Dietary nitrate supplementation: physiological responses during prolonged exercise and optimizing nitric oxide bioavailability

Rachel Tan

Submitted to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Sport and Health Sciences

July 2019

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: .....

Abstract

Dietary nitrate supplementation has been evidenced to lower the oxygen cost of submaximal exercise and, in some circumstances, to be ergogenic. Recent advances indicate that the mechanistic bases involve the independent or combined effects of nitric oxide-mediated improvements of contractile efficiency, mitochondrial efficiency, tissue perfusion, and/or redox signalling with effects perhaps being greater in type II muscle fibres. The aims of this thesis were: 1) to explore the potential effects of dietary nitrate supplementation on physiological responses in prolonged endurance exercise; and 2) to investigate potential supplementation strategies to enhance nitric oxide bioavailability during such exercise. In all four original investigations of the present thesis, the subjects consisted of healthy recreationally active adults who volunteered to participate. The subjects underwent various nitrate supplementation regimens, invasive and non-invasive physiological measurements, and a battery of exercise tests to assess the influence of dietary nitrate supplementation on attenuating fatigue during prolonged type exercise and enhancing subsequent exercise performance. The original findings from the present thesis indicate that the favourable effects of dietary nitrate supplementation are likely ascribed to its nitrate content and that nitrate supplementation may be a strategy to attenuate the decline in physiological function (i.e. intramuscular glycogen depletion, phosphocreatine depletion, and decline in muscle excitability) during prolonged cycling exercise lasting 1 to 2 h. In addition, results also showed that nitric oxide bioavailability can be influenced by ingesting an additional dose of nitrate during exercise and/or co-ingesting nitrate with a reduced thiol donor (i.e. N-acetylcysteine), which may subsequently influence the efficacy of nitrate. Together, the findings from the present thesis extend knowledge by indicating that dietary nitrate supplementation holds potential for aiding individuals in performing endurance exercise lasting ≥ 1 h and that altering the supplementation procedure has potential to increase nitric oxide bioavailability. These novel contributions have important implications for the application of dietary nitrate supplementation, particularly in endurance events.

Table of contents

EXPERIMENTAL CHAPTERS

List of tables

List of figures

Chapter 2 Literature Review

example, NO reacts with $O_2^{\bullet-}$ to form reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) (Halliwell, 1994; Reid & Durham, 2002). Excessive ROS and RNS production may impair contractile function by altering Ca^{2+} handling and myofilament function (Reid, 2016a).

Figure 2.4 The pulmonary $\dot{V}O_2$ response following the onset of constant work-rate exercise in the moderate-, heavy-, and severe-intensity domains. Abbreviations: GET (gas exchange threshold), CP (critical power), $\dot{V}O_{2max}$ (maximal aerobic capacity). Adapted from Wilkerson et al. (2004).

Figure 2.5 A schematic diagram of the pathways for nitric oxide (NO) production. In the NOS-dependent pathway, nitric oxide synthase (NOS) enzymes catalyse the production of NO using substrates L-arginine and oxygen (O_2), along with cofactors calmodulin/calcium (Ca^{2+}), nicotinamide dinucleotide phosphate (NADPH), flavin adenine nucleotide (FAD), flavin mononucleotide (FMN), haem iron (Fe) and 5,6,7,8-tetrahydrobiopterin (BH_4). In the NOS-independent pathway, NOS-derived and exogenous sources of nitrate (NO_3^-) undergoes reduction to form nitrite (NO_2^-), a reaction catalysed by anaerobic facultative bacteria in the oral cavity. NO_2^- is further metabolized in a process facilitated by xanthine oxidase (XOR), deoxyhaemoglobin (deoxy-Hb), deoxymyoglobin (deoxy-Mb) and mitochondrial respiration complexes into NO and S-nitrosothiols (RSNO) which can induce signalling and post-translational protein modifications. This reduction process is potentiated by hypoxic and acidic environments. In addition, RSNO may decompose and release NO. Endogenous and exogenous (i.e. dietary derived) NO will recycle to form higher nitrogen oxides NO_2^- and NO_3^-. Through the NO_3^--NO_2^--NO pathway, ingestion of NO_3^--rich foods may increase NO_2^- and NO production.

Figure 2.6 A simplified schematic diagram redrawn from Gilchrist et al., 2011. The enterosalivary circulation of NO_3^- from dietary sources in the NO_3^-–NO_2^-–NO pathway: 1) endogenous NOS-derived NO_3^- mixed with exogenous NO_3^- from the diet is converted into NO_2^- by facultative anaerobic bacteria in the oral cavity and is swallowed; 2) a portion of the NO_2^- is reduced into NO in the stomach (McKnight et al., 1997) by acid solutions and a portion of NO_2^- enters systemic circulation undergoes a single electron reduction to form NO in reactions catalysed by NO_2^- reductases (see text for details); 3) rest of NO_3^- is absorbed by the kidneys; and 4) NO_3^- is excreted in the urine.

