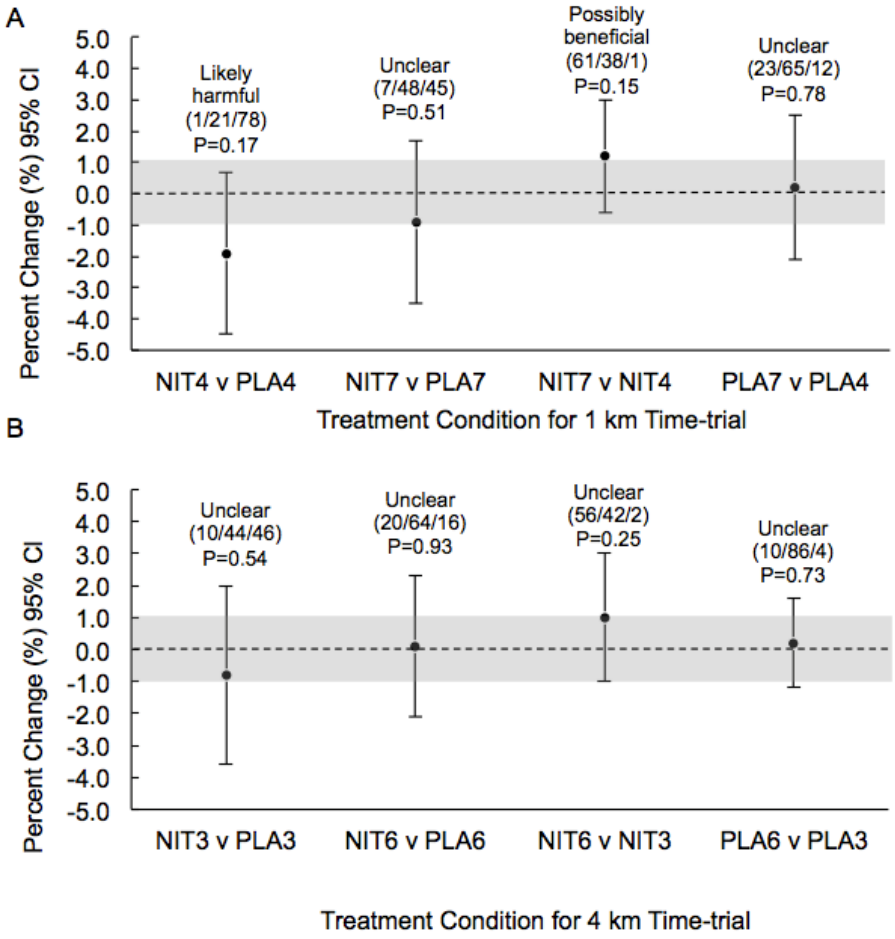


Figure 11: Percent outcome ±95% CL, qualitative inference and chances (beneficial/trivial/harmful) and significance (P<0.05) of intra- and inter-treatment comparisons for 1 km and 4 km TT performance. Where 4 km time-trial performance represented at d 3 (NIT3 and PLA3) and 6 (NIT6 and PLA6) [Figure A]. Likewise, 1 km time-trial performance comparison is represented by nitrate (NIT) or placebo (PLA) at d 4 (NIT4 and PLA4) and 7 (NIT7 and PLA7) [Figure B]. Error bars indicate uncertainty in the true mean with 95% confidence intervals; if error bars overlap the opposing positive or negative smallest worthwhile effect (shaded area representing ±1%), changes are deemed *unclear*. NIT3 and NIT6 = days 3 and 6 of nitrate; PLA3 and PLA6 = days 3 and 6 of placebo.

Perceptual Measures

Gut comfort perception prior to all time-trials was *unclear* for any dose duration.

Discussion

To our knowledge, this is the first study to investigate the effects of dose duration of dietary NO_3^- supplementation on cycling time-trial performance across two different distances in highly-trained endurance cyclists. Utilising a magnitude-based inferences

(MBI) approach the main finding of this study was that short duration (≤4 d) NO_3^- supplementation impaired 1 km time-trial performance but not 4 km time-trial performance. Longer-term NO_3^- supplementation (≥6 d) resulted in *unclear* effects for 1 and 4 km distances.

While the mechanisms underpinning endurance performance enhancement resulting from NO_3^- supplementation are well-documented (Jones, 2014), it is difficult to reconcile mechanisms for performance impairment based on current understanding of the role of NO_3^- in humans. The 1 km time-trial in the present study likely required high utilisation of glycolytic pathways resulting in substantial acidosis which theoretically should provide favourable conditions for NO_3^- to exert its physiological effects given the reduction of NO_2^- to NO is enhanced within lowered PO_2 and pH environments (Lundberg & Weitzberg, 2009). Furthermore, in rats, it has been shown that NO_3^- supplementation positively influences type II muscle fibres (IIB and d/x) (Ferguson, Hirai, et al., 2014; Ferguson, Holdsworth, et al., 2014) potentially providing a mechanistic basis for further potential for enhancements in humans (Jones et al., 2016). However, lack of performance enhancement in the present study could have been due to participants not being short-duration specialists, such as the 500 m specialists employed by Peeling et al. (2015), and did not possess a well-developed level of event-specific fitness. Indeed, endurance training appears to reduce the volume of type II fibres (Coyle et al., 1992), speeds oxygen uptake kinetic responses, enhances economy and improves hypoperfusion (Jones & Carter, 2000), physiological adaptations which may negate the effects of NO_3^- supplementation in endurance trained populations (Jones et al., 2016). Conversely, kayak training has been shown to increase type II fibres in upper body musculature (Baker & Hardy, 1989), which might explain the tendency for outcomes to be positive in studies utilising upper-body sports in the high-intensity domain (Bond et al., 2012; Peeling et al., 2015). Unfortunately, as with other studies involving highly-trained athletes (Hoon, Hopkins, et al., 2014; Peeling et al., 2015), we were not able to take muscle biopsies from our participants therefore are unable to gain insight of potential fibre-specific effects of NO_3^- and whether variations in fibre distribution between athletes contributed to physiological and performance outcomes in our cohort of highly-trained cyclists.

Previous work has shown positive adaptations to mitochondria architecture (Larsen et al., 2011) following 3 d and augmentation of muscle contractile proteins (Hernandez et al., 2012) following 7 d of NO_3^- supplementation. While extending the dosing period to 7 d in the present study substantially reduced the chance of harm (78% to 45%), the small improvement in 1 km performance after 7 d, relative to 4 d, alongside the *unlikely* chance of benefit (7%) is not large enough to warrant use of NO_3^- supplementation in highly-trained endurance athletes in events of ~80 s (**Figure 11**).

With respect to the longer 4 km time-trial, however, we observed *unclear* effects on 4 km time-trial MPO following 3 and 6 d of NO_3^- supplementation. These findings are consistent with previous cycling (Hoon, Hopkins, et al., 2014) and running (Boorsma et al., 2014) studies of similar duration and athlete calibre, but in contrast to those of (Lansley, Winyard, Bailey, et al., 2011) whose lesser-trained cyclists (56 $ml.kg^{-1}.min^{-1}$) showed a *very likely* 2.8% improvement in 4 km time (~6.30 min at 292 W) following acute NO_3^- supplementation. Athletic calibre (refer Tables 2, 3, 4 and 7) has been proposed as a factor influencing the efficacy of NO_3^- to enhance performance (Porcelli et al., 2015).

A commonly reported and relevant physiological enhancement following dietary beetroot juice consumption is an improvement in economy (Lansley, Winyard, Bailey, et al., 2011; Wylie et al., 2016). The mechanisms postulated for these enhancements following beetroot juice consumption are improvements in muscular contraction and mitochondrial oxidative phosphorylation (Bailey, Vanhatalo, Winyard, & Jones, 2012; Lundberg et al., 2008) with both acute and chronic NO_3^- loading strategies enhancing economy (Wylie et al., 2016). However, in the present study, despite employing near identical NO_3^- loading strategies and submaximal intensities to previous studies (Cermak, Gibala, et al., 2012) economy at 40, 50 and 60% PPO was predominantly unchanged following 6 d of dietary NO_3^- supplementation (**Figures 8 and 9**). Relative to sedentary populations, endurance trained athletes possess greater resting levels of NOS and more specifically neuronal NOS (nNOS) than those of sedentary populations (McConnell et al., 2007). In highly-trained endurance athletes such elevated levels are postulated to blunt the effectiveness of NO_3^- supplementation regardless of the increase in basal levels of NOS (Porcelli et al., 2015) which may explain our findings.

Finally, it is acknowledged there are some potential limitations to our study, and further research is proposed to address these shortcomings. Firstly, while our sample size was consistent with previous studies reporting performance enhancement (Bond et al., 2012; Lansley, Winyard, Bailey, et al., 2011) following NO_3^- supplementation, a larger sample size would reduce the possibility of type II error. Secondly, in this predominantly applied study we adopted a limited range of mechanistic measures. Specifically, in light of the present and previous findings reporting performance impairment (Hoon, Hopkins, et al., 2014) further research is required to elucidate the mechanism/s leading to harmful outcomes in performance in well-trained endurance athletes following NO_3^- consumption. In this regard it would be pertinent to assess the effects of NO_3^- supplementation on a variety of upper-, lower- and whole-body sporting events across a range of durations and intensities.

Conclusion

In highly-trained cyclists a short (3 to 4 d) dosing period negated 1 km performance whilst 4 km time-trial performance was unchanged. Extending the dosing period (6 to 7 d) improved 1 km performance, however, overall neither 1, nor 4 km distances were enhanced following the longer supplementation period. Therefore, NO_3^- supplementation of 3 to 7 d is not recommended for highly-trained endurance athlete populations seeking to enhance performance in short-duration, supramaximal cycle exercise.

Acknowledgements

We thank our participants for their time, enthusiasm and effort over the duration of this research project. We also wish to thank Professor Will Hopkins and Joe Chang for their invaluable guidance with statistical and nitrite analysis, respectively. This project was supported with a student stipend for J.A.M from High Performance Sport NZ.

Declaration of Funding Sources

HPSNZ Student Scholarship (J.A.M).

CHAPTER 5. THE EFFECT OF NITRATE SUPPLEMENTATION ON CYCLING PERFORMANCE IN THE HEAT IN WELL-TRAINED CYCLISTS

Abstract

Purpose: The aims of this study were to determine the effect of NO_3^- consumption on measures of perception, thermoregulation and cycling performance in hot conditions. **Methods:** Using a randomised, double-blind, crossover-design, 8 well-trained cyclists (mean ± SD: age: 25 ± 8 y, VO_2peak: 64 ± 5 $ml \cdot kg^{-1} \cdot min^{-1}$) performed 2 separate trials, in hot (35°C; 60% RH) environments, having ingested either 140 ml NO_3^--rich beetroot juice ~8 mmol NO_3^- (NIT), or placebo (PLA), daily for 3 d with a 7 d washout period separating trials. Trials consisted of 2 x 10 min bouts at 40 and 60% peak power output (PPO) to determine physiological and perceptual responses in the heat, followed by a 4 km cycling time-trial. **Results:** Basal [nitrite] was substantially elevated in NIT (2.70 ± 0.98 µM) vs PLA (1.10 ± 0.61µM) resulting in a *most likely* (ES = 1.58 ± 0.93) increase after 3 d. There was a *very likely trivial* increase in rectal temperature [T_{re}] in NIT at 40% (PLA;37.4 ± 0.2ºC vs NIT;37.5 ± 0.3ºC, 0.1 ± 0.2°C) and 60% (PLA;37.8 ± 0.2ºC vs NIT;37.9 ± 0.3ºC, 0.1 ± 0.2°C) PPO. Cycling performance was similar between trials (PLA;336 ± 45 W vs NIT;337 ± 50 W, CV±95%CL; 0.2 ± 2.5%). Outcomes for heart rate, and perceptual measures were *unclear* across the majority of time-points. **Conclusions:** Three d of NO_3^- supplementation, resulted in small increases in T_{re} during low- to moderate-intensity exercise, however this did not appear to influence 4 km cycling time-trial performance in hot climates.

Key Words: Nutrition, time-trial, heat-stress, competitive, beetroot juice

Introduction

Athletes commonly use dietary supplements in an effort to enhance athletic performance. Recently, consumption of NO_3^- by way of either nitrate salt ($NaNO_3^-$) or beetroot juice has been found to improve both economy and performance (Cermak, Gibala, et al., 2012) potentially due to improved efficiency of mitochondrial coupling (Bailey, Winyard, et al., 2009) and enhanced phosphorylation ratio (Larsen et al., 2011). Furthermore, NO_3^- has conferred muscular related enhancements in calcium handling (Hernandez et al., 2012), blood flow (Ferguson et al., 2013), and microvascular pressure (Ferguson, Holdsworth, et al., 2014), in type II, but not type I skeletal muscle (Jones et al., 2016). However, while performance enhancement has been reported across a range of sports/events and durations/intensity, studies to date have predominantly been conducted in temperate (18 to 21°C; 20 to 50% RH conditions (Cermak, Gibala, et al., 2012; Lansley, Winyard, Bailey, et al., 2011; Porcelli et al., 2015), with little consideration of how additional heat stress and NO_3^- might interact and influence physiological responses and performance in athletic populations.

Recently, the effects of NO_3^- supplementation in hot environments using either beetroot juice (Kuennen et al., 2015) or L-arginine (Tyler et al., 2016) has been assessed in healthy, non-athletic populations. Specifically, while consumption of beetroot juice over a 6 d period reduced oxygen consumption, an increase in core body temperature in the final third of a 45 min march in a hot (41ºC; 20% RH) environment was observed (Kuennen et al., 2015). The authors projected that NO_3^- would cause participants to reach critical core body temperature (40ºC) earlier, and subsequently reduce distance marched by 10% due to hyperthermic stress (Kuennen et al., 2015). Conversely, Tyler et al. (2016) reported that an acute dose of L-arginine, which is the precursor of nitric oxide synthase derived production of nitric oxide (NO), had no effect on oxygen consumption or rectal temperature (T_{re}) during moderate intensity exercise in 35ºC; 40% RH environments. Given athletes are often exposed to hot environments during competition, it would be of interest to assess the effects of NO_3^- consumption on exercise performance in hot conditions. In regards to dosing duration, although we are yet to demonstrate the effectiveness of chronic (>3 d) dosing strategy on cycling performance in well-trained cyclists, the only investigation to show positive response as a result of NO_3^- employed a chronic dosing period. Previous research suggests that at least 3 d of NO_3^- supplementation is required for mitochondrial changes to take place (Larsen et al., 2011),

yet a number of authors have reported improvements in high-intensity endurance performances following acute doses of NO_3^- (Bescos et al., 2011; Peeling et al., 2015). Enhanced physiological and/or improved performances in light of dosing durations <3 d suggests that mitochondrial enhancements are not the sole basis by which NO_3^- imparts physiological alterations. Despite this, we do not wish to discount the potential for mitochondrial alterations to occur by using a shorter dosing strategy to assess the effects of NO_3^- supplementation on performance in hot conditions. Therefore, the aims of this study involving well-trained cyclists were to determine 1) the effects of 3 d of NO_3^- supplementation on thermoregulatory and perceptual responses during low-, moderate- and, maximal-intensity exercise in the heat and 2) the effects of NO_3^- on maximal-intensity exercise performance in the heat.

Methods

Participants

Eight well-trained, competitive male endurance cyclists (mean ± SD: age: 25 ± 8y, body mass: 74.9 ± 7.3 kg, height: 180 ± 6 cm, VO_2peak: 64 ± 5 $ml \cdot kg^{-1} \cdot min^{-1}$) provided written consent to participate in this study which had received ethical approval from the Auckland University of Technology Ethics Committee (10/309). All participants were free of injury and illness for at least six weeks prior to study commencement, and were carrying out regular weekly training sessions amounting to ≥200 $km \cdot wk^{-1}$. Participants were asked to maintain their usual training and racing schedule, but on the day prior to any laboratory assessments, complete no more than a 2 h low intensity session. Verbal confirmation of this fact was given to the lead investigator the day of the testing and participants received a schedule of dates and times which included instructions for management of training load in the 24 h prior to assessments. Data collection took place during the competitive phase (November through to January) of each athlete's periodised season, coinciding with the final month of spring and first two months of summer. Participants were told that the aim of study was to investigate the effects of beetroot supplementation on cycling physiology and performance. Additionally participants were fully informed about the possible risks of all experimental procedures prior to informed consent being obtained. Participants were asked to maintain their habitual diet, and record and repeat their dietary intake in the 24 h leading into the initial experimental trial, with the exception of abstinence from caffeine and alcohol in the 24 and 48 h prior to testing, respectively. All

experimental trials took place in a sea-level altitude laboratory and occurred at the same time of day (± 2 h) to control for biological variation.

Preliminary Testing – Procedures and Assessments

To ensure the minimum peak oxygen uptake (VO_2peak >55 $ml \cdot kg^{-1} \cdot min^{-1}$) for study inclusion was attained, prospective participants were required to undertake an incremental ramp assessment on an electromagnetically-braked cycle ergometer (Velotron, Racermate, Seattle, USA), fitted with the participant's own pedals, 10 to 14 d before the main trials began. Measurements of seat and handle bar position were recorded from the participant's own bike and replicated on the ergometer prior to all subsequent assessments. The protocol consisted of 3 min at 50 W, followed by a 20 $W \cdot min^{-1}$ increase until volitional exhaustion or when participants' cadence fell below 70 $rev \cdot min^{-1}$. Gas-exchange and ventilatory measures were assessed using a mixing chamber metabolic system (TrueOne 2400, Parvo Medics, Sandy, UT). Calibration of the gas and turbine sensors took place prior to each test using alpha standard gases (BOC Gases, Auckland, NZ) and known volumes (3L syringe, Hans Ruldolph, Shawnee, USA) respectively. Peak oxygen uptake was defined as the highest 30 s mean VO_2 value achieved during the test. Incremental peak power output (PPO) was also determined.

Over the following 7 d participants returned to the lab for familiarisation purposes to complete two 4 km laboratory-based time-trials in a temperate (20°C; 60% RH) environment, located at sea level. Participants were instructed to complete the 4 km distance in the shortest time possible. The time-trial mode used by the Velotron cycle ergometer allowed for the use of self-selected gearing and cadence to best reflect actual individual competition performance. Duration, mean power output (MPO), and average heart rate were recorded during each time-trial in the familiarisation phases.

Experimental Protocol

Participants were randomly assigned to one of two 3 d experimental nitrate (NIT) or placebo (PLA) groups and acted as their own controls in this double-blind, crossover-design study. After each 3 d trial, participants completed a performance trial in hot conditions, preceded by two sub-maximal exercise bouts. The two 3 d experimental periods were separated by a 7 d washout period. On d 3 of each treatment, participants completed a performance trial in hot conditions, preceded by two x 10 min sub-maximal

exercise bouts. Participants were supplied with 3 x 140 ml doses of either NO_3^--rich beetroot juice [~8.0 mmol] or a NO_3^- free placebo of identical taste, smell and appearance, as previously used (Cermak, Gibala, et al., 2012). The same manufacturer (James White Drinks, Ipswich, UK) produces an identical looking and tasting NO_3^- depleted [~0.003 mmol] placebo juice, and we adopted the supplement and administration procedures of a recent study (Cermak, Gibala, et al., 2012) using organic beetroot juice. Over the course of the study, participants were administered the juice at a rate of 2 x 70 ml daily and asked to consume the juice 90 min prior to their scheduled arrival at the laboratory for trials, whereas on non-trial days, participants were asked to consume it as close to the time on trial days. The tester administering the beverage was unaware of whether the drink was NO_3^--rich or placebo beetroot juice. Participants were instructed to avoid spitting, chewing gum or using antibacterial mouthwash during the supplementation interventions, as these actions are associated with a lowering of plasma [NO_2^-] (Govoni et al., 2008).

Blood collection

On arrival at the laboratory, participants were asked to sit motionless in a reclined position for 15 min. Thereafter, a venous blood sample from an antecubital vein was collected via an evacuated SST monovette tube (Becton Dickinson Biosciences). The 10 ml of extracted blood was then left to clot for 20 min before being centrifuged at 1218 x g for 10 min at 4°C **(**Heraeus Megafuge 16R, Thermo Scientific) as per the manufacturer's instructions. Serum was then aliquoted into micro containers and stored at -80°C for later analysis.

Experimental Exercise Trials, Blood Pressure and Plasma NO_2^-

To begin all trials, and following blood collection procedures and voiding of bladder, participants nude body mass was measured (Seca Ltd, Germany) followed by self-insertion of a disposable rectal thermistor (Monatherm Thermistor, 400 Series, Mallinckrodt Medical, St. Louis, MO) approximately 12cm past the anal sphincter. Rectal temperature, measured to 0.01ºC, was continuously recorded with a data logger (Squirrel SQ2020, Cambridge, UK) connected to the rectal probe. Afterwards, participants were equipped with a heart rate monitor, which recorded every 5s, and entered an environmental chamber (Design Environmental Ltd., Gwent, United Kingdom), set to 35ºC; 60% RH, and equal to a Wet Bulb Globe Temperature (WBGT) of 37ºC. To begin each trial, participants were seated on a chair in the heat for a 10 min period in order to

achieve core-temperature stabilisation (Schulze et al., 2015). Following this preliminary phase, participants performed 20 min of cycling at 40% and 60% PPO. Forward facing convective wind movement was simulated with 2 industrial fans (FS-75, FWL, Auckland, New Zealand), generating a wind speed of ~33 $km.h^{-1}$ (3000 Wind Meter, Kestrel, Sylvan Lake, MI); an appropriate wind speed for reducing heat storage and comparable to outdoor cycling as used in previous trials (Schulze et al., 2015). Thereafter, participants remained in the chamber and were given 5 min of seated rest before they commenced the 4 km time-trial. During this time the Velotron was calibrated and set into time-trial mode. Participants were asked to perform a maximal effort and to complete the trial as quickly as possible. During each trial, the lead researcher provided consistent encouragement to participants while elapsed time, power output, gear selection and distance completed, was provided visually using the Velotron software. Participants were asked to rate their perceived exertion (Borg, 1982) (6 = no exertion at all, to 20 = maximal exertion), modified thermal comfort (1 = comfortable, to 10 = extremely uncomfortable) and modified thermal sensation (1 = unbearably cold, to 13 = unbearably hot) (Gagge et al., 1967), and feeling (Hardy & Rejeski, 1989) (5 = very good, to -5 = very bad). These occurred at 4 (ambient conditions, following 10 min T_{re} stabilisation, prior to, and post time-trial) time-points. Participants then exited the chamber, towelled dry before re-weighing nude body mass with subsequent data used to establish effect of NO_3^- supplementation on sweat rate. Heart rate was measured using a heart rate monitor (Garmin 410, Garmin Ltd., USA). Body mass alterations was calculated as: body mass alterations = Δbody mass + fluid ingestion. During the first trial, participants were provided water ad libitum, and the timing and amount of water consumed was recorded and replicated in the following trial.

Serum NO_2^- Analysis

NO_2^- analysis was carried out using the Griess Method (Chae et al., 2003) with a commercially available kit (Promega, Wisconsin, USA). Samples were deproteinized by adding 400 µL trichloroacetic acid (TCA) to 400 µL serum, vortexed and centrifuged at 14,500 x rpm for 5 min **(**Espresso, Thermo Scientific) (Ghasemi, Hedayati, & Biabani, 2007). Thereafter, 150 µL of supernatant was added to 130 µL of deionized water followed by 20 µL of Griess reagent, prior to an incubation period of 30 min, as per manufacturer's instructions. Absorbance was measured at 548 nm. The resulting [NO_2^-]

were reported in µM, consistent with previous research using the Griess analysis method (Tsikas, 2007).

Statistical Analysis

All data were reported as means ± standard deviations (SD), and mean (95% confidence limits; CL) as appropriate. The differences in performance, physiological and subjective measurements were analysed using a magnitude-based inference approach (Hopkins et al., 2009) and were determined using published spreadsheets (xParrallelGroupsTrial.xls) from sportsci.org (Hopkins et al., 2009). Time-trial, heart rate and body mass data were log-transformed prior to statistical analysis. Uncertainties for time-trial performance effects were expressed as probabilities of harm or benefit in relation to the coefficient of variation (±1.0%) in each condition. To make inferences for performance measures an estimate of the smallest worthwhile change (SWC) in power output is required. The SWC is based on published literature on between competition performance variability of top cyclists (Paton & Hopkins, 2006). Cohen effect sizes (ES) were used to express the magnitude of differences in the changes between trials for heart rate and body mass and were reported as standardised differences. The criteria used for interpreting the magnitude of the ES for these variables were: <0.2, trivial; 0.2-0.5, small; 0.5-0.8, moderate; and >0.8, large (Hopkins et al., 2009). A novel approach for magnitude thresholds was used to determine the SWC for T_{re} and perceptual measures in which the possible range of change was transformed into a full scale of deflection (Hopkins, 2010). Briefly, each range was made from 0-100% and magnitude thresholds were defined as 10%, 30%, 50%, 70% and 90% for small, moderate, large, very large and extremely large changes. Quantitative chances of NO_3^- affecting measurement outcomes were assessed qualitatively as follows: <1%, *most unlikely*; 1-5%, *very unlikely*; 5-25%, *unlikely*; 25-75%, *possible*; 75-95%, *likely*; 95-99%, *very likely*; >99% *most likely* (Hopkins et al., 2009). When an effect was >5% for both benefit and harm, the true value of the difference was described as unclear.

Results

Self-reported adherence to supplementation was 100% for both treatment periods for the 8 participants, with four of the cohort consuming NIT in the first treatment round. One participant elected not to have their blood testing performed, therefore serum [NO_2^-]

analysis was conducted with n=7. All participants experienced beeturia during the supplementation phases, however no other side effects were reported.

Relative to PLA, there was a *most likely* greater increase (factor ×/÷95%CL: 2.8 ×/÷1.8) in mean [NO_2^-] following 3 d NO_3^- supplementation (**Figure 12**).

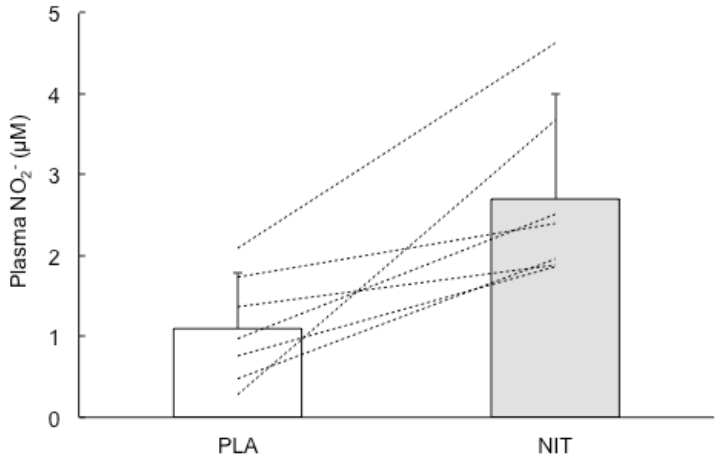


Figure 12: Serum [NO_2^-] levels at baseline, during placebo (PLA; mean + SD; white column), and nitrate (NIT; mean + SD; shaded column) treatments.

Sweat loss (via Δbody mass) for both PLA (0.66 $L \cdot h^{-1}$; mean ± SD%; ES ± 95%CL; -0.6 ± 1.1%; 0.04 ± 0.06) and NIT (0.72 $L \cdot h^{-1}$; -0.8 ± 0.3%; -0.06 ± 0.02) were both *most likely trivial*, whilst the differences between the two outcomes (-0.2 ± 0.6 kg; -0.23 ± 0.87) were *unclear*.

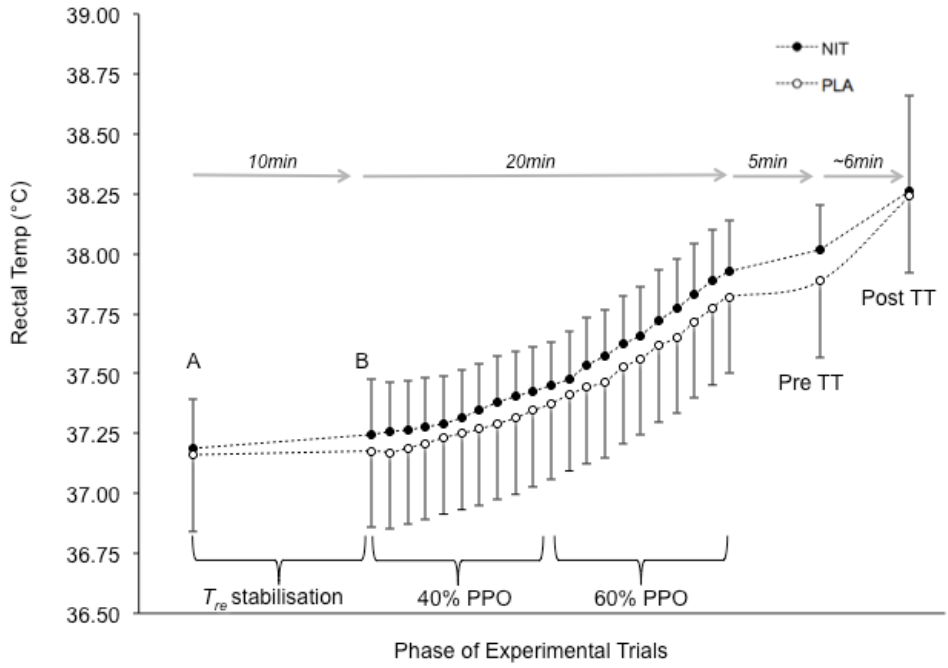


Figure 13: Mean group response during nitrate (NIT; closed circles) + SD, and placebo (PLA; open circles) – SD conditions, for rectal temperature (Tre) for A (ambient), B (end 10 min stabilisation), 20 min priming (40 and 60% peak power output), prior to, and following 4 km time-trial performance.

Measurement outcomes for group mean T_{re} (±95%CL) are shown in **Figure 13**. Relative to PLA, the NIT trial revealed small changes (range 0.09 to 0.12ºC) in T_{re}, equating to a *very likely trivial* increase (PLA;37.8 ± 0.2ºC vs NIT;37.9 ± 0.3ºC, 0.1 ± 0.2°C) at end 20 min priming, and prior to the 4 km time-trial (PLA;37.9 ± 0.2ºC vs NIT;38.0 ± 0.2ºC, 0.1 ± 0.2°C), however effects for T_{re} were *unclear* (PLA;38.2 ± 0.4ºC vs NIT;38.2 ± 0.2ºC, 0.0 ± 0.3°C) following time-trial performance.