Chapter 4 **Dietary nitrate supplementation and the oxygen cost of exercise: the influence of the number of transitions and nitrate-depleted beetroot juice**

Figure 1 Mean ± SE plasma nitrate (NO_3^-; panel A) and nitrite (NO_2^-; panel B) at rest following: 1) CON, water supplementation consumed before exercise (solid circle and solid line); 2) PL, nitrate-depleted beetroot juice consumed before exercise (open circle and dashed line); and 3) BR, nitrate-rich beetroot juice consumed before exercise (solid triangle and dotted line). * = significantly different from CON and PL (P<0.05).

Figure 2 Individual responses of plasma nitrate (NO_3^-; panel A, B, C) and nitrite (NO_2^-; panel D, E, F) on day 1 and 2 following: 1) CON, water supplementation consumed before exercise; 2) PL, nitrate-depleted beetroot juice consumed before exercise; and 3) BR, nitrate-rich beetroot juice consumed before exercise.

Figure 3 Mean ± SE and individual responses of pulmonary O_2 uptake averaged over 4 step transitions (panel A) following: 1) CON, water supplementation consumed before exercise; 2) PL, nitrate-depleted beetroot juice consumed before exercise; and 3) BR, nitrate-rich beetroot juice consumed before exercise.

Chapter 5 **Beetroot juice ingestion *during* prolonged moderate-intensity exercise attenuates progressive rise in O_2 uptake**

Figure 1 Data are means ± SE of plasma nitrate (A) and nitrite (B) concentrations over 120 min of moderate-intensity cycle exercise and a subsequent 4-km time trial (TT) following PL+PL: placebo consumed before and during exercise (solid triangle and dotted line); BR+PL: NO_3^--rich beetroot juice consumed before and placebo consumed during exercise (open circle and solid line); and BR+BR: NO_3^--rich beetroot juice before and during exercise (solid circle and solid line) (n=9). * = Significantly different from PL+PL. ** = BR+BR significantly different from BR+PL. # = Significantly different from 120 min to end of TT in BR+BR. † = Significantly different from 60 min to end of TT in BR+PL. ‡ = Significantly different from 90 min to TT in PL+PL.

Figure 2 Data are means ± SE of O_2 uptake over 120 min of moderate-intensity cycle exercise (A) and the change in O_2 uptake from 30 to 120 min (B) following PL+PL, PL+BR, and BR+BR. * = Significantly different in BR+BR compared with PL+PL.

Figure 3 Data are means ± SE of muscle glycogen concentration ([glycogen]) at

rest (PRE), after 120 min of moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n = 9). DW, dry weight. * = Significantly different from PRE to POST. ** = Significantly different from POST to TT. There were no significant differences among the three conditions at any discrete time point but the change in muscle [glycogen] was significantly less in BR+BR compared with PL+PL ($P<0.05$; see text for details).

Figure 4 Data are means ± SE of muscle PCr concentration ([PCr]; A), ATP concentration ([ATP]; B), and lactate concentration ([lactate]; C) at rest (PRE), after 120 min of moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n 9). * = Significantly different from PRE to POST. ** = Significantly different from POST to TT.

Figure 5 Data are means ± SE of O_2 uptake (A), power output (B), and completion time (C) over the 4-km time trial in PL+PL (black bars), BR+PL (gray bars), and BR+BR (white bars). Completion times for individual subjects shown in gray lines.

Chapter 6 The effect of dietary nitrate supplementation on skeletal muscle contractile efficiency and O_2 cost of prolonged single-leg knee extensor exercise

Figure 1 Schematic diagram of the experimental protocol (panel A) and exercise protocol (panel B). BR = beetroot supplementation; PL = placebo supplementation; MRS = magnetic resonance spectroscopy.

Figure 2 Mean ± SEM of plasma nitrate concentrations ($[NO_3^-]$) during the laboratory visit over a 2-h single-leg knee extensor exercise protocol (panel A), and at rest during the MRS visit (panel B) following nitrate-rich beetroot juice (BR; open circle and bar), and nitrate-depleted beetroot juice supplementation (PL; solid circle and bar). Mean ± SEM of plasma nitrite concentrations ($[NO_2^-]$) during the laboratory visit over a 2-h single-leg knee extensor exercise protocol (panel C), and at rest during the MRS visit (panel D) following BR (open circle and bar), and PL (solid circle and bar) supplementation. * = significantly different from PL.