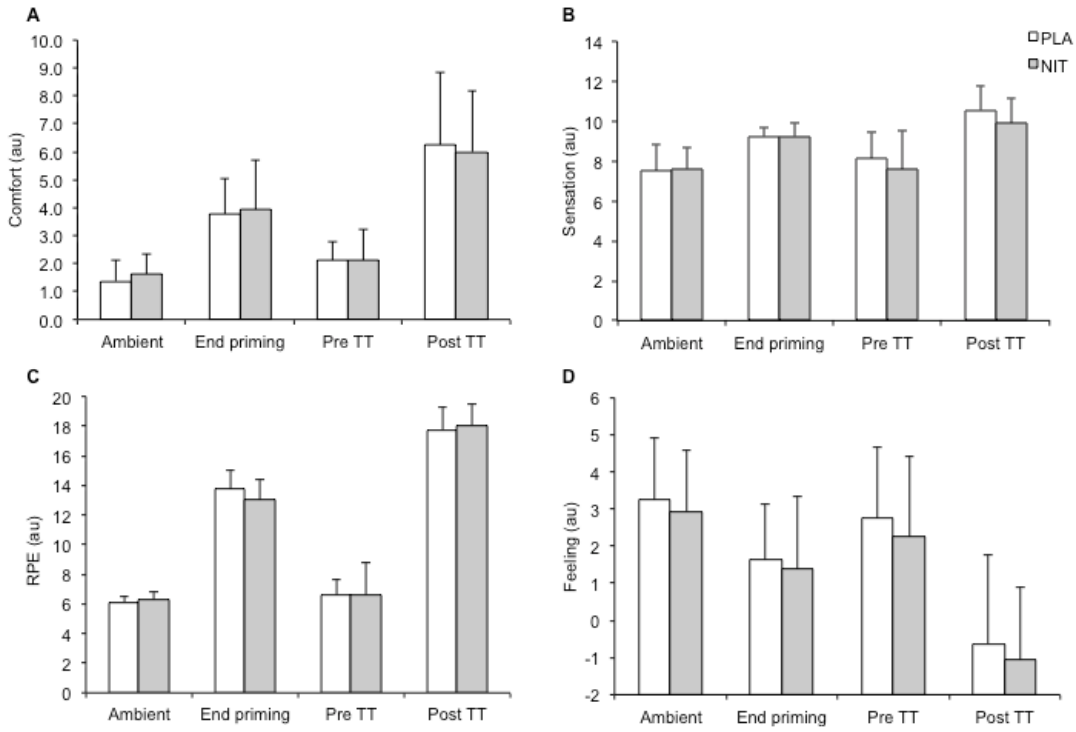


Figure 14: Mean group response (+ SD) for perceptual measures of A) Comfort; 1 = comfortable to 10 = extremely uncomfortable, B) Sensation; 1 = unbearably cold, to 13 = unbearably hot, C) RPE (rate of perceived exertion); 6 = no exertion at all, to 20 = maximal exertion, and, D) Feeling; 5 = very good, to -5 = very bad for ambient, end priming, pre time-trial and post time-trial reference points following nitrate (NIT; shaded columns) and placebo (PLA; white columns) treatments. AU = arbitrary units, TT = time-trial.

Mean outcomes and standardised effects for comfort, sensation, feeling, and RPE are shown in **Figures 14** and **15** respectively. Three d of NO_3^- supplementation had *trivial* or *unclear* effects on the majority of subjective measures of perception (**Figure 14**). Relative to PLA, a *small possible* increase (ES=0.27 ± 0.42) was reported for NIT in ambient conditions for thermal comfort, whilst a *small likely* decrease, and *small possible* decrease were reported for RPE at end priming (ES=-0.45 ± 0.46), and feeling prior to time-trial (ES=-0.20 ± 0.20), respectively.

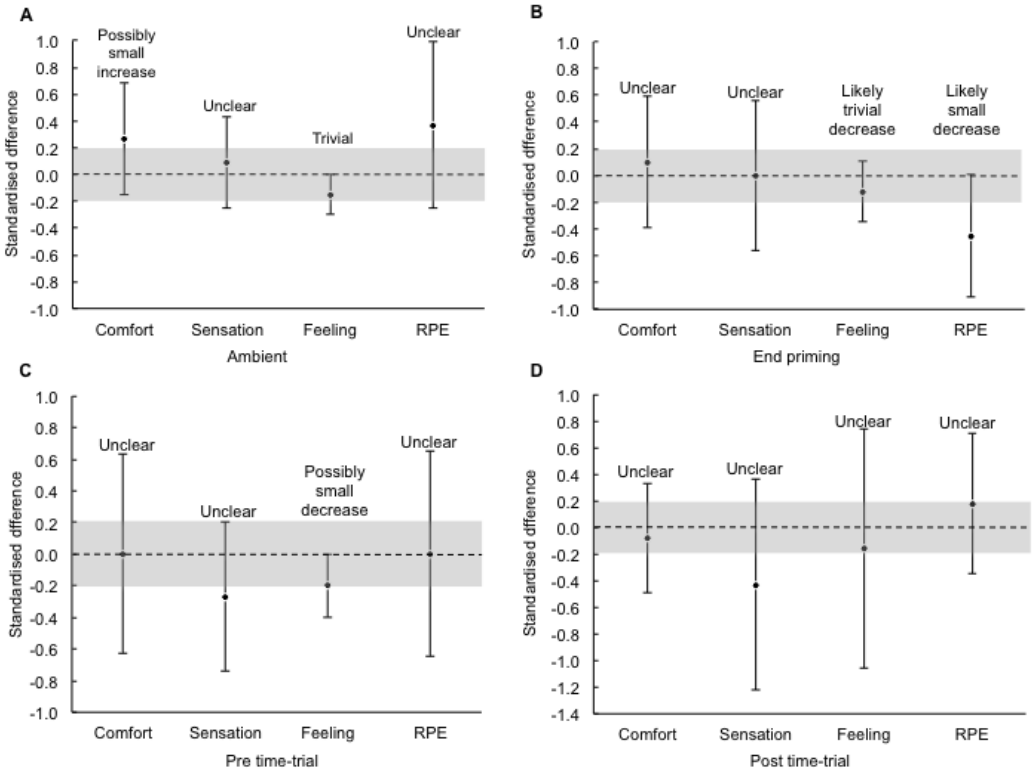


Figure 15: Standardised difference in comfort, sensation, feeling and rate of perceived exertion (RPE) in ambient (A), end-priming (B), pre time-trial (C), and post time-trial (D). Error bars indicate uncertainty in the true mean value with 95% confidence interval; if error bars overlap both the opposing increased and decreased *trivial* (shaded area representing ± 0.2 SD) values, changes are deemed *unclear* (see Methods section). Effects above the black dotted line indicate an increase in measure as a result of nitrate (NIT) vs. placebo (PLA), whereas effects below the line indicate decrease, as a result of NIT. Magnitude and quantitative chances of change are described qualitatively in the text above their respective error bars.

Following NIT supplementation, effects for heart rate resulted in a *likely trivial* increase (PLA;69 ± 12 bpm vs NIT;71 ± 12 bpm, ES=0.10 ± 0.12) in ambient conditions, a *likely trivial* decrease (PLA;158 ± 9 bpm vs NIT;157 ± 10 bpm, ES=-0.10±0.19) at the conclusion of priming, and a *likely trivial* increase (PLA;99 ± 10 bpm vs NIT;102 ± 11 bpm, ES=0.26 ± 0.17) prior to time-trial. Post time-trial effects were *unclear* (PLA;186 ± 10 bpm vs NIT;186 ± 12 bpm, ES=-0.06 ± 0.27) following NIT.

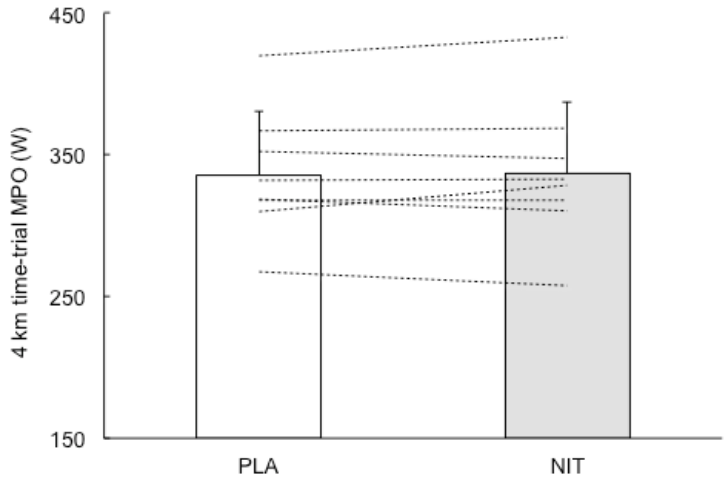


Figure 16: Mean group response for 4 km time-trial mean power output (± SD) and individual responses (dashed lines) for nitrate (NIT; shaded) and placebo (PLA; white) conditions.

Mean power output for the 4 km time-trial is shown in **Figure 16**. In relation to PLA, NIT showed *unclear* effects for both 4 km performance time (mean ±95%CL: -0.1 ±0.9%) and MPO (0.2 ±2.5%).

Discussion

To our knowledge, this is the first study to report on the effects of NO_3^--rich beetroot juice on thermoregulatory and perceptual responses in cyclists in hot environments and extends the current body of literature regarding the supplementation of NO_3^- supplementation in well-trained endurance athletes. The main findings of this study indicated that although a 3 d NO_3^- supplement period increased T_{re} at various time-points during low- to moderate-intensity exercise in the heat, subsequent time-trial performance in well-trained cyclists was unchanged.

Whilst NO_3^- supplementation has previously been shown to increase T_{re} and reduce estimated exercise tolerance (Kuennen et al., 2015), thus suggesting a potentially negative effect of dietary NO_3^- on walking intensity exercise in hot environments, our results indicate no harm or benefit effects following NO_3^- supplementation in the heat (**Figure 16**), despite small increases in T_{re} (**Figure 13**). It is acknowledged that increases in core (Peiffer & Abbis, 2011) and muscle (Cairns, 2013; Sargeant, 1987) temperature may serve to improve (Sargeant, 1987), or reduce (Peiffer & Abbis, 2011; Tatterson, Hahn, Martin, & Febbraio, 2000) performance depending on the magnitude of the temperature

rise, exercise type/duration and invoked physiological mechanistic responses (Cheung & McLellan, 1998; Peiffer & Abbis, 2011; Tatterson et al., 2000). For instance, relative to cooler (23ºC; 60% RH) environments, Tatterson et al. (2000) demonstrated increased blood lactate and pH reduction in exercising muscle of elite cyclists during the first third of a 30 min time-trial performance in hot (32ºC; 60% RH) environments (Tatterson et al., 2000). While an increase in muscle acidosis is negatively associated with exercise related fatigue in high-intensity events (Cairns, 2013), acidic conditions appear to enhance the bioavailability and bioactivity of NO (Modin et al., 2001) and therefore NO_3^- supplementation may serve to maintain or enhance high-intensity performance trials in hot conditions. Conversely, based on the findings of Kuennen et al. (2015), despite the initial positive effects on walking economy, there is potential for negative outcomes in longer term exercise following NO_3^- supplementation. These initial physiological related improvements, may have resulted due to the larger influence that NO_3^- appears to impart on recreational level cohorts, relative to those of higher training status (Porcelli et al., 2015). Similarly, the sensitivity to NO_3^- may also, in part, explain the subsequent negative effects witnessed in lesser-trained populations (Kuennen et al., 2015).

Most likely trivial (range 0.09 to 0.12ºC) increases in T_{re} during the first 20 min of exercise in hot environments were reported as a result of NO_3^- supplementation (**Figure 13**). The 0.3ºC increase is slightly less than that of Kuennen et al. (2015) who reported a significant (0.14ºC) increase ($p<0.05$) in T_{re} at the conclusion of a longer (45 min), though lower, fixed intensity exercise protocol after consuming NO_3^-. In hyperthermic inducing environments, blood flow is distributed to the periphery of the body in an attempt to maintain thermal equilibrium through evaporative cooling (Tripathi et al., 1990). Under exercising conditions temperature gradient increases of as little as 9ºC has been shown to elevate skin temperature by as much as 22% (Tatterson et al., 2000). As such, blood flow to the skin heavily influences the rate at which the body is able to dissipate stored heat (Crandall & Gonzalez-Alonso, 2010), therefore any diversion of this sympathetic mediated response may have negative consequences on homeostasis. The mechanistic basis for an increase in T_{re} following NO_3^- supplementation appears to be consequent to the step-wise reduction of NO_2^- to NO enabling whole body vasodilation (Modin et al., 2001), muscle-fibre specific enhancements (Jones et al., 2016), and the over-riding of the standard homeostatic response following exposure to hot environments (Tripathi et al., 1990). Interestingly, our small rise in T_{re} under NO_3^- conditions may have been partially

due to the fixed exercise workload, given that previous research has shown participants choose to reduce work output in order to manage increasing core temperatures during longer and more intense exercise than our 20 min sub-maximal exercise protocol (Tatterson et al., 2000).

Whilst the majority of perceptual measures were *unclear* or *trivial*, we reported several negative mean qualitative outcomes following NO_3^- consumption in ambient conditions for thermal comfort, and for feeling, prior to the time-trial (**Figures 14** and **15**), although exercise exertion (RPE) following end priming equated to a positive outcome under NO_3^- supplemented conditions. In relation to our findings, Kuennen et al. (2015) reported increased RPE, general discomfort and thermal sensation from 10 to 45, 25 to 45, and 40 to 45 min respectively, as a result of NO_3^- under hyperthermic conditions. Perceptually, trained populations cope with the effects of exercise in hyperthermic environments to a better extent than less-trained (Cheung & McLellan, 1998) and as such athletes in the present study are likely to have managed the perceptual effects of heat stress better in hot environments as a result of their aerobic (64 $ml \cdot kg^{-1} \cdot min^{-1}$) capabilities, relative to lesser-trained populations (Kuennen et al., 2015). Additionally, it is also important to note that the current study took place in the summer months, whereas Kuennen et al. (2015) assessed participants during winter months, which may have further predisposed their participants to the negative effects of a hot environment. Clearly further research is required to better understand the balance between desirable increases in core and muscle temperature for optimal performance and athletes' perceived thermal load following NO_3^- supplementation in the heat, especially during trials of varying duration.

Exercise in hot conditions led to a *most likely trivial* reduction in body mass via sweat rate for both NIT (0.72 $L \cdot h^{-1}$) and PLA (0.66 $L \cdot h^{-1}$) treatments. The between treatments comparisons were *unclear* suggesting that the consumption of NO_3^- did not lead to elevated sweat rate despite higher T_{re} under NIT conditions. Whilst both we, and Kuennen et al. (2015) have shown no clear difference in sweat response between NIT and PLA treatments within 45 min total heat exposure, it would be pertinent to explore how maximal intensity exercise over longer durations, e.g. Olympic distance cycling time-trial events in similarly challenging conditions (Ross et al., 2011), might influence sweat response, with regards to performance, particularly as sweat-rates of higher-trained

athletes appear to be elevated relative to lesser-trained populations (Cheung & McLellan, 1998).

Finally, it is acknowledged that there are some limitations to the present study that should be considered. Firstly, we did not compare the effectiveness of NO_3^- and placebo in temperate conditions and therefore we are unable to state to what extent heat exposure may have influenced performance. However, previous work in this area suggests that, in ambient conditions, well-trained male cyclists (63 $ml.kg^{-1}.min^{-1}$) demonstrated a *likely beneficial* improvement in 4 km time-trial PPO (2.4 ±2.5%) following 8 d of NO_3^- (McQuillan, Dulson, Laursen, & Kilding, 2017b). Based on these outcomes, the current findings indicate that either the addition of heat stress impairs the ability of NO_3^- to improve short-term, high-intensity performance, or, that athletes are recommended to supplement for a longer period of time prior to competition in hot environments. Secondly, while the possibility of type II error may exist due to a smaller cohort, the sample size in the present study was similar with previous studies reporting performance enhancement (Bond et al., 2012; Hoon, Jones, et al., 2014; Lansley, Winyard, Bailey, et al., 2011; Shannon et al., 2017) and impairment (McQuillan, Dulson, Laursen, & Kilding, 2017a) following NO_3^- supplementation. Furthermore, the reliability of our primary outcome measure (4 km time-trial time) in our laboratory is high (0.7 and 1.2%),[31,32] and participants were well-trained competitive cyclists, who train and race frequently and were familiar with cycle ergometer based exercise. Taken together, the study design, and participant characteristics employed help reduce the chance of type II error. Clearly, given our findings, further research is required to better inform practitioners of the risk to benefit ratio of prescribing NO_3^- supplementation prior to exercise and/or competition in hot environments.

Practical Applications

- Three days of NO_3^- supplementation elevated core body temperature, but not perceptual responses, during fixed intensity sub-maximal exercise in the heat. Practitioners therefore should be aware of the small additional thermal load following NO_3^- consumption, particularly during longer duration exercise bouts.
- In well-trained cyclists, NO_3^- supplementation over a 3 d period has no effect on short-term, high-intensity time-trials performed in hot conditions. However, practitioners

are encouraged to investigate the performance effects of NO_3^- supplementation during longer durations, as well as greater dose durations, as these may influence the ergogenic potential of NO_3^- in the heat.

Conclusions

This is the first investigation to report on the thermoregulatory, perceptual and ergogenic effects of NO_3^- supplementation on maximal-intensity cycling performance in a hot environment in well-trained athletes. Relative to placebo, daily NO_3^-supplementation of 140 ml beetroot juice [~8.0 mmol NO_3^-] over a 3 d period produced small increases in T_{re} during sub-maximal exercise in the heat. However, subsequent 4 km time-trial performance, cardiovascular, and perceptual responses were unaltered following NO_3^- supplementation. Given the tendency for NO_3^- supplementation to elevate T_{re}, future studies should explore the effects of NO_3^- on endurance events of longer duration in the heat.

Acknowledgements

We thank the participants for their time, enthusiasm and effort over the course of this research project.

CHAPTER 6: OVERALL DISCUSSION AND CONCLUSION

A comprehensive review of the literature at the outset of this work highlighted a number of limitations in regards to the current understanding of the effectiveness of NO_3^- supplementation on physiology and short-term, high-intensity endurance performance in well-trained endurance athletes. Despite the positive effects of NO_3^- supplementation in untrained populations, it would appear that the effects of NO_3^- supplementation are somewhat limited in athletic populations. The reduction in effects in athletic populations may be as a consequence of training mediated responses such as greater resting levels of nitric oxide synthase (Jungersten et al., 1997; Tordi et al., 2006), and/or elevated intakes of exogenous nitrate due to greater dietary intakes to meet greater energy expenditure (Jonvik et al., 2016). Few studies had assessed the impact of NO_3^- on physiology and/or short-term, high-intensity endurance performance in athletic populations via a range of dosing strategies (Bescos et al., 2012; Nyakayiru et al., 2016). Furthermore, evidence supporting effective ways of using NO_3^- was vague, in part due to the wide range of supplementation regimes employed in the literature, and the wide variation of performance trial duration employed to assess performance outcomes. Specifically, there was a lack of information surrounding the impact of NO_3^- dose duration on shorter duration maximal intensity performance in endurance-trained populations. Additionally, despite the positive effects witnessed in recreationally trained populations in thermo-neutral conditions, the effectiveness of NO_3^- as an ergogenic aid in hot environments was unclear, given previous neutral (Tyler et al., 2016) and even adverse findings (Kuennen et al., 2015) in non-athletic populations. Accordingly, this thesis attempted to address these gaps in the literature, according to the main paradigm of interest: what is the efficacy of NO_3^- supplementation in competitive cycling populations on physiology and short-term, high-intensity endurance performance? A summary of the chapters included as part of this thesis is presented in Table 8. Subsequent discussion in this section articulates the main findings of this thesis in regards to the individual research questions underpinning the aim of this thesis.

Table 7: Summary of key findings from the chapters included as part of the thesis

Chapter	Title	Participants	Study Design	Intervention	Performance and Physiological Assessments	Key Findings
Chapter 2	The effectiveness of nitrate supplementation as an ergogenic aid in competitive athletes	n/a	Literature Review	n/a	n/a	• In moderately-trained athletes NO_3^- supplementation appears to be effective in events 6.30 to 27 min duration • In highly-trained populations shorter duration (~80 s) events may be enhanced, however the majority of findings suggest that NO_3^- supplementation has little influence on performance • The influence of NO_3^- on VO_2peak are inconclusive • Economy appears enhanced in moderately-trained athletes, but unchanged in athletes of greater aerobic ability, as a result of NO_3^- supplementation • Regardless of aerobic ability, heart rate and blood lactate appear not to be influenced by consumption of NO_3^- • While the effectiveness of NO_3^- on exercise in hypoxic environments has been assessed, information on the influence of NO_3^- in hot environments is currently lacking
Chapter 3	Effect of nitrate supplementation on physiology and performance in well-trained cyclists	Eight well-trained male cyclists (VO_2peak = 63 ml.kg^{-1}.min^{-1})	Randomised, placebo-controlled, cross-over design	8 d of 70 ml NO_3^- BJ 4 mmol NO_3^- daily	Day 6 = VT_1, VT_2; d 7 = incremental PPO, VO_2peak; Days 8 = 4 km TT	NO_3^-supplementation resulted in *unclear* effects for VT_1, and VO_2peak, whilst VT_2, were *trivial* at d 6. A 7 d period resulted in *trivial* effects for economy, however 8 d of NO_3^- had a *likely beneficial* effect on 4 km TT cycling performance. In well-trained cyclists performance enhancements, but not changes in physiological determinants of performance, result with 6 to 8 d NO_3^- supplementation.

Chapter 4	Dietary nitrate fails to improve 1 and 4 km cycling performance in highly-trained cyclists	Nine highly-trained male cyclists (VO_2peak = 68 ml.kg^{-1}.min^{-1})	Randomised, placebo-controlled, cross-over design	7 d of 140 ml NO_3^- BJ 8.0 mmol NO_3^- daily	Day 3 and 6 = 4 km TT and economy at 40, 50, and 60%; day 4 and 7 = 1 km TT	Following 3 and 6 d of NO_3^- supplementation effects for 4 km TT and economy were *unclear*. In the same condition, 1 km TT performance was *likely harmful* following 4 d, but *unclear* after 7 d. For highly-trained endurance athletes, NO_3^- supplementation did not positively influence cycling economy or TT performance.
Chapter 5	The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists	Eight well-trained male cyclists (VO_2peak = 63 ml.kg^{-1}.min^{-1})	Randomised, placebo-controlled, cross-over design	3 d of 140 ml NO_3^- BJ 8.0 mmol NO_3^- daily	Day 3 = T_{re} during 40 and 60% PPO and 4 km TT in hot environments (35°C; 60% RH)	Three days of NO_3^- supplementation, had a small *most likely trivial* increase in T_{re} at moderate intensity exercise, however this did not appear to influence (either negatively or positively) 4 km cycling time-trial performance in hot conditions. Relative to PLA, NO_3^- supplementation had no effect on performance, sweat rate or perceptual responses in hot conditions.
BJ = beetroot juice; d = day; min = minute; NO_3^- = nitrate; PLA = placebo; PPO = peak power output; RH = relative humidity; s = seconds; T_{re} = rectal temperature; TT = time-trial; VO_2peak = peak oxygen uptake; VT_1 = first ventilatory threshold; VT_2 = second ventilatory threshold						

Research Questions Addressed in this Thesis

Does 6 to 8 days of NO_3^- supplementation influence VO_2peak, ventilatory threshold, and economy in well-trained male endurance cyclists?

In elite endurance athletes, measures of VO_2peak, VT_2, and economy are synonymous with performance ability (Coyle, 1999; Holloszy & Coyle, 1984; Joyner & Coyle, 2008), therefore changes in either of these measures is likely to influence performance outcomes in endurance events (Hawley & Noakes, 1992; Hopkins et al., 1999). In Chapter 3 a *trivial* (-1.0 ±3.0%) decrease in oxygen utilisation at VT_2 resulted following 6 d of NO_3^-. These findings are congruent with the findings of Bescos et al. (2012) who reported a reduction in VT_2 following $NaNO_3^-$ supplementation in a trained cycling population (Bescos et al., 2012). Conversely, the *unclear* (mean ±95%CL: 1.8 ±5.5%) increase in VO_2peak following NO_3^- supplementation oppose those showing no change (Porcelli et al., 2015), or reduced (Bescos et al., 2011) VO_2peak in endurance trained athletes as a result of NO_3^-. In recreationally-active participants, VO_2peak was unchanged regardless of the duration (1 to 15 d) of NO_3^- (Vanhatalo et al., 2010). With regards to measures of economy, we report *trivial* (-1.0 ±1.6%) effects following NO_3^- supplementation (~4.0 $mmol.L^{-1}$) over a 7 d period. Recent work by Porcelli et al. (2015) suggests that despite significant increases ($P < 0.001$) in [NO_2^- and NO_3^-], supplementation of NO_3^- failed to confer any improvement in running economy or 5 km performance in highly-trained athletic populations, yet, the same dosing strategy improved both outcomes in lesser-trained populations (Porcelli et al., 2015). Moreover, the authors reported strong (r^2=-0.75 to -0.73) inverse relationships between aerobic ability (VO_2peak) and Δ[NO_2^- and NO_3^-] (Porcelli et al., 2015) suggesting that elevations in [NO_2^- and NO_3^-] are greater in populations with lower aerobic ability. Therefore it would appear that the effects of NO_3^- on measures such as VO_2peak, VT_2, and economy in wel-trained cycling populations are still to be elucidated.

Does 8 days of NO_3^- supplementation influence 4 km cycling TT performance in well-trained male endurance cyclists?

Chapter 3 reported that consumption of NO_3^- by well-trained cyclists had a *likely beneficial* effect on 4 km time-trial performance. Improved mitochondrial function (Larsen et al., 2011), and increased efficiency of muscular force generation (Bailey, Fulford, et al., 2010), have been reported following 3 and 6 d of NO_3^- supplementation,

respectively. However, a single acute dose of NO_3^- has been shown to improve short-term, high-intensity performance activity by 2 to 3% in both moderately- (Lansley, Winyard, Bailey, et al., 2011) and well-trained (Peeling et al., 2015) athletes. These outcomes suggest that performance improvements mitigated by NO_3^- may not be fully dependent on previously identified mechanistic alterations and/or the duration of dosing. In Chapter 3, a *likely beneficial* (2.4 ± 2.5%) improvement in 4 km time-trial performance was reported following an 8 d period of NO_3^- (4 $mmol.d^{-1}$). The performance improvement occurred despite a lack of substantial alterations in a range of performance indicators (**Table 8, Figure 6**). Despite a number of previous studies demonstrating a lack of effects in short-term, high-intensity endurance performance following a 6 to 8 d supplementation period (Boorsma et al., 2014; Nyakayiru et al., 2016), our findings are similar, if not as large as those of Cermak, Gibala, et al. (2012) who reported 1.2% improvements in 10 km cycling performance in a trained cycling population.

Does the duration of NO_3^- supplementation have any influence on a range of economy measures in well-trained male endurance cyclists?

Chapter 4 investigated the effects of 3 to 6 d NO_3^- supplementation on economy over a range of intensities. Relative to placebo conditions, effects for nitrate NO_3^- supplementation were either *unclear* or *trivial* for all measures, regardless of intensity (**Figures 8 and 9**). Our findings appear to be congruent with previous findings in well-trained endurance athletes (Carriker et al., 2016; Porcelli et al., 2015), but oppose the positive outcomes witnessed in trained-cyclists of ~60 $ml.kg^{-1}.min^{-1}$ following either acute (Wilkerson et al., 2012), or 6 d (Cermak, Gibala, et al., 2012) NO_3^- supplementation. The lack of effects in well-trained athletes relative to less fit populations, would suggest that athletic calibre plays a part in the efficacy of NO_3^- supplementation to alter physiology, though there are exceptions to this generalisation. For example, Peeling et al. (2015), reported that elite male kayak paddlers improved their economy following an acute dose of NO_3^-. Interestingly, the discrepancy between physiological enhancements between upper and lower body sports may explain this given the predominance of type II fibres in upper body muscle (Baker & Hardy, 1989), relative to the oxidative nature of type I muscle fibre typically utilised in cycling activity (Ivy, Costill, & Maxwell, 1980).

Does the duration of NO_3^- supplementation have any influence on 1 and 4 km time-trial performance in well-trained male endurance cyclists?

Chapter 4 assessed the effects of an 8 mmol.d^{-1} NO_3^- dose on 1 and 4 km time-trial performance in a highly-trained cycling cohort (68 ml.kg^{-1}.min^{-1}). Using a novel approach, 4 km time-trial performance was assessed on day 3 and 6, while 1 km performance was assessed on day 4 and 7 of the supplementation period. The main finding of this study was that NO_3^- supplementation over a 4 d period resulted in a 1.9% *likely harmful* reduction in mean power output over a 1 km distance, whilst a 7 d period resulted in *unclear* effects. To date this is only the second study in the wider literature to suggest performance impairment under NO_3^- supplemented conditions following that of Hoon, Hopkins, et al. (2014). Moreover, we are the first to cite impairment in highly-trained athletic populations in events ≤4 min duration, thus opposing the findings of Peeling et al. (2015) in a 2 min event, for elite, flat-water female kayakers and those of Bond et al. (2012) following a 6 x 90 s interval session in trained rowers. With regards to 4 km TT performance, neither 3 nor 6 d of NO_3^- supplementation influenced 4 km time-trial ability, with effects for both durations reported as *unclear*. In contrast, the findings of Vanhatalo et al. (2010) suggest that in recreationally active populations an acute dose of NO_3^- enhances a range of exercise capacity measures to the same extent that 5 and 15 d did. In well-trained (65 ml.kg.$^{-1}$min^{-1}) cyclists neither 1 or 6 d NO_3^- influenced 10 km cycling time-trial performance or economy (Nyakayiru et al., 2016). Therefore, in conjunction with the findings in this thesis, and in particular Chapter 4, the findings of several investigations (Nyakayiru et al., 2016; Vanhatalo et al., 2010; Whitfield et al., 2015) suggests that the duration of dosing does not influence performance outcomes over and above that of an acute dose. Recently, the findings of Shannon et al. (2017) appear to confirm that event duration, and therefore muscle fibre recruitment plays a role the efficacy of NO_3^- supplementation. Despite 5- to 6-fold elevations in [NO_2^-] following an acute dose of 12.5 mmol NO_3^-, relative to PLA, outcomes for 1500 (~325 s) and 10000 (2643 s) m running events were quite divergent. Equating to 86% VO_2peak, effects for the 1500 m event following NO_3^- supplementation were *very likely beneficial*, however in contrast, over a 10000 m distance (~78% VO_2peak), effects for NO_3^- were *unclear*. The higher workload for the 1500 m, would reflect a greater utilisation of type II fibres, which would appear to benefit the greatest extent from NO_3^-. Clearly, the opposing findings in maximal intensity events of ~2 to 6 min duration require further investigation

to elucidate the impact of NO_3^- on performance, given the proposed range of positive influences on type II muscle (Jones et al., 2016).

What is the impact of 3 days of NO_3^- supplementation on heart rate and rectal temperature in hot environments in well-trained male endurance cyclists?