Figure 3 Group mean of representation of a 2-h continuous low-intensity prolonged single-leg knee extensor exercise protocol for the determination of intramuscular [phosphocreatine] ([PCr], panel A), [inorganic phosphate] ($[P_i]$, panel B), and pH (panel C), interspersed with four 24-s high-intensity bouts for the determination of [PCr] τ from baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min). Intramuscular [PCr], [Pi] and pH were analyzed in 1 min bouts

from 14 to 15 min, 30 to 31 min, 48 to 49 min, 69 to 70 min, 88 to 89 min, and 103 to 104 min. † = significantly different from 14 to 15 min in PL (P<0.05), ** = significantly different from 69 to 70 min in PL (P<0.05), # = significantly different from 88 to 89 min in PL (P≤0.05), ‡ = significantly different from 103 to 104 min in PL (P<0.05), a = significantly different from 98 to 99 min in BR (P<0.05), b = significantly different from 69 to 70 min in BR (P<0.05), c = significantly different from 30 o 31 min (P<0.01), d = significantly different from 30 to 31 min (P<0.05).

Figure 4 Mean ± SEM of muscle [PCr] recovery kinetics (τ) averaged over the four 24-s high-intensity bouts during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min). In BR, [PCr] recovery τ sped up from baseline to end-exercise (35 ± 6 vs. 27 ± 4-s, P=0.001), baseline to half-way (35 ± 6 vs. 29 ± 5-s, P<0.01), and was not different from half-way to end-exercise (29 ± 5 vs. 27 ± 4-s, P>0.05). In PL, [PCr] recovery τ sped up from baseline to end-exercise (37 ± 8 vs. 25 ± 6-s, P<0.0001), and tended to speed up from baseline to half-way (37 ± 8 vs. 31 ± 8-s, P=0.06), and from half-way to end-exercise (31 ± 8 vs. 25 ± 6-s, P=0.06). * = significantly different from baseline (P<0.01), # = significantly different from half-way (P<0.01).

Figure 5 Mean ± SEM of pulmonary $\dot{V}O_2$ measured during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min) during the four 24-s high-intensity bouts following nitrate-rich beetroot juice (BR; open bar) and nitrate-depleted beetroot juice (PL; solid bar) supplementation (panel A), and individual pulmonary $\dot{V}o_2$ responses over time (panel B). * significantly different from baseline (P<0.001); # = significantly different from half-way (P<0.05).

Figure 6 Group mean ± SEM GE calculated during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min) during the four 24-s high-intensity bouts interspersed with 2 min 36 s of recovery following nitrate-rich beetroot juice (BR; open bar) and nitrate-depleted beetroot juice (PL; solid bar) supplementation. # = significantly different to baseline (P<0.05); ‡ = significantly different to half-way (P<0.05).

Figure 7 Change in plasma nitrate ($[NO_3^-]$; panel A) and nitrite ($[NO_2^-]$; panel B) following BR (BR – PL; μM, nM) in males (n=5) and females (n=5) and the change in pulmonary $\dot{V}O_2$ measured over time in BR (120 – 0 min; $ml \cdot kg^1 \cdot min^{-1}$; panel C) between males (n=5) and females (n=6).

Chapter 7 The influence of co-ingesting nitrate and N-acetylcysteine on nitric oxide bioavailability, neuromuscular function and respiratory muscle fatigue during prolonged heavy-intensity exercise.

Figure 1 Individual representative curve for plasma cysteine and glutathione (panel A) and plasma malondialdehyde (panel B) assessed via high performance liquid chromatography.

Figure 2 Mean ± SE plasma nitrate (NO_3^-; panel A) and nitrite (NO_2^-; panel B) over 1-h of heavy-intensity exercise and a subsequent TTE following: PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise (solid circle and solid line); PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise (open circle and solid line); BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise (solid triangle and dotted line); and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise (open triangle and dotted line). * = significantly different from 0 min (P<0.05), † = significantly different from TTE (P<0.05), § = significantly different in BR+NAC compared to PL+MAL and PL+NAC (P<0.01), # = significantly different in BR+MAL compared to PL+MAL and PL+NAC (P<0.01), ‡ = significantly different in BR+NAC compared to BR+MAL (P<0.05). Data shown for a subset of n=14 from 0 to 60 min and for n=13 for post-TTE.

Figure 3 Mean ± SE plasma cysteine (panel A), glutathione (panel C), and malondialdehyde (panel E) over 1-h of heavy-intensity exercise and a subsequent TTE. Change from 0 to 60 min in plasma [cysteine] (panel B), change in plasma [glutathione] (panel D), and change in plasma [MDA] (panel F). PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise. * = significantly different from 0 min (P<0.01). § = significantly different to PL+NAC and PL+MAL (P<0.05). Data shown for a subset of n=14 for time points 0 to 60 min and n=13 for time point post-TTE for plasma [cysteine] and [glutathione], and a subset of n=13 for plasma [malondialdehyde].

Figure 4 Compound muscle action potential (M-wave) amplitude and M-wave area (normalized to maximum M-wave during baseline pedalling)

indicating peripheral neuromuscular excitability (panel A and B); voluntary electromyographic root mean square (EMG RMS) amplitude (normalized to M-wave amplitude at 1 min of exercise) indicating muscle activation level (panel C); and RMS/M-wave (normalized to corresponding M-wave amplitude at each measurement time point) indicating central fatigue (panel D) during 1-h of heavy-intensity cycling at 0, 30, and 57 min, as well as at the end of the time-to-exhaustion (Post-TTE). * significantly different from 0 min (P<0.01); ‡ = significantly different from 30 min; † = significantly different from post-TTE (P<0.01). Data shown for subset of n=12.

Figure 5 Change from 0 to 60 min in compound action potential (M-wave) amplitude indicating peripheral neuromuscular excitability. * = significantly different to PL+NAC. Data shown for subset of n=12.

Figure 6 Mean ± SE MEP measured at 0 and 50 min during 1-h of heavy-intensity cycle exercise and post-TTE. PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise.

Figure 7 Mean ± SE completion time during the time-to-exhaustion at 70%Δ following 1-h of heavy-intensity cycle exercise. PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise. Data shown for a subset of n=15.

Symbols and abbreviations

[ ]	concentration
Δ	Difference/change
%Δ	% difference between GET and $\dot{V}O_{2peak}$
^{31}P-MRS	^{31}Phosphorus magnetic resonance spectroscopy
5,6,7,8-tetrahydrobiopterin	BH_4
Acetyl CoA	acetyl coenzyme A
ADP	adenosine diphosphate
AMP	adenine monophosphate
ANOVA	analysis of variance
ANT	adenine nucleotide translocase
ATP	adenosine triphosphate
BP	blood pressure
BR	nitrate-rich beetroot juice
Ca^{2+}	calcium
CK	creatine kinase
cAMP	cyclic adenine monophosphate
CASQ1	calsequestrin 1
cGMP	cyclic guanosine monophosphate
CP	critical power
CPT1	carnitine palmitoyl transferase complex 1
CPT2	carnitine palmitoyl transferase complex 2
Cr	creatine
CYS	cysteine
CV	coefficient of variation (expressed as a percentage (%))
DHPR	dihydropyridine receptor
EMG	electromyogram
eNOS	endothelial nitric oxide synthase
ETC	electron transport chain
FAD/$FADH_2$	flavin adenine dinucleotide
FABP	fatty acid binding protein
FATP	fatty acid transport protein
FFA	free fatty acid
GE	gross efficiency
FMN	flavin mononucleotide
GET	gas exchange threshold

GLY	glycogen
GSH	glutathione
H^+	hydrogen ion/proton
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HR	heart rate
HSL	hormone-sensitive lipase
iNOS	inducible nitric oxide synthase
K^+	potassium
KNO_3^-	potassium nitrate
kJ	kilojoules
MAL	maltodextrin
MAP	mean arterial pressures
MDA	malondialdehyde
MEP	maximum expiratory pressure
Mg^{2+}	magnesium
MRS	magnetic resonance spectroscopy
mV	millivolt
Na^+	sodium
NAC	N-acetylcysteine
NAD^+/NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
$NaNO_3^-$	sodium nitrate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2^-	nitrite
NO_3^-	nitrate
NOS	nitric oxide synthase
O_2	oxygen
$O_2{\cdot}^-$	superoxide
$ONOO^-$	peryoxynitrite
PCr	phosphocreatine
P_i	inorganic phosphate
PL	nitrate-depleted beetroot juice (placebo)
PO_2	O_2 tension
P/O	oxygen cost of ATP resynthesis

PPO	peak power output
RER	respiratory exchange ratio
RNS	reactive nitrogen species
ROS	reactive oxygen species
RONS	reactive oxygen and nitrogen species
RSNO	S-nitrosothiols
RyR	ryanodine receptor
SEM	standard error of the mean
SERCA	sarcoplasmic endoplasmic reticulum calcium ATPase
SD	standard deviation
SOD	superoxide dismutase
SH-	sulfhydryl
SR	sarcoplasmic reticulum
τ	phosphocreatine recovery time constant
τ_p	primary/phase II time constant
TCA	tricarboxylic acid cycle
TTE	time-to-exhaustion
TT	time trial
UCP	uncoupling protein
$\dot{V}E$	pulmonary ventilation (expired)
VL	vastus lateralis
VCl_3	vanadium chloride
VM	vastus medialis
$\dot{V}O_2$	pulmonary oxygen uptake
$\dot{V}O_{2sc}$	pulmonary oxygen uptake slow component
$\dot{V}O_{2max}$	maximal oxygen uptake/maximal aerobic capacity
$\dot{V}O_{2peak}$	peak oxygen uptake
W	Watt

Declaration

The material contained within this thesis is original work, conducted and written by Rachel Tan. The following publications and communications are a direct consequence of this work.

Peer-reviewed journal articles

Tan R, Wylie LJ, Thompson C, Blackwell JR, Bailey SJ, Vanhatalo A, Jones AM. Beetroot juice ingestion *during* prolonged moderate-intensity exercise attenuates progressive rise in O_2 uptake. *J Appl Physiol.* 124(5): 1254-1263, 2018.