In Chapter 5 we reported that a 3 d NO_3^- supplementation period had *unclear* effects on T_{re} during low intensity (40% PPO) exercise, but had a *very likely trivial* increase in T_{re} during moderate intensity (60% PPO) exercise (**Figure 9**). The small ~0.10°C increase in T_{re} did not appear to influence measures of perception or 4 km cycling time-trial performance. The reported increase in T_{re} is similar, but not as substantive, as previous findings in recreationally active participants engaged in marching (Kuennen et al., 2015). Nevertheless, further work is required to understand the effects of NO_3^- on T_{re} in hot conditions given that whole-body vasodilation occurring following NO_3^- consumption (Govoni et al., 2008) opposes the homeostatic response of internal vasoconstriction (Tripathi et al., 1990). Additionally, recreationally active participants exercising in hot environments under NO_3^- supplemented conditions have demonstrated increased T_{re} which was likely to lead to a reduction in exercise capacity (Kuennen et al., 2015). To date, it is unknown if, or to what extent NO_3^- might affect core temperature measures in athletic populations. Therefore, given that numerous Olympic and World Championship competitions take place in hot environments it would be important to address this question.

With regards to heart rate, following NO_3^- supplementation, heart rate demonstrated a *likely trivial* decrease at rest in ambient conditions (**Figure 17**). Under thermally challenging conditions a *likely trivial* decrease following pre-time-trial priming was found, while a *likely trivial* increase in heart rate was reported prior to the time-trial. At the conclusion of the 4 km time-trial, relative to placebo, effects for heart rate were *unclear* following NO_3^- consumption. No comparison was made for heart rate response under thermo-neutral conditions in Chapter 5, however the non-substantive changes resulting from NO_3^- are in agreement with previous research in temperate environments in non-athletic populations (Kuennen et al., 2015). NO_3^- has been shown to reduce blood pressure in healthy, normotensive populations (Kapil et al., 2010; Webb et al., 2008) and

sufferers of COPD (Berry et al., 2015; Kenjale et al., 2011) via NO mediated vascularisation of exercising tissues highlighting the vasodilating capacities of NO.

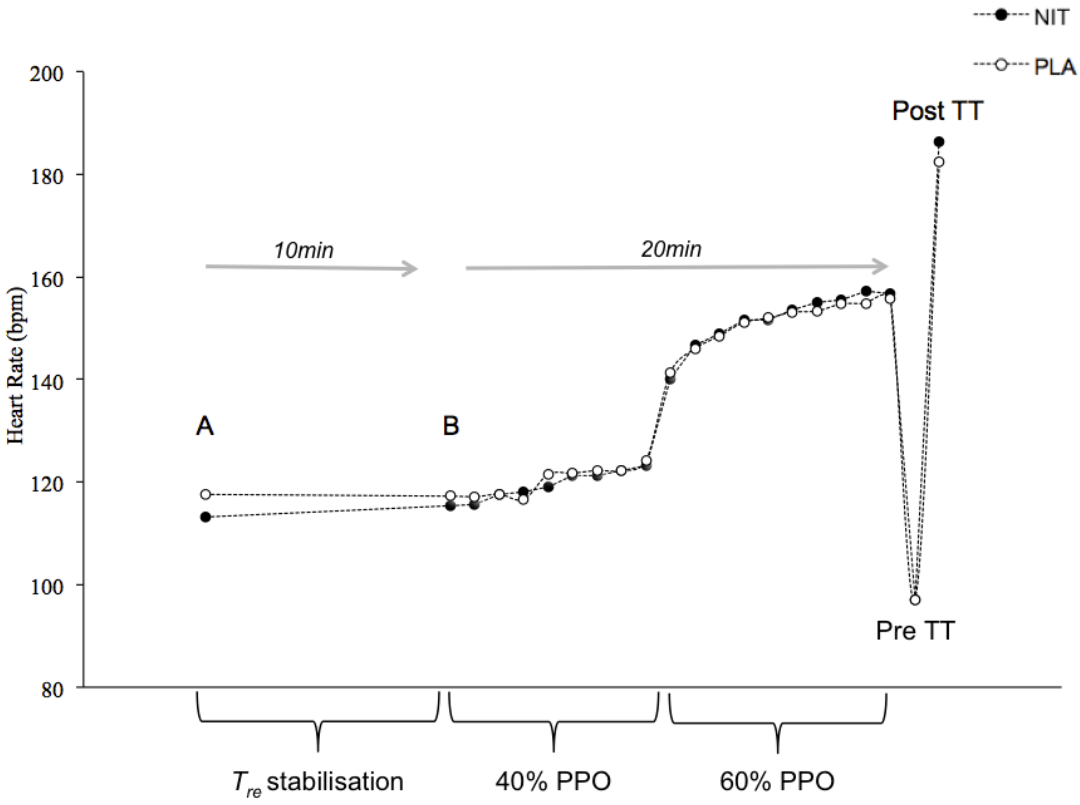


Figure 17: Mean group response during nitrate (NIT; closed circles), and placebo (PLA; open circles) conditions, for heart rate for A (ambient), B (end 10 min acclimation), 20 min priming (40 and 60% PPO), prior to, and following 4 km time-trial performance, with standard deviations removed for clarity.

What is the impact of 3 days of NO_3^- supplementation on short-term, high-intensity performance in hot environments in well-trained male endurance cyclists?

During exercise in hot environments, blood flow is distributed to the body's periphery in order to maintain core temperature equilibrium (Tripathi et al., 1990). In order to shuttle blood to the outer reaches of the body, internal vasoconstriction must occur. Given that bolus doses of NO_3^- lead to whole-body vasodilation (Bloomer, 2010), and alter blood flow (Ferguson et al., 2013), the interplay of NO_3^- supplementation by athletic populations competing in hot-environments is of interest. In recent times, the majority (Sydney 2000, Athens 2004, Beijing 2008 and Rio 2016) of Olympic Games have been held in countries in which thermally challenging environments (>30ºC) may be present. In contrast, the majority of NO_3^- studies have been conducted in temperate (~18 to 20ºC) standardised laboratory climates. As harmful effects for NO_3^- supplementation in

recreationally trained participants have been reported during exercise in hyperthermic (41ºC; 20% RH) conditions (Kuennen et al., 2015), it was of interest to investigate the effects of NO_3^- on performance in athletic populations in climates in which they could realistically be exposed to.

Chapter 5 reported that the effects of NO_3^- on 4 km time-trial performance in well-trained cyclists under hot (35ºC; 65% RH) conditions were *unclear*. These findings align with those of Chapter 4, whereby NO_3^- supplementation for 3 and 6 d had no effect on 4 km time-trial performance in ambient conditions. The outcomes for Chapters 4 and 5 contrasts with the *likely beneficial* performance improvements in 4 km time-trial reported in Chapter 3. These performance differences occurred despite all three cohorts possessing similar cycling ability through out the three investigations. Despite cycling under thermally challenging conditions, the short 6 min duration for the completion of the 4 km performance reported in Chapter 5 is unlikely to induce a rise in core body temperature near those that would be considered critical (Nielsen et al., 1993; Tatterson et al., 2000). However, given our findings of a small increase in core body temperature occurring with moderate intensity activity, it would be pertinent to investigate the effects of NO_3^- supplementation on thermoregulatory responses over a longer duration assessment. These findings suggests that in hot environments, elevated basal [NO_2^-] resulting from beetroot juice consumption, raises core-body temperature during steady state exercise, but does not influence short-term, high-intensity performance. The elevation in body temperature as a result of NO_3^- supplementation is a unique finding that suggests that athletes be wary of the potential negative consequences of whole-body vasoregulation as a result of the NO_2^- to NO mediated vascular response. For athletes competing over longer distance events in hot environments this is particularly important, given that the performance intensity (pacing strategy) is lowered in order to reduce the rate of heat gain within the body (Peiffer & Abbis, 2011). Therefore, further work is required to understand the NO_3^- mediated responses to exercise in hot environments where the inability to regulate core body temperature may negatively affect performance.

Practical Applications

The overall aim of this thesis was to determine the efficacy of various NO_3^- supplementation strategies on well-trained endurance cyclists in an attempt to provide coaches and athletes with relevant information relating to the use of NO_3^- prior to competition. To this end, this thesis employed several 'novel' NO_3^- supplementation methods by utilising a range of experimental strategies, which were structured progressively using placebo-controlled, double-blinded, cross-over designed studies. Based on the thesis findings, and current state of the literature, a summary of practical applications is provided to assist coaches, sports scientists and athletes when deciding on NO_3^- supplementation strategies prior to short-term, high-intensity athletic performance.

- Supplementation of 4 mmol NO_3^--rich beetroot juice over an 8 d period may improve 4 km cycling time-trial performance in well-trained (63 $ml.kg^{-1}.min^{-1}$) endurance cyclists.
- In well-trained (68 $ml.kg^{-1}.min^{-1}$) endurance cyclists' supplementation of 8 mmol NO_3^- does not appear to improve 4 km time-trial performance following a 3 or 6 d period of NO_3^-.
- In this same group it would appear that 4 d of 8 mmol NO_3^- supplementation may reduce 1 km time-trial performance.
- Increasing the dose duration to 7 d has little influence on baseline performance for 1 km time-trial performance.
- Regardless of aerobic ability, the supplementation of NO_3^- appears to have little influence on oxygen utilisation (economy) or a range of performance indicators during low- to moderate-intensity exercise in well-trained (63 to 68 $ml.kg^{-1}.min^{-1}$) cyclists.
- Likewise, in the same population, NO_3^- supplementation appears to have little influence on a range of perceptual responses.
- NO_3^- supplementation demonstrates a small increase in T_{re} during moderate-intensity exercise in hot environments.
- NO_3^- does not appear to influence high-intensity cycling events of 6 min in hot (35°C; 60% RH).
- It would appear that the individual responses to the effects of NO_3^- supplementation are not solely dependent on aerobic calibre of the athlete.

- Sports scientists and coaches of endurance athletes should trial the effects of NO_3^- on individual athletes using the specific environmental conditions, and distances to assess the efficacy on performance.

Thesis Limitations

It is acknowledged that there are some limitations to the thesis findings that should be considered, specifically:

- Between eight and nine participants were employed across the 3 studies, which, while low in number, is consistent with previously published NO_3^- supplementation papers (MacLeod et al., 2015) and notably greater than other NO_3^- related research (Peeling et al., 2015). However, the smaller populations used is due, in part, to the focused nature of the overall thesis topic, and highlights the limitations in targeting higher calibre participants for research trials. As such, the inclusion criteria severely limited the pool of potential volunteers. The benefits of employing a more homogeneous, regularly trained population is that trained athletes will: 1) have a lower between subject variation, 2) have less variation in week-to-week performance, 3) be more familiar with maximal performance than untrained, 4) be aware of the need to pace time-trial efforts, and 5) make the findings of the thesis applicable to a specific population. In addition to the familiarisation sessions ahead of each study, these particular participant characteristics will have contributed to a lowered CV for trials, thus improving the accuracy of the study, and reducing the likelihood of type II error (Hopkins, 2000) relative to other NO_3^- studies. For instance, Cermak, Gibala, et al. (2012) and Lansley, Winyard, Bailey, et al. (2011) report similar (1%) CVs for familiarisation trials to those for 4 km mean time-trial performance (0.7 to 1.2%) under ambient conditions. While the between subject variations in placebo trials (2.9 to 3.6%) is not as low as the 1.8% reported by both Cermak, Gibala, et al. (2012) or Peeling et al. (2015), it is substantially better than the 6.2% of Lansley, Winyard, Bailey, et al. (2011). Our lower CV suggests that our findings are applicable to the subject population studied, and continue the trend of lowered responsiveness to NO_3^- in line with increases in aerobic ability. As a result, it is believed that our lowered participant numbers would not have detracted from the findings reported in this thesis.

- All 3 studies reported effects at 95% confidence limits, resulting in default ranges of 12.5% benefit and 0.25% harm in order to assess the impact of NO_3^- on a range of physiological and performance measures in competitive cyclists. A 95% confidence interval provides a greater degree of certainty around the outcome of the measure. The default >66% odds benefit ratio provides a useful guide, however athletes and coaches want to know with greater certainty that a treatment designed to enhance performance is more likely to be effective or not. Chapter 4 demonstrated that nitrate supplementation may actually decrease performance in participants with greater aerobic fitness and findings from this thesis highlights that populations of ~63 $ml.kg^{-1}.min^{-1}$ may be at the tipping point at which the effectiveness of NO_3^- supplementation reduces substantially from cycling populations of lower aerobic and performance ability. Therefore performance related outcomes were assessed using a threshold for harm of greater (0.25%) sensitivity. As such it is likely that athletes would employ a strategy which provides them with a 95% chance that there is >12.5% or greater chance of benefit relative to <0.25% chance of harm.
- Chapter 5 reported that 3 d of NO_3^- supplementation resulted in a small increase in T_{re} however, the duration of the time-trial while consistent with other studies in the thesis, was probably too short to ascertain whether these changes would have carried over to longer duration time trial performance. Given that study one identified improvements in 4 km time-trial performance after an 8 d supplementation period, it may have been prudent to extend the dosage out to 8 d. However, previous findings suggest that 3 d of NO_2^- supplementation are enough to mediate positive effects on mitochondria and exercise capacity (Larsen et al., 2011). Moreover, relative to placebo, basal [NO_2^-] had a *most likely* (factor of 2.8) increase following three d of NO_3^- supplementation, suggesting the bioavailability of NO_2^- did not limit performance outcomes. Economy was not measured in study 3, but the previous two studies indicated that NO_3^- supplementation did not alter economy in similarly trained populations.
- Economy was assessed in two of the three studies; while an alternate metabolic cart was used for these two studies the CVs for both study 1 (3.5%) and study 2 (2.7%) are within acceptable limits. At all times calibration procedures were carried out as per manufacturer's instructions and therefore we are confident measurements were as accurate as they could have been.

- In reference to study 3, the measurement of 4 km time-trial performance in ambient conditions would have allowed comparison of the effects of NO_3^- on both hot and ambient environments in endurance trained cyclists. Incorporating a trial in ambient conditions would have been particularly pertinent as it would have allowed the combining of several variables from the former studies. For instance, similarly to study 1, mean VO_2peak of trained cyclists in study 3 was almost identical (63 vs. 64 $ml.kg^{-1}.min^{-1}$) and, unlike study 1, which employed a daily intake of 4 mmol NO_3^-, a greater (8 mmol NO_3^-) dosing strategy was employed for study 2. As doses of 8 but not 4 mmol NO_3^- have been shown to enhance performance over a similar duration to 4 km cycling time-trial (Hoon, Jones, et al., 2014), this would have made for interesting comparison. Therefore, the combination of these (athlete calibre and greater NO_3^- dose) factors to study 3 would have enabled a contrast of performance in both ambient and hot conditions. Furthermore, our knowledge relating to the effects of NO_3^- on performance responses in trained athletes would have been broadened.
- Throughout all three experimental chapters, basal [NO_2^-] was assessed using the Griess analysis method. We adopted the Griess method of NO_2^- determination as that was the technique available to us at the time of the studies. The standard procedures for that method were followed, and our resulting basal [NO_2^-] measures, reported in μM, are consistent with previous work adopting the Griess method in human plasma (da Silva Pereira et al., 2010; Moshage, Kok, Huizeng, & Jansen, 1995). Conversely, others have employed chemiluminescence (Bailey, Vanhatalo, Wilkerson, DiMenna, & Jones, 2009; Kenjale et al., 2011; Muggeridge et al., 2014), gas chromatography/mass spectrometry (Jungersten et al., 1997) and high performance liquid chromatography (Glaister et al., 2015) with values for [NO_2^-] typically reported in mM. In any case, recent research has shown nitrate supplementation to have a much more moderated effect on nitrite measures (Bescos et al., 2011), while more recent findings have failed to show any improvement in performance in highly trained athletes despite substantial increases in basal [NO_2^-] (Porcelli et al., 2015) suggesting that an elevation in either basal [NO_2^- and/or NO_3^-] does necessarily confer to an improvement in performance in highly-trained athletes.
- While basal [NO_2^-] was measured and reported during all three studies, basal [NO_3^-] was not measured. The fact that basal [NO_3^-] was not measured is

due to the fact that whole bolus doses of NO_3^- elevate basal [NO_3^-], the circulating pool of bioactive NO_2^- alongside exercise activity appears to be most responsible for imparting physiological enhancements during exercise (Webb et al., 2008) and is most sensitive to altered physiological states (Lundberg & Govoni, 2004).