Clark I, Vanhatalo A, Thompson, C, Joseph C, Black, MI, Blackwell JR, Wylie L J, **Tan R,** Bailey SJ, Wilkins B, Kirby B, Jones AM. Plasticity of the power-duration relationship during prolonged endurance exercise: influence of carbohydrate ingestion. *J Appl Physiol.* (*submitted).*

Conference communications

Tan R, Wylie, LJ, Thompson C, Blackwell JR, Vanhatalo A, Jones AM. (2017) [oral]. Dietary nitrate supplementation attenuates progressive loss of efficiency during prolonged moderate-intensity exercise. ECSS annual conference, Essen, Germany 2017.

Acknowledgements

I wish to express my gratitude to the following individuals, without whom my journey through academic training and early career research so far would not have been possible.

I decided to leave the comfort of home in Canada and pursue my Ph.D. at the University of Exeter in England because of the research that Professor Andrew Jones was accomplishing. Indeed, Andy's research team was, is, and will remain in the upper echelons of the research world, to which I feel extremely lucky to be a part of. It was a dream to be accepted into the team by you, Andy. Therefore, I would like to firstly thank Professor Andrew Jones, for giving me the opportunity to explore my interest in integrative physiology and for supporting me throughout my academic development. This experience has allowed me to grow more than I ever thought I could achieve, both academically and as a person. My hope is that my thesis achieves the quality of research that you are known to produce.

Secondly, I would like to thank Professor Anni Vanhatalo, who served as my secondary supervisor. No matter how busy you were, you were available for our weekly Friday meetings which I learned a lot from. Thank you for giving me the opportunity to learn from you and for taking the time to explain your perspective on how to approach research. I am extremely grateful for your academic and pastoral support, and not to mention the early morning biopsy support you gave which occurred no matter what day of the week it was. In addition, your high standard in the lab setting inspired me to pursue perfection over the years. I hope my future work, shaped by your prestigious training, will make you proud in the future.

I would like to thank my third supervisor, Dr. Lee Wylie. Thank you for being there for me at every stage of my thesis, from the start to the very end. You have always made time for me despite your ever-increasing roles within the University. You have also been a kind friend to speak to about important life choices. Your role on my supervisory team has been crucial pastorally and academically. In conclusion to my entire supervisory team, I would like to thank you all for providing unwavering support and constructive feedback at critical points of my research development, without which I would not have succeeded.

I would like to thank an all-star research group for helping me during my thesis; I could not have done it without you: Dr. Stephen Bailey, Dr. Christopher Thompson, Dr. Matthew Black, Adam Linoby, Dr. Ida Clark, Dr. Sinead McDonagh, and Stefan Kadach. In addition, thank you to the experts Mr. Jamie Blackwell and Dr. Jonathan Fulford. To Sam Bailey, Luke Connolly, Mike Cooke, Sabrina Wright and Garry Massey, thank you for your technical assistance and help with analysis over the years. I am extremely grateful for everyone's time and efforts.

I would also like to thank all participants and especially the undergraduates who have been vital to my PhD: Joseph Home, Felix Leonard-Fox, James Lewis, Tuesday Platt, Jordan Ley, and Becca Dewhurst-Trigg. I appreciate how quickly you had to learn daunting experimental techniques, yet you all approached it with enthusiasm, professionalism, and dedication. To this entire research team, thank you for sharing the 1000+ hours of data collection time with me.

I would not have had the confidence to pursue a Ph.D. if Dr. Bryan Taylor had not been my supervisor during my M.Sc. degree at Exeter. Bryan, your teachings carried throughout my Ph.D., and I hope you know the enormous impact you had on me as I progressed from undergraduate to postgraduate studies.

Finally, thank you to my family who supported my decision to leave Canada to complete this degree. Mom and Dad, you both saw my desire to become an academic and reminded me of this drive when I (often) doubted myself. Grandparents and aunts, you always financially supported me without question. To my second family: Rowena, Sapphie, Tobes, Francis, Kev, Charlotte, Wade; you are my family in England and have shared all of my experiences with me. Thank you for taking me in, for showing me the wonderful English life (and food), and for a great four years. Thank you to my support from England (Chloe, Bobbie, YX, Eva, Nick, Flips, James, Jo, Steve, Felix, Richie), Japan (Rup), Canada (Kelsey, Lowell), USA (Lindsey, Breanna, Merlin, Paul, Cooker, Hunter, Jeff); lastly, K and Sweep, you both arrived in my life at the perfect moment and your impact on my journey will not be forgotten.

I dedicate this thesis to everyone who has had an impact on my journey.