- Assessment of the influence of NO_3^- on mitochondrial efficiency, muscle [NO_2^-/NO_3^-], or participants muscle fibre distribution was not undertaken during any of three studies in this thesis. Information regarding these potential confounders would have been pertinent to the current literature given that participants were a homogenous population. Measurement of mitochondrial efficiency and/or muscle [NO_2^-/NO_3^-] may have provided an opportunity to identify physiological alterations purported to either enhance or blunt physiological and/or performance outcomes. Additionally, the identification of participant's muscle fibre type would have allowed the role of fibre type to be assessed. To date, in highly-trained endurance athletes, it would appear more likely that NO_3^- positively influences events of predominantly upper (Bond et al., 2012; Peeling et al., 2015), but not lower (Boorsma et al., 2014; Hoon, Hopkins, et al., 2014) body activity. Moreover, as per the recent findings of Whitfield et al. (2015), who reported improved economy despite no change in mitocohondrial efficiency, thereby opposing findings of Larsen et al. (2011), we may have been able to challenge current opinions. Time and resourcing allowing, further research should be undertaken to identify the mechanisms that influence the efficacy of NO_3^- supplementation in both trained and untrained populations.
- Although we report Cohens effect sizes for Chapters 3, 4 and 5 and used ANOVA for Chapter 4, we mostly employed a Magnitude Based Inference (MBI) approach as our primary statistical analysis for the three experimental chapters which is consistent with other nitrate studies (Bond et al., 2012; Hoon, Hopkins, et al., 2014; Lansley, Winyard, Bailey, et al., 2011). A MBI approach provides a description of the degree to which the size, or magnitude of an effect, or outcome, lies in relation to the smallest worthwhile change (SWC) of the measure. As a result of an intervention, the span of the confidence interval for an effect may be positive, negative, a combination of both, or trivial. Additionally, MBI also allows the interpretation in the uncertainty of the confidence interval using qualitative terminology such as *possibly harmful, likely beneficial,* or in the case of an interval crossing both the positive and negative threshold, '*unclear*'. In simple terms, the

use of MBI allows the researcher to state the level of confidence in the outcome. In practical terms, the use of MBI allows a sport scientist to interpret outcomes for coaches and athletes to weigh up by using qualitative terms that offer more scope than null hypothesis significance testing. For instance, a coach may be better placed to make a call on the use of a dietary supplement if it is found to have a '*likely beneficial*' effect on performance, as opposed to whether the result is 'significant' or 'non-significant'.

Despite the advocated benefits of the MBI approach, it is acknowledged that prior to, and following submission of this thesis, MBI has been criticised on a number of fronts including: 1) log-transformation of data even though it may be evenly distributed, 2) over-interpretation of the confidence interval, and 3) potential for elevated type 1 error rates (false-positive) (Sainani, published ahead of print; Welsh & Knight, 2015). These suggested shortfalls are rigorously defended by proponents of MBI (Batterham & Hopkins, 2015; Buchheit, 2016; Hopkins & Batterham, 2016). No doubt, scientific debate will continue with respect to the 'pros and cons' of MBI relative to more traditional statistics approaches. Regardless, MBI appears to be favoured by numerous researchers and practitioners in a non-clinical context for the flexibility that it provides over more traditional statistical methods and the qualitative interpretation of outcomes (non-clinical benefit and harm) relevant to sport performance.

Recommendations for Future Research

This thesis examined the influence of NO_3^- supplementation on short-term, high-intensity performance and a range of physiological measures using a range of dosing strategies. Additionally, the effectiveness of NO_3^- on 4 km performance was assessed in both ambient, and hot environments.

- Wherever possible, populations of greater aerobic calibre should be used to determine the effectiveness of NO_3^- supplementation on effects for performance and economy given that the majority of research to date demonstrates reduced effects as athlete ability increases. Furthermore, the use of a reference group of recreationally trained participant as a secondary cohort may provide more

evidence as to what factors and/or characteristics determine the effectiveness of NO_3^- as a performance aid.

- As with other nutritional or training interventions, it is apparent that there are responders and non-responders to treatments. As such, analysis of individual response(s) to NO_3^- supplementation should be considered inline with mean group outcomes. Furthermore, researchers should endeavour to elucidate the mechanisms underpinning positive responders to NO_3^- by measuring characteristics, such as fibre type, which may influence the responsiveness of NO_3^-. Additionally, given several investigations have demonstrated strong correlated changes in basal plasma [NO_2^-] with performance changes as a result of NO_3^-, the influence of basal [NO_2^-], in addition to muscle [NO_2^-] on performance outcomes should be encouraged.
- While the majority of NO_3^- related research has employed male participants, relatively few studies have assessed the effectiveness of NO_3^- on female populations. To date, consumption of NO_3^- has been shown to have substantial positive effects in elite female athletes in performances of 2 min, whereas in longer duration events their appears to be little support for NO_3^-.
- In study 3 (Chapter 5), we determined that NO_3^- supplementation increased rectal temperature in moderate activity cycling in hot environments, however, this increase did not alter time-trial performance relative to placebo conditions. Therefore, research assessing the effects of NO_3^- on longer duration endurance events in hot climates is warranted.

Conclusions

In summary, it can be concluded that NO_3^- supplementation improves short-term, high-intensity performance in well-trained, but not highly-trained cyclists in ambient temperatures. Moreover, a range of physiological measures are unchanged regardless of dose duration and athlete calibre. However, there is a potential for performance decrement following short duration dosing in very short (80 s) cycle time trials suggesting some caution is needed with NO_3^- prescription in highly-trained athletes. Finally, despite small alterations in thermoregulation during moderate intensity cycling in the heat, 4 km time-trial performance in a hot environment is not altered with NO_3^- supplementation in well-trained cyclists. Collectively these findings provide useful information to coaches,

practitioners and researchers as to the usefulness (and relative risk) of NO_3^- supplementation in well-trained competitive cyclists in ambient and hot environments.

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APPENDICES

Appendix 1: Approved Ethics Application

AUCKLAND UNIVERSITY OF TECHNOLOGY
TE WĀNANGA ARONUI O TAMAKI MAKAU RAU

MEMORANDUM

Auckland University of Technology Ethics Committee (AUTEC)

To: Andrew Kilding

From: **Dr Rosemary Godbold and Madeline Banda** Executive Secretary, AUTEC

Date: 12 April 2011

Subject: Ethics Application Number 10/309

1. The effect of nitrate supplementation on physiology and performance in well trained cyclists.

2. The dose response effect of supplemented nitrate on physiology and performance in well trained cyclists.

3. A comparison of natural vs pharmacological supplemented nitrate on physiology and performance of well trained cyclists.

Dear Andrew

Thank you for providing written evidence as requested. We are pleased to advise that it satisfies the points raised by the Auckland University of Technology Ethics Committee (AUTEC) at their meeting on 13 December 2010 and that on 8 March 2011, we approved your ethics application along with an amendment altering the participants from kayakers to cyclists. This delegated approval is made in accordance with section 5.3.2.3 of AUTEC's *Applying for Ethics Approval: Guidelines and Procedures* and is subject to endorsement at AUTEC's meeting on 9 May 2011.

Your ethics application is approved for a period of three years until 8 March 2014.

We advise that as part of the ethics approval process, you are required to submit the following to AUTEC:

- A brief annual progress report using form EA2, which is available online through http://www.aut.ac.nz/research/research-ethics/ethics. When necessary this form may also be used to request an extension of the approval at least one month prior to its expiry on 8 March 2014;
- A brief report on the status of the project using form EA3, which is available online through http://www.aut.ac.nz/research/research-ethics/ethics. This report is to be submitted either when the approval expires on 8 March 2014 or on completion of the project, whichever comes sooner;

It is a condition of approval that AUTEC is notified of any adverse events or if the research does not commence. AUTEC approval needs to be sought for any alteration to the research, including any alteration of or addition to any documents that are provided to participants. You are reminded that, as applicant, you are responsible for ensuring that research undertaken under this approval occurs within the parameters outlined in the approved application.

Please note that AUTEC grants ethical approval only. If you require management approval from an institution or organisation for your research, then you will need to make the arrangements necessary to obtain this.

When communicating with us about this application, we ask that you use the application number and study title to enable us to provide you with prompt service. Should you have any further enquiries regarding this matter,

you are welcome to contact Charles Grinter, Ethics Coordinator, by email at ethics@aut.ac.nz or by telephone on 921 9999 at extension 8860.

On behalf of AUTEC and ourselves, we wish you success with your research and look forward to reading about it in your reports.

Yours sincerely

Dr Rosemary Godbold and Madeline Banda

Executive Secretary

Auckland University of Technology Ethics Committee

Cc: Joe McQuillan , Paul Laursen

Appendix 2: Chapter 4 Supplementary Materials

Application for High Performance Sport New Zealand Research Award

Background:

The use of dietary nitrate supplementation by way of inorganic beet juice has been heralded for lowering the oxygen cost of exercise and increasing stamina (Bailey, Vanhatalo, et al., 2009; Larsen et al., 2010), enhancing cycle time-trial performance (Cermak, Gibala, et al., 2012) and contributing towards improved overall health through numerous therapeutic mechanisms (Benjamin et al., 1994; Lundberg et al., 2006; Wootton-Beard & Ryan, 2011). It is therefore no surprise that the use of dietary supplementation of nitrate is growing in popularity with endurance athletes (Maughan, Greenhaff, & Hespel, 2011).

The use of dietary nitrate supplementation by way of inorganic beet juice has been heralded for lowering the oxygen cost of exercise and increasing stamina, enhancing cycle time-trial performance and contributing towards improved overall health through numerous therapeutic mechanisms. It is therefore no surprise that the use of dietary supplementation of nitrate is growing in popularity with endurance athletes.

In an attempt to extend the body of knowledge in nitrate supplementation we have selected to use well-trained cyclists (VO_2max >60 ml/kg/min, age 18 – 39 y) as our research population. As yet there are only 2 published nitrate supplementation studies on trained athletes (VO_2max ~58 ml/kg/min) and currently, there is no published data on the effects of dietary nitrate supplementation on elite athletes. Despite this, numerous professional athletes and teams use nitrate supplementation via beetroot juice on a regular basis due to the research profile, therapeutic benefits and antioxidant capacity it provides.

We are interested in investigating changes in performance in short-term, high-intensity endurance activities with durations between 3 and 8 min. This time band encompasses a number of sporting disciplines including:

- All rowing events
- Mens 4000 m Team Pursuit - Cycling
- Womens 3000 m Team Pursuit - Cycling

- Mens 1000 m K1, K2 and K4 flat-water kayak events
- 400 m Swimming events
- 1500 m athletics track event

Why these events?

- These events will likely contribute the greatest portion of medals for New Zealand at the London 2012 Olympics
- These events require ~70 to 80% aerobic contribution to overall performance and require athletes to be at optimal aerobic capacity
- VO_2peak is reached in the initial stages of the event and this steady state is held for the duration of the event
- When similar intensity demands are made on nitrate supplemented subjects time to steady state lowering of VO_2 for the same workload and reaching the required steady state quicker thus reducing the reliance on energy from the anaerobic system

Overall Research Aims:

The aim of this series of research studies is to:

- Establish effective loading and washout protocols for sports
- Determine the impact of nitrate supplementation across a range of performance and physiology measures

Physiological and Performance Enhancements:

Following consumption of 500 mL [~5.2 mmol] of exogenous nitrate (NO_3^-) and subsequent absorption via the stomach wall, ~25% is reduced to nitrite (NO_2^-) and re-circulated via dorsal surfaces of the tongue (McKnight et al., 1997) with subsequent nitrate and nitrite blood plasma levels rapidly peaking ~2.5 h following ingestion (Webb et al., 2008). Continued supplementation over a 5 to 15 day period has shown a substantially moderated rise in NO_3^- and NO_2^- blood plasma levels (Vanhatalo et al., 2010) with similar enhancements in economy and kinetics of oxygen to previous studies employing identical dosing over a 6 day period (Bailey, Vanhatalo, et al., 2009; Larsen et al., 2007; Larsen et al., 2010). Similarly a 6 day supplementation period utilising 140

mL [8.0 mmol] NO_3^- has improved 10 km time-trial performance in trained cyclists by 1.3% (Cermak, Gibala, et al., 2012).

Mechanistically, three days of nitrate dietary supplementation may be required to affect changes in mitochondrial efficiency, however these changes are not likely due to either alterations in the density or biogenesis of the mitochondria (Larsen et al., 2011).

How Could These Findings Contribute to Success at London 2012?

While there is acknowledged potential for performance enhancement following dietary nitrate supplementation there is a clear lack of agreement over loading protocols or if in fact elite athletes will be advantaged when using it. This could be overcome under the following proposal:

Firstly, the establishment of loading protocols and knowledge of the washout time for this supplement will ensure that optimal levels of nitrite are available to allow maximum benefit from loading and ensure safety for the athlete.

Secondly, ongoing review of relevant literature will establish a comprehensive knowledge base of nitrate supplementation, leading to continued refinements of any currently established protocols.

This knowledge will be transferred to the field via presentations, fact sheets, emails and verbally enabling sport support personal to establish the optimal strategy for nitrate supplementation and evaluate the supplements effectiveness on elite athletes. This ongoing cycle ensures that practical application is backed by scientific research.

Research Methodology & Outline of Research

The series of studies that will contribute to the research outcomes are at various stages of completion. Ethics has been granted for all studies. In all studies we employ a double blinded, cross-over research design and recruit well-trained cyclists (VO_2peak >60 $ml.kg.^{-1}min^{-1}$), training >300 $km.week^{-1}$, aged between 18 and 39 years. All cycling assessments are carried using a Velotron cycle ergometer allowing for replication of the participants reach and height from their own bike. The Velotron has a reported accuracy of ±1.5%. As with all data collection procedures the sampling, processing, storage and analysis of blood for measurement of plasma nitrate and plasma nitrite assays will be carried out on premises at the AUT SPRINZ lab.

Physiological measurements of interest include VO_2, VCO_2, V_E and RER along with oxygen kinetic response at moderate and high domain intensity workloads. To determine the impact of nitrate supplementation on performance measures of incremental peak power and time-trial distances of 1 and 4 km will be used.

The Effect of Nitrate Supplementation on Physiology and Performance in well-trained Cyclists

This study will investigate the impact of six days of nitrate supplementation on measures of peak power, exercise economy, on- and off- kinetic response at moderate and heavy domain intensity workloads and 4 km time-trial performance. Following baseline testing subjects will consume either placebo or nitrate rich beet-it juice over a six d period. On day four of the loading phase participants completed an incremental ramp test to exhaustion. On days five and six subjects complete two x 6 min moderate- and one x 6 min heavy- domain intensity bout of cycling. The step-wise design incorporated active rest periods of six min prior to, between and following each of the three workloads. On the seventh day subjects will complete a 4 km time-trial.

The aim of this study is to:

1. Determine time to peak nitrate/nitrite blood plasma concentrations following six days of nitrate supplementation
2. Determine the washout time of nitrate/nitrite blood plasma following six days of nitrate supplementation
3. Determine the effect of six days of nitrate supplementation on peak power, exercise economy, oxygen kinetic response and 4 km time-trial performance

Progress

Ethics has been granted and an initial group of cyclists identified for the research project.