“True knowledge exists in knowing you know nothing.”
“I cannot teach anybody anything, I can only make them think.”
-Socrates

Chapter 1: Introduction

Nitric oxide (NO) has become known as a 'miracle molecule' since scientists began unravelling its functional importance to physiology and medicine over three decades ago (Furchgott & Zawadzki, 1980; Ignarro et al., 1987; Murad et al., 1978). It is now understood that NO is a ubiquitous free radical signalling molecule involved in biological processes at the tissue and cellular level, regulating vasodilation (Joyner & Tschakovsky, 2003; Moncada & Higgs, 1993), skeletal muscle glucose uptake (Merry et al., 2010), mitochondrial cellular respiration (Brown & Cooper, 1994; Shiva et al., 2007), neurotransmission (Garthwaite, 2008), skeletal muscle contraction (Reid, 1998; Stamler & Meissner, 2001; Viner et al., 2000), and fatigue development (Percival et al., 2010). The functional importance of NO is highlighted by the complementary O_2-dependent and O_2-independent pathways which enable continual recycling of nitrate (NO_3^-) and nitrite (NO_2^-) into NO and NO intermediates in the blood and in multiple tissues including skeletal muscle (Gilliard et al., 2018; Lundberg et al., 2008; Piknova et al., 2015). Importantly, the discovery of the O_2-independent pathway provides a mechanism by which exogenous NO_3^- sources could further increase NO bioavailability and thus, increase its associated functional consequences.

A little over a decade ago, two independent physiology laboratories in Sweden (Karolinska Institutet, Professor Björn Ekblom *et al*.) and in the United Kingdom (University of Exeter, Professor Andrew M. Jones *et al*.) recognized the potential of manipulating NO bioavailability through dietary and pharmacological sources of NO_3^- to enhance physiologic function during exercise, given the myriad of regulatory roles of NO in skeletal muscle. Specifically, both research groups identified that NO_3^- supplementation, administered as NO_3^- salts and/or beetroot juice, could lower the O_2 cost of exercise (i.e. improve exercise efficiency) for the same given submaximal work rate, which was a surprising effect given that the O_2 cost of exercise cannot be altered by any other intervention (Bailey et al., 2009; Larsen et al., 2007). With respect to exercise performance, the lowering in the absolute O_2 cost at a given work rate in relation to the maximal aerobic capacity (i.e. $\dot{V}O_{2max}$) could be expected to enable improved exercise performance (Bassett & Howley, 2000; Jones & Burnley, 2009). Accordingly, over the past 12 years, there has been a surge in publications which have focused on the physiological and ergogenic effects of NO_3^- supplementation (Bailey et al.,

2010; Cermak et al., 2012a; Lansley et al., 2011b), the application of NO_3^- supplementation to various exercise modalities (Bond et al., 2012; Murphy et al., 2012; Nybäck et al., 2017; Peeling et al., 2015; Thompson et al., 2015), and on identifying the mechanisms of action (Bailey et al., 2010; Ferguson et al., 2013; Hernández et al., 2012; Larsen et al., 2011; Whitfield et al., 2016; 2017).

To date, multiple candidates for the mechanistic underpinnings for the effects of NO_3^- supplementation have been identified and remains the focus of intense research due to equivocal findings. The literature suggests that the mechanistic bases of the physiological effects of NO_3^- supplementation in exercise are due to the independent or combined effects of: 1) improved mitochondrial efficiency (Larsen et al., 2011); 2) improved muscle contractile efficiency (Bailey et al., 2010); 3) redox signalling (Whitfield et al., 2016); and/or 4) improved muscle contractile function and blood flow in type II muscle fibres (Hernández et al., 2012; Ferguson et al., 2013). The elevation in NO bioavailability, reflected in the increase of plasma [NO_2^-], is widely accepted as a key determinant in the efficacy of NO_3^- supplementation (Dreissigacker et al., 2010; Porcelli et al., 2015; Wilkerson et al., 2012). While the mechanistic underpinnings remain to be resolved, these recent advances have provided rationale to explore the potential influence of NO_3^- supplementation in exercise modalities that require a greater proportional contribution of type II muscle fibres (Hernández et al., 2012; Ferguson et al., 2013), and/or exercise protocols whereby a decline in exercise efficiency (e.g. caused by a decline in mitochondrial and/or contractile efficiency) manifests. Accordingly, given that a decline in exercise efficiency occurs over prolonged type exercise (Black et al., 2017; Hopker et al., 2017; Paris et al., 2019), and that there is potential for the additional progressive recruitment of type II muscle fibres over time (Vøllestad et al., 1984), it could be reasoned that dietary NO_3^- supplementation would improve and/or attenuate the decline in the physiological responses in prolonged exercise. However, the potential for NO_3^- supplementation in prolonged endurance-type exercise has yet to be explored. In addition, considering that the muscle metabolic demands of short continuous endurance type exercise employed in the earlier studies are different to the metabolic demands of prolonged type exercise (e.g. the decline in intramuscular finite glycogen reserves over prolonged exercise; Hermansen et al., 1967),

investigating NO_3^- supplementation during prolonged type exercise could reveal novel mechanisms by which NO_3^- exerts physiological effects.