Sport Personnel and HPSNZ Discipline Leads in Support of Application

*Bike NZ – Andy Reid, Craig Palmer, Daniel Healey

*Rowing NZ – Christel Dunshea-Mooij

Paul Laursen – Exercise Physiology Discipline Lead

Ien Hellemens – Sport Nutrition Discipline Lead

**Sports in conjunction with support staff currently trialling nitrate supplementation in training and/or competition with loading protocol suggestions via primary researcher.*

Investment Required

Completion date for research aims and reporting to sports is Monday 4th June 2012. An investment of $7000 is sought as a research assistant scholarship for the primary researcher to be paid in two equal installments of $3500 initially, at the agreement of the contract and subsequently, at the conclusion of the research finding presentation to sports (4th June 2012).

Appendix 3: Chapters 3 to 5 Supplementary Materials

Subject Information Packs

Participant Information Sheet

Date Information Sheet Produced:
26th November 2010

Project Title
The Effect of Nitrate Supplementation on Physiology and Performance in well-trained Cyclists

An Invitation
Hi, my name is Joe McQuillan and I am a PhD student at AUT University. Along with my supervisors Associate Professor Andrew Kilding and Adjunct Professor Paul Laursen I am inviting you to help with a project that looks at the role of nitrate supplementation in enhancing cycling physiology and performance. You should decide whether or not you would like to be involved. You don't have to be involved, and you can stop being involved in the study at any time.

What is the purpose of this research?
The purpose of the research is to investigate the impact of beetroot supplementation on cycling performance and physiology. Numerous supplements are promoted as being beneficial for enhancing sport performance. While research does support the use of a number of supplements the majority of supplements promoted to aid performance have little if any supporting research to support the manufacturers claims. This study aims to establish whether nitrate supplementation in the form of beetroot juice is a beneficial

supplement for elite athletes. As yet no research exists into the impact of nitrate supplementation on well-trained athletes therefore this study provides an opportunity to be involved in novel and unique research.

How was I chosen for this invitation?

You (or your coach) have expressed interest in the research and you meet the criteria for the study.

What will happen in this research?

The study will consist of an 8 d supplementation period (beetroot juice or placebo) followed by a 10 d wash-out period before the second 8 d of supplementation (beetroot juice or placebo). You will be given either 70 cL·day of beetroot juice (nitrate concentration of 6.2 mM) or a placebo to consume following your morning training session. During the study you will be required to undergo a number of performance measures as described below. You will perform these tests before and after the two x 8 day periods of supplementation. During one of the two supplementation periods you will be randomly placed in either a control group and you will receive a placebo drink in place of the nitrate-containing supplemented drink. Your blood pressure and plasma nitrate levels (from a sample of your blood) will be measured on days 1, 4 and 7 of the familarisation/baseline assessment period, days 9, 12 and 15 of the first supplement period, days 17, 19 and 24 of the washout period and days 27, 29, 32 and 35 of the second supplement period. The figure below will help you understand the time line and testing requirements for this study.

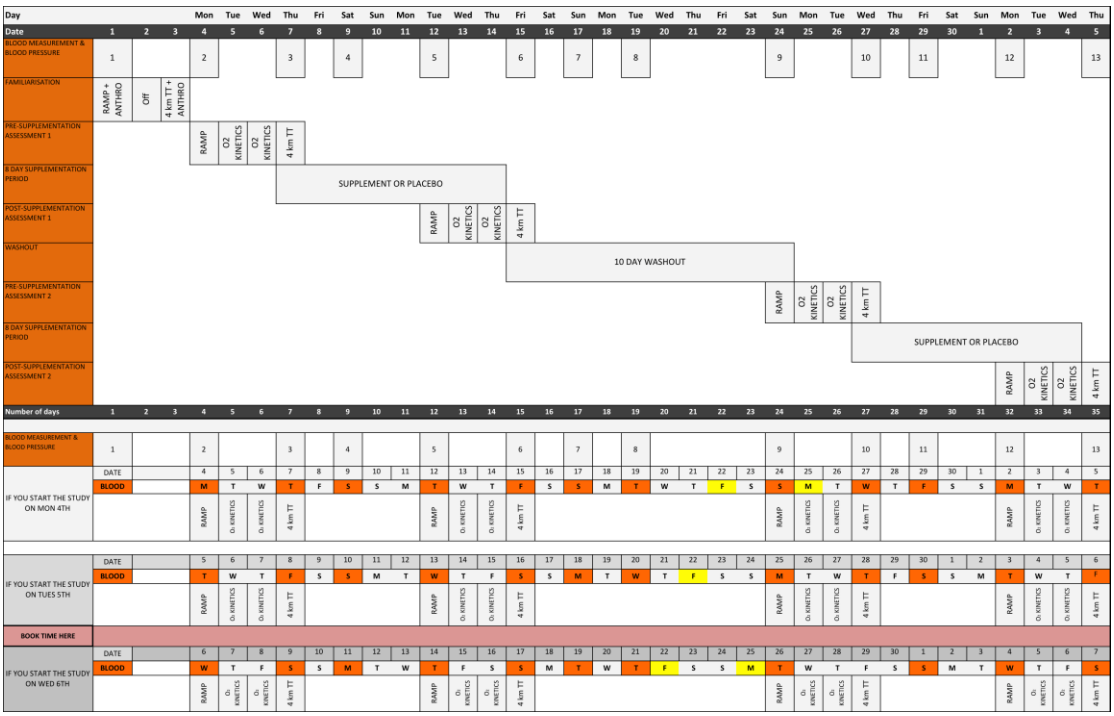

Fig 1. Schematic of study design

Testing

Incremental Ramp Assessment, Oxygen Kinetics and 4 km time-trial performance

Prior to, and after each of the two supplementation periods you will undergo four consecutive days of assessments. Day one consists of an incremental ramp assessment to determine your ventilatory threshold and VO_2peak. On days two and three you will ride at fixed workloads (wattages) based on day one results and on day four you will carry out a 4 km time-trial to establish time and average power over the distance. All testing will be carried out on a Velotron cycle ergometer which we will adjust to replicate your bike set-up. During the incremental ramp assessment we will measure your submaximal and maximal VO_2 using a metabolic cart, which will involve you wearing a mask for the duration of the test. Throughout the assessment heart rate will be recorded using a heart rate monitor while oxygen and carbon dioxide will be measured using the metabolic cart. Towards the completion of the ramp assessment the intensity will be similar to what you experience in a competitive hill ascent.

During the two days of fixed workloads (days two and three) you will be required to cycle for 42 min at a cadence of 70 RPM to enhance the accuracy of the data collected over this time. There are 7 x 6 min blocks to be completed with four of these at 50 W which serve

as a baseline from which we will measure the change of oxygen requirement and carbon dioxide build up during the three workload periods.

Monitoring of training and competition

During periods of training and competition it is necessary to record power output, heart rate, speed and distance. To do this your bike will be fitted with an SRM power meter to record training volume and intensity. You will have access to SRM software to download your training and racing and will also be given access to Training Peaks on-line to upload your training data. This data will be used to quantify your training throughout the research study. Outside of the assessment schedule you should train and race as normal. It is vitally important that you do not increase or decrease your training volume or training intensity over the period of the study as this may affect the physiological response and there for the outcome of your data.

Skinfolds

During one of the first four assessment days we will measure your body dimensions using skinfold callipers and a tape measure. This will take place at the University lab at the Millennium Institute for Sport and Health in Mairangi Bay.

Blood Nitrate and Blood Pressure Measures

We will measure the amount of nitrate in your blood and your blood pressure a total of 13 times during the study. Four of these occasions will be on the day of an assessment in the lab with the remaining seven being completed during the supplementation period. For all these assessments you will need to come to the Millennium Institute for Sport and Health in Mairangi Bay. A trained phlebotomist will use a small needle to draw a small sample of blood from your forearm vein. We will send your sample away to an independent laboratory.

What are the discomforts and risks?

You may experience some temporary discomfort (exertion) during the incremental ramp assessment and the 4 km time-trial. This will be similar to what you feel during hard training and racing (heavy breathing, tired muscles). However, if you experience any excessive discomfort you will be able to stop the test at anytime. Temporary discomfort

will also be experienced when taking blood. This usually subsides within 1 min of the blood sample being taken.

How will these discomforts and risks be alleviated?

The research student is a qualified first aid responder and a medical clinic is located within the building where the lab testing will take place. Cool water will be offered at the end of the assessments and adequate measures will be taken if you feel at all dizzy during the assessments. You will have sufficient time to warm-up prior to starting the assessments. Trained phlebotomists are experienced in minimising stress and discomfort during physical measures.

What are the benefits?

You will benefit from this study by:

- understanding how nitrate supplementation may affect your cycling performance
- establishing markers of fitness such as VO_2peak at this current period in your cycling career
- receiving free blood pressure measurements and a free skinfold assessment
- having access to a SRM power meter and Training Peaks for the duration of the study

What compensation is available for injury or negligence?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

All information related to you will be coded in order to ensure that you cannot be identified. The information will remain in locked storage and will only be accessible to the people of the cycling assessment project. No-one will be able to identify you from any of the summary findings for the report of the project.

What are the costs of participating in this research?

The only cost to you to participate in this study is that of time. Aside from training and racing that make up part of your normal routine, there are a total of 22 occasions on which you will be required to visit the University lab. The first day of the study you should allow 90 min to complete forms, have blood drawn and have your skinfold measured prior to the ramp assessment. On subsequent days when you have blood pressure and blood nitrate measures along with testing to carry out you should allow 75 min. These are days 4, 7, 12, 15, 24, 27, 32 and 35. On the days when you have only blood pressure and blood nitrate measures to carry out you should allow 30 min. These are days 9, 17, 19 and 29. The remaining days where you have oxygen kinetic measure being measured you should allow 60 min. The performance, blood pressure and blood nitrate volume assessments will take place at the University lab at the Millennium Institute for Sport and Health in Mairangi Bay.

How will being involved in the research impact on my training during the study?

During the training there will be times when you will not be able to train as an example during the assessment phases. Periods when this will occur will be covered on your first visit to the lab.

What opportunity do I have to consider this invitation?

- You may take the time you need and decide whether or not you would like to be involved
- You can stop being involved in the project at any point

How do I agree to participate in this research?

If you agree to participate please fill in the attached consent form and return to myself.

Will I receive feedback on the results of this research?

Yes, feedback will be provided to you, if you request it. The results will also be passed onto your coach if you request it.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor:

Assoc. Prof. Andrew Kilding, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, *madeline.banda@aut.ac.nz*, Ph 921 9999 ext 8044.

Whom do I contact for further information about this research?

Researcher contact details:

Joe McQuillan, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Auckland 0637, Ph 921 9999 ext. 7119, joe.mcquillan@aut.ac.nz

Project supervisor contact details:

Assoc. Prof. Andrew Kilding, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee on 8th March 2011, AUTEC Reference number 10/309.

Participant Information Sheet

Date Information Sheet Produced:
10th January 2012

Project Title
The Effects of Nitrate Supplementation on Time-Trial Performance in Well Trained Cyclists.

An Invitation
Hi, my name is Joe McQuillan and I am a PhD student at AUT University. Along with my supervisors Associate Professor Andrew Kilding and Adjunct Professor Paul Laursen I am inviting you to help with a project that looks at the effect of nitrate supplementation on cycling time-trial performance over distances of 1 and 4 km. You should decide whether or not you would like to be involved. You don't have to be involved, and you can stop being involved in the study at any time.

What is the purpose of this research?
The purpose of the research is to establish whether supplementing your normal diet with an increased amount of nitrate by way of natural beetroot juice reduces the time to complete 1 and 4 km time trial distances and improves cycling economy. There is evidence to suggest that ingestion of nitrate enhances time trial performance and lowers the cost of exercise in sedentary and moderately trained populations however there are no current studies reporting the effects of nitrate loading on these measures in well-trained cyclists.

How was I chosen for this invitation?
You or your coach expressed interest in this study and you fit the study criteria.

What will happen in this research?
The study will consist of a 7 d supplementation period of 140 ml beetroot juice. The 140 ml will be made up of either 140 ml nitrate rich beetroot juice (A) or 70 ml nitrate rich

beetroot juice and 70 ml placebo (B). The first treatment period (A or B) will be followed by a 7 d wash-out period before a second 7 d supplementation period (A or B). During the study you will be required to undergo a number of assessments which are described below. You will perform these tests prior to, and during the two x 7 d periods of supplementation. These assessments will be carried out using a Velotron cycle ergometer which will be set-up as closely as possible to your own bikes specifications. Your blood pressure and plasma nitrate levels (from a sample of your blood) will be measured twice prior to the supplementation periods and prior to each of the eight time-trials held during the supplementation period. During the washout week you will not be required to complete any assessments or undergo any testing.

Incremental Ramp Assessment

Utilising a starting power of 50 W and increasing linearly each min by 25 W, the ramp assessment will be used to determine ventilatory thresholds (VT_1 and VT_2), VO_2max and peak power output, all of which will be used towards description of study participant characteristics. Establishment of peak power will allow the calculation of the wattages for exercise economy measures. Over the course of the ramp assessment we will record your breathing response by having you breathe into a mask.

Exercise Economy Measurement

Using your peak power output from the incremental ramp assessment, workloads of 40, 50 and 60% will be used to measure your exercise economy prior to the start of *all* 1 km and 4 km time trials. This 30 min measurement with workloads ascending every 10 min and will require a mask to be worn during this time in order that we record ventilatory responses. The exercise economy measurement will act as the warm-up prior to all the 1 km and 4 km TT during the familiarisation, baseline testing and supplementation periods.

1 km and 4 km Time-Trial Performance

On days *three* and *six* of the supplementation periods you will be required to complete a 4 km time-trial and on days *four* and *seven* you will be required to complete a 1 km time-trial.

Both the 1 km and 4 km TT will be completed on the same cycle ergometer as previously described and involve covering the 4 km distance as quickly as possible. At the

conclusion of the time-trial we will record average power, average cadence, average heart rate and time for completion of the distance.

Skinfolds

During the first week of the study we will measure your body dimensions using skinfold callipers and a tape measure. This will take place at the University lab at the Millennium Institute for Sport and Health in Mairangi Bay prior to an assessment.

Blood Nitrate and Blood Pressure Measures

We will measure the amount of nitrate in your blood and your blood pressure prior to and after all time-trial performances over the course of the study. This will take place at the University lab at the Millennium Institute for Sport and Health in Mairangi Bay. A trained phlebotomist will use a small needle to draw 10 ml of blood from your forearm vein. This will later on be analysed for concentrations of plasma nitrate and nitrite.

What are the discomforts and risks?

You may experience some temporary discomfort (exertion) towards the end of the incremental ramp assessment and during the 1 km and 4 km time-trials. This will be similar to what you feel during hard training and racing (heavy breathing, tired muscles). However, if you experience any excessive discomfort you will be able to stop the test at anytime. Temporary discomfort will also be experienced when taking blood. This usually subsides shortly after the blood sample is taken.

How will these discomforts and risks be alleviated?

The research student is a qualified first aid responder and a medical clinic is located within the building where the lab testing will take place. Cool water will be offered at the end of the assessments and adequate measures will be taken if you feel at all dizzy during the assessments. Warm-up protocols are built into all assessment and performance measures.

What are the benefits?

You will benefit from this study by understanding how nitrate supplementation may affect your cycling performance. You will also establish markers of fitness at this current period in your cycling career.

What compensation is available for injury or negligence?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

All information related to you will be coded in order to ensure that you cannot be identified. The information will remain in locked storage and will only be accessible to the people involved in the project. No-one will be able to identify you from any of the summary findings for the report of the project.

What are the costs of participating in this research?

The only cost to you is that of time. Aside from training and racing which are a part of your normal routine, there will be the initial skinfold measure during the first week. On the first day you should allow 90 min for the ramp test, have blood and blood pressure taken and to fill in forms. Subsequently, the *twelve* days of blood measurement, blood pressure recording, exercise economy measures and 1 km and 4 km TT performance will require 80 min each of your time. All time-trials, blood pressure and blood assessments will take place at the University lab at the Millennium Institute for Sport and Health in Mairangi Bay.

What opportunity do I have to consider this invitation?

- You may take the time you need and decide whether or not you would like to be involved
- You can stop being involved in the project at any point

How do I agree to participate in this research?

If you agree to participate please fill in the attached consent form and return to myself.

Will I receive feedback on the results of this research?

Yes, feedback will be provided to you, if you request it.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor, Assoc. Prof. Andrew Kilding, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, *madeline.banda@aut.ac.nz*, Ph 921 9999 ext 8044.

Whom do I contact for further information about this research?

Researcher Contact Details

Joe McQuillan, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Auckland 0637, Ph 921 9999 ext. 7119, joe.mcquillan@aut.ac.nz

Project Supervisor Contact Details

Assoc. Prof. Andrew Kilding, Institute of Sport & Recreation Research New Zealand, School of Sport and Recreation, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee on 8th March 2011, AUTEC Reference number 10/309

Participant Information Sheet

Date Information Sheet Produced: 5th December 2013.

Project Title

The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists.

An Invitation

Hi, my name is Joe McQuillan and I am a PhD student at AUT University. Along with my supervisors Assoc. Prof. Andrew Kilding and Assoc. Prof. Paul Laursen I am inviting you to help with a project that looks at the role of nitrate supplementation in enhancing cycling physiology and performance in the heat. You should decide whether or not you would like to be involved. You don't have to be involved, and you can stop being involved in the study at any time.

What is the purpose of this research?

The purpose of the research is to investigate the impact of beetroot supplementation on cycling performance in warm environments. There are a number of practices used prior to, and during competition to assist athletes to better cope with competing in hot climates. Often this will involve a number of days training in such temperatures, but sometimes this is not practical or affordable option for athletes or teams. This study aims to establish whether nitrate supplementation in the form of beetroot juice is a beneficial supplement for athletes competing in hot conditions. As yet no research exists into the impact of nitrate supplementation on well-trained athletes in the heat therefore this study provides an opportunity to be involved in novel and unique research.

How was I chosen for this invitation?

You (or your coach) have expressed interest in the research and you meet the entry criteria for an invitation to the study.

What will happen in this research?

The experimental phase of the study will consist of a 3 d supplementation period (beetroot juice or placebo) followed by a 6 d wash-out period before the second and finally third 3 d period of beetroot juice or placebo supplementation. You will be given either 140 ml

daily of beetroot juice (nitrate concentration of ~8.0 mmol) or a placebo (nitrate concentration of <0.1 mol) and asked to drink the beverage over each of the three days. On the third day of each period you will be asked to consume the 140 ml of juice 1.5 h prior to your scheduled appointment. During the study you will be required to undergo a number of physiological and performance assessments which are described below. You will perform these tests prior to, and during the three 3 day supplementation periods. All 4 km time-trial assessments will take place in a heat and humidity controlled chamber. The assessments prior to supplementation will be at ambient temperature. Your blood pressure and plasma nitrate levels (from a sample of your blood) will be measured on the days of each of the performance tests during each supplementation phase. The figure below will help you understand the experimental testing requirements for this study and indicates the first 2 of 3 experimental trials.

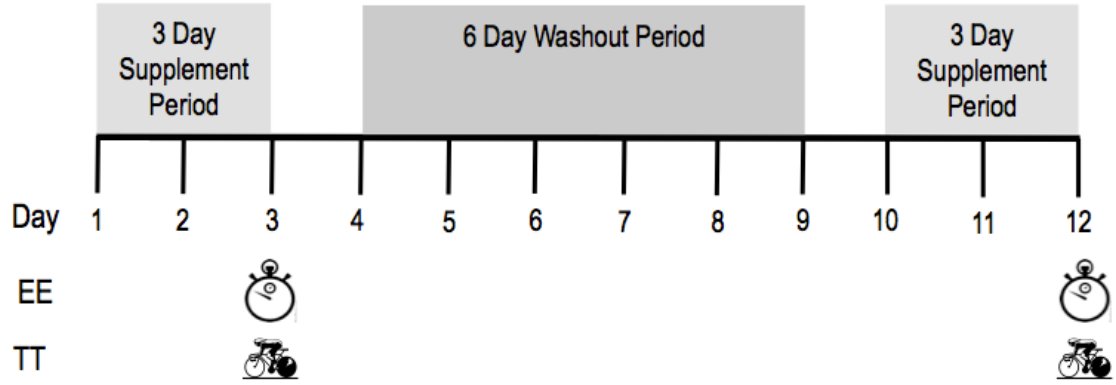

Figure 1. Schematic representation of experimental design.

EE = Exercise economy

TT = 4 km time-trial

Incremental Step Test and Exercise Economy

Prior to the start of the study you will undergo an incremental step test to determine your peak aerobic power (VO_2max), and peak power output (PPO) to ensure you meet the minimum fitness criteria for study inclusion ($VO_2max \geq 60$ ml.kg.$^{-1}$min^{-1}). This one-off test involves cycling on a cycle ergometer over continuous, multiple 3 min duration stages. At the end of each 3 min stage, the intensity (wattage) will increase until you can no longer maintain your cadence at the required power output. Your perceived effort for the final stages will similar to what you experience in a competitive hill ascent.

Exercise economy measurement will involve cycling for a total of 20 min at two intensities based on your PPO from the incremental assessment and will take place on five occasions, prior to each 4 km time-trial, thus serving as a controlled warm-up.

During both the incremental assessment and exercise economy measurements we will record your submaximal VO_2 using a metabolic cart, which will involve having a breathing tube in your mouth for the duration of the test. The incremental assessment will also establish your VO_2max. To ensure testing accuracy, a Velotron cycle ergometer will be used for all assessments in place of your own bike. We will replicate your own bikes measurements during the set-up of the Velotron. Heart rate will be assessed throughout all physiological tests using a heart rate monitor.

4 km Time Trial

The 4 km time trial will be carried out on the same cycle ergometer as the assessments described above. Prior to the time-trial you will complete a pre-programmed warm-up with the intensity of efforts based on the exercise economy assessment. The cycle ergometer will have a gearing arrangement that you can use to alter cadence and effort as required. On all occasions the time-trial should be completed as quickly as possible. Heart rate will be assessed throughout all time-trials with mean power, duration and average heart rate being recorded for analysis. The first two familiarisation time-trials will be carried out in the environmental chamber in the same conditions as the experimental trials.

Monitoring of Training

During your periods of training it is necessary to record your current volume (hr/wk) and perceived intensity of training. A training diary will be provided for you to note your daily training activity in. At all times you will compete and train as written in your training program recording all your cycling sessions in your training log.

Skinfold Measurement

During one of your first three visits we will measure your body dimensions using skinfold callipers and a tape measure. This will take place at the University lab at the AUT-Millennium in Mairangi Bay.

Blood Nitrate and Blood Pressure Measures

We will measure the amount of nitrate in your blood and your blood pressure twice during the study. On these occasions will be on the day of each experimental 4 km time-trial under either NIT or PLA conditions. For all these assessments you will need to come to the University lab at AUT-Millennium in Mairangi Bay. A trained phlebotomist will use a small needle to draw a small sample of blood from your forearm vein. We will later analyse this sample.

What are the discomforts and risks?

You may experience some temporary discomfort (exertion) during the incremental step test and 4 km time-trial performances. This will be similar to what you feel during hard training and racing (heavy breathing, tired muscles). However, if you experience any excessive discomfort you will be able to stop the test at anytime. Temporary discomfort will also be experienced when taking blood. This usually subsides within a few seconds of the blood sample being taken.

How will these discomforts and risks be alleviated?

The research student is a qualified first aid responder and a medical clinic is located within the building where the lab testing will take place. Cool water will be offered at the end of the assessments and adequate measures will be taken if you feel at all dizzy during the assessments. You will have sufficient time to warm-up prior to starting the assessments. Trained phlebotomists are experienced in minimising stress and discomfort during physical measures.

What are the benefits?

You will benefit from this study by understanding how nitrate supplementation may affect your competitive performance in the heat. You will also establish markers of fitness at this current period in your cycling career.

What compensation is available for injury or negligence?

In the unlikely event of a physical injury as a result of your participation in this study, rehabilitation and compensation for injury by accident may be available from the Accident Compensation Corporation, providing the incident details satisfy the requirements of the law and the Corporation's regulations.

How will my privacy be protected?

All information related to you will be coded in order to ensure that you cannot be identified. The information will remain in locked storage and will only be accessible to the people of the cycling assessment project. No one will be able to identify you from any of the summary findings for the report of the project.

What are the costs of participating in this research?

The only cost to you is that of time. Aside from training and racing which are part of your normal routine, there will be the initial skinfold measure during one of your six visits. The incremental step assessment will be approximately 30 min in length. You should allow 60 min for the exercise economy assessment warm-up and time-trial procedures. All performance, blood pressure and blood nitrate volume measurements will take place at the University lab at the AUT-Millennium in Mairangi Bay.

What opportunity do I have to consider this invitation?

- You may take the time you need and decide whether or not you would like to be involved
- You can stop being involved in the project at any point

How do I agree to participate in this research?

If you agree to participate please fill in the attached consent form and return to myself.

Will I receive feedback on the results of this research?

Yes, feedback will be provided to you, if you request it.

What do I do if I have concerns about this research?

Any concerns regarding the nature of this project should be notified in the first instance to the Project Supervisor:

Assoc. Prof. Andrew Kilding, Sport Performance Research Institute of New Zealand, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Concerns regarding the conduct of the research should be notified to the Executive Secretary, AUTEC, Madeline Banda, *madeline.banda@aut.ac.nz*, Ph 921 9999 ext 8044.

Whom do I contact for further information about this research?

RESEARCHER CONTACT DETAILS:

Joe McQuillan, Sport Performance Research Institute of New Zealand, AUT University, Auckland 0637, Ph 921 9999 ext. 7119, joe.mcquillan@aut.ac.nz

PROJECT SUPERVISOR CONTACT DETAILS:

Assoc. Prof. Andrew Kilding, Sport Performance Research Institute of New Zealand, AUT University, Private Bag 92006, Auckland 1020, Ph 921 9999 ext. 7056, andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee on 8th March 2011, AUTEC Reference number 10/309.

Appendix 3: Consent to participate in research

AUCKLAND UNIVERSITY OF TECHNOLOGY
TE WĀNANGA ARONUI O TAMAKI MAKAU RAU

Consent to Participation in Research

Title of Project: The Effects of Dietary Nitrate Supplementation on Physiology and Performance in Trained Cyclists**.**

Project Supervisor: **Associate Professor Andrew Kilding**

Researcher: **Joe McQuillan**

- I have read and understood the information provided about this research project (Information Sheet dated 26th November 2010)

  Yes/No

- I have had an opportunity to ask questions and to have them answered

  Yes/No

- I am not suffering from any injury or illness which may impair my physical performance Yes/No

- I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way Yes/No

- If I withdraw, I understand that all relevant information will be destroyed

  Yes/No

- I consent to my data being shared with my coach Yes/No

- I understand that the information collected will be used for academic/feedback purposes only and will not be published in any form outside of this project without my written permission Yes/No

- I agree to take part in this research Yes/No

- I understand that I can request to have any blood/biological fluid taken returned to me Yes/No

- I wish to receive a copy of the report from the research: ***tick one***: Yes O No O

Participant signature:.....……..

Participant name: …….

Date: …….

Participant's Contact Details:

……..

……..

Project Supervisor Contact Details:

Associate Professor Andrew Kilding

Sport Performance Research Institute of New Zealand

School of Sport and Recreation

Auckland University of Technology

Private Bag 92006

Auckland 1020

Ph 921 9999 ext. 7056

Andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee 12th April 2011

AUTEC number 10/309

Consent to Participation in Research

Title of Project: The Effects of Single and Double Dosing Strategy of Nitrate Supplementation on Time-Trial Performance in Well Trained Cyclists**.**

Project Supervisor: **Associate Professor Andrew Kilding**

Researcher: **Joe McQuillan**

- I have read and understood the information provided about this research project (Information Sheet dated 10th January 2012) Yes/No
- I have had an opportunity to ask questions and to have them answered Yes/No
- I am not suffering from any injury or illness which may impair my physical performance Yes/No
- I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way Yes/No
- If I withdraw, I understand that all relevant information will be destroyed Yes/No
- I consent to my data being shared with my coach Yes/No
- I understand that the information collected will be used for academic/feedback purposes only and will not be published in any form outside of this project without my written permission Yes/No
- I agree to take part in this research Yes/No
- I understand that I can request to have any blood/biological fluid taken returned to me Yes/No
- I wish to receive a copy of the report from the research: ***tick one***: Yes O No O

Participant signature:.....……..

Participant name: …….

Date: …….

Participant's Contact Details:

……..

……..

Project Supervisor Contact Details:

Associate Professor Andrew Kilding

Sport Performance Research Institute of New Zealand

School of Sport and Recreation

Auckland University of Technology

Private Bag 92006

Auckland 1020

Ph 921 9999 ext. 7056

Andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee 12th April 2011

AUTEC number 10/309

Consent to Participation in Research

Title of Project: The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists

Project Supervisor: **Associate Professor Andrew Kilding**

Researcher: **Joe McQuillan**

- I have read and understood the information provided about this research project (Information Sheet dated 5th December 2013)
  Yes/No
- I have had an opportunity to ask questions and to have them answered
  Yes/No

- I am not suffering from any injury or illness which may impair my physical performance Yes/No
- I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way Yes/No
- If I withdraw, I understand that all relevant information will be destroyed
  Yes/No
- I consent to my data being shared with my coach
  Yes/No
- I understand that the information collected will be used for academic/feedback purposes only and will not be published in any form outside of this project without my written permission
  Yes/No
- I agree to take part in this research Yes/No
- I understand that I can request to have any blood/biological fluid taken returned to me
  Yes/No
- I wish to receive a copy of the report from the research: ***tick one***: Yes O No O

Participant signature:.....……..

Participant name: …….

Date: …….

Participant's Contact Details:

……..

……..

Project Supervisor Contact Details:

Associate Professor Andrew Kilding

Sport Performance Research Institute of New Zealand

School of Sport and Recreation

Auckland University of Technology

Private Bag 92006

Auckland 1020

Ph 921 9999 ext. 7056

Andrew.kilding@aut.ac.nz

Approved by the Auckland University of Technology Ethics Committee 12th April 2011

AUTEC number 10/309

Appendix 4: Chapter 4 Supplementary Materials

SRM Download & Maintenance Instructions

Recommend using the PC software when using SRM software

Download the latest software:
Version # 6.42.06
http://www.srm.de/index.php/us/support/phoca-down-test/category/17-srm-trainings-system-software

Download PC7 manual
http://www.srm.de/index.php/us/support/phoca-down-test/category/26-pc-7

Download PC6 manual
http://www.srm.de/index.php/us/support/phoca-down-test/category/9-powercontrol

Download SRM Manual
http://www.srm.de/index.php/us/support/phoca-down-test/category/14-powermeter

Maintenance of the Power Meter and Power Control

Power Meter

- If washing your bike with a high-pressure hose avoid cleaning the SRM power meter as it will force water into the housing and ruin the circuit board
- Avoid travelling with the bike on top of the car in rain – if you have to travel and can't put the bike inside the car place a plastic bag over the SRM power meter for the travel.

Power Control

- Keep the battery charged. This means plugging in every 4 days for a PC6 and every 14 days for a PC7. The batteries do not like being run down as it will shorten their effective life

- Take the power control off the handle bar clip before travelling with the bike on the outside of the car as it may come off during travel
- Download regularly or bring your power control in to download in the lab on your visits.