Therefore, the purpose of this thesis was to investigate the potential influence of dietary NO_3^- supplementation, administered as concentrated NO_3^--rich beetroot juice, on the physiological responses during prolonged endurance exercise (≥ 1 h) and to examine the potential of dietary NO_3^- supplementation to enhance exercise performance immediately following such exercise. A secondary aim of this thesis was to investigate potential supplementation strategies to further enhance and/or preserve NO bioavailability during prolonged exercise given the importance in the elevation of plasma [NO_2^-] in the efficacy of NO_3^- supplementation, and the potential for plasma [NO_2^-] to decline over exercise.

Chapter 2: Literature Review

2.1. Metabolic pathways for ATP resynthesis in skeletal muscle

Muscle contraction requires the coordination of multiple physiological systems to provide energy in a timely manner to meet dynamic muscle metabolic demands. As energetic demands increase, the supply of oxygen (O_2) must increase to support the production of adenosine triphosphate (ATP), the high-energy molecule providing energy to drive muscle contraction and other biochemical processes (Cain et al., 1962). ATP releases chemical energy in an exergonic reaction when the terminal phosphate is cleaved from the molecule and the end products are adenosine diphosphate (ADP) and inorganic phosphate (P_i). This reaction is reversible, such that ATP may be resynthesized by combining ADP and P_i (Cain & Davies, 1962). The ability to rapidly resynthesize ATP in response to muscular contraction is critical given that ATP molecules are heavy and thus, storage capacity is limited, with only several seconds of exercise worth of stored energy available. Therefore, it is unsurprising the human body has several pathways for ATP resynthesis which together contribute to the total energy supply (Fig. 2.1). Importantly, the contribution of energy production from each pathway is dependent on the exercise intensity and duration (Howlett et al., 1998).

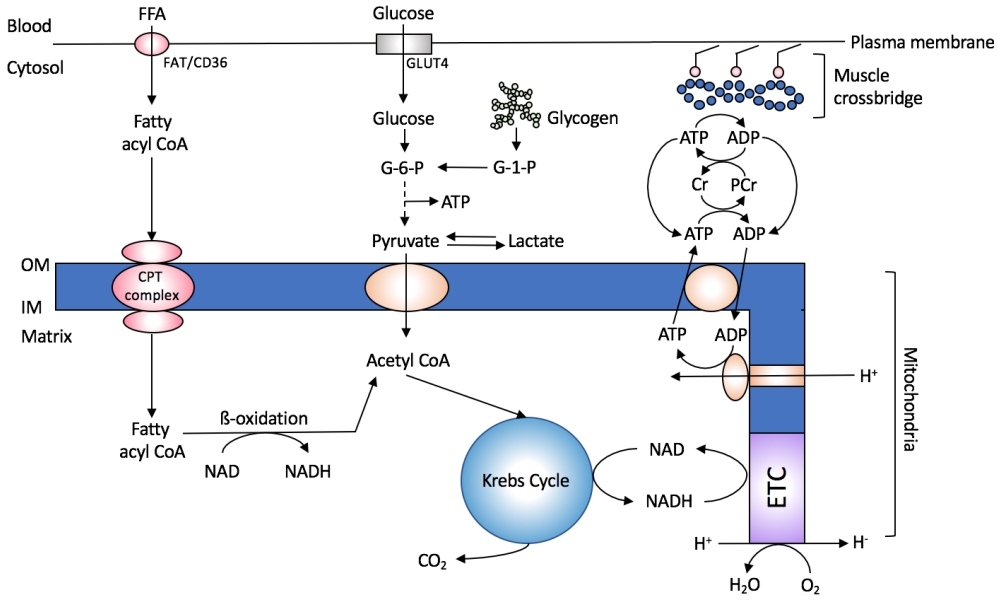

Figure 2.1. A simplified schematic diagram redrawn from Spriet, 2007. Abbreviations: ADP = adenosine triphosphate; ATP = adenosine triphosphate; CoA = coenzyme A; CPT = carnitine palmitate transferase; Cr = creatine; ETC = electron transport chain; FAT/CD36 = fatty acid translocase; FFA = free fatty acid; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; GLUT4 = glucose transporter; IM = inner mitochondrial membrane; NAD =

nicotinamide adenine dinucleotide; NADH = nicotinamide adenine dinucleotide phosphate; OM = outer mitochondrial membrane; PCr = phosphocreatine.

2.1.1. Substrate level phosphorylation

Substrate level phosphorylation consists of two anaerobic pathways which rapidly synthesize ATP in response to metabolic demand: PCr hydrolysis and anaerobic glycolysis. The immediate source of energy in response to the onset of muscular contraction is generated anaerobically (i.e. without the presence of O_2) through phosphocreatine (PCr) hydrolysis, a process catalyzed by creatine kinase (CK) such that a water (H_2O) molecule cleaves P_i from PCr and P_i is donated to phosphorylate ADP to form 1 ATP molecule (Parolin et al., 1999). Importantly, the accumulation of intramuscular metabolites (i.e. P_i, ADP, adenosine monophosphate, AMP) and changes to contraction-induced calcium (Ca^{2+}) stimulate glycolysis and thus have a role in regulating mitochondrial respiration (Walsh et al., 2001). The simplicity of this reaction allows for the rapid resynthesis of ATP for contraction but it can only be sustained for short periods (~1 to 10 seconds) given that intramuscular PCr stores are limited (Bogdanis et al., 1996; Boobis et al., 1983; Hultman et al., 1990). However, during recovery, PCr resynthesis occurs with the presence of O_2, restoring resting concentration within 1 min of rest (Haseler et al., 1999). Although PCr hydrolysis is always utilized to some extent, it is predominantly utilized during short bursts of high-intensity exercise (Cheetham et al., 1986; Hultman et al., 1990).

Anaerobic glycolysis is an anaerobic pathway that involves 10 to 12 reactions and takes place in the cytoplasm of the cell (Chasiotis et al., 1982). Energy from this pathway is produced from the breakdown of stored glycogen into glucose molecules and subsequently the conversion into pyruvate (Febbraio et al., 1998; Watt et al., 2001). A total of 3 ATP molecules is produced through this process with glycogen, and 2 ATP molecules with glucose. This pathway is predominant in providing energy for sustained high-intensity exercise (Cheetham et al., 1986; Nevill et al., 1989). Anaerobic glycolysis can provide energy to sustain muscular contraction for longer durations than PCr hydrolysis but cannot be sustained for longer than 1 to 2 minutes of contraction. Importantly, hydrogen ions (H^+) are produced which increases muscle acidosis (i.e. reduces pH).

2.1.2. Oxidative phosphorylation

Oxidative phosphorylation, also known as cellular respiration, oxidative metabolism or aerobic metabolism, is an aerobic pathway (i.e. requires the presence of O_2) that occurs in the mitochondria. ATP production occurs at a relatively slower rate compared to substrate level phosphorylation as a greater number of reactions are required to convert carbohydrate and fat stores into ATP (Spriet et al., 2002; Watt & Spriet, 2004). Acetyl coenzyme A is produced from these processes which enters the Krebs or tricarboxylic acid (TCA) cycle, followed by the electron transport chain (Mitchell, 1961) to yield a substantially greater amount of ATP relative to substrate phosphorylation (Spriet et al., 2002). A total of 38 ATP molecules are produced from glycolysis and oxidative phosphorylation combined. The number of ATP molecules produced with FFA is dependent on the length of the carbon chain. For example, a 16 carbon chain can yield around 131 ATP through oxidative phosphorylation.

The increase in oxygen uptake ($\dot{V}O_2$) during exercise indicates increased substrate oxidation (i.e. carbohydrate and fat) for oxidative phosphorylation. However, substrate oxidation is never fully coupled to ATP production (Brand et al., 1994). Changes to the P/O ratio (i.e. the amount of O_2 consumed per ATP molecule produced; Hinkle et al., 2005) indicates the efficiency of oxidative phosphorylation. For example, an increased P/O ratio would indicate increased ATP synthesis per O_2 molecule. In contrast, a decreased P/O ratio suggests a reduction in ATP synthesis per O_2 molecule. A decreased P/O ratio could be caused by proton leakage, whereby protons flow back into the mitochondrial matrix in a futile process that does not cause ATP synthesis. Proton leakage is in part thought to be induced by uncoupling proteins (UCP) and/or adenine nucleotide translocase (ANT) (Bevilacqua et al., 2010; Brand et al., 2005; Parker et al., 2008; Rolfe et al., 1994). A decreased P/O ratio could also be caused by a redox slip reaction, whereby the electron transfer across the ETC complexes occurs without pumping protons across the inner mitochondrial membrane or creating the electrochemical proton gradient.

2.2. ATP utilization in skeletal muscle

2.2.1. Skeletal muscle contraction

ATP within skeletal muscle is used for muscle contraction. Voluntary contraction is initiated upon the delivery of a nerve impulse from the brain to the spinal cord to the muscle at the point of the neuromuscular junction (Fleischer & Inui, 1981). Excitation-contraction coupling is the process through which the propagation of an action potential, muscle membrane depolarization via the influx of sodium (Na^+) and an efflux of potassium (K^+), is coupled to the release of Ca^{2+} from Ca^{2+} voltage sensing release channels (i.e. dihydropyridine receptor, DHPR and ryanodine receptors, RyR) in the sarcoplasmic reticulum (SR, Imagawa et al., 1987; Schneider & Chandler, 1973), resulting in the ATP-driven contraction by the shortening action of actin and myosin filaments (Ashley et al., 1991) (Fig. 2.2). Disruptions anywhere along these pathways lead to muscle fatigue, which is defined as the exercise-induced reduction in the ability of muscle to sustain a force output, and can be reversible through rest (Bigland-Ritchie & Woods, 1984).

Contraction ceases when neural activity stops. Upon cessation, the enzyme sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) utilizes ATP to actively pump cytosolic Ca^{2+} back into the SR (Periasamy & Kalyanasundaram, 2007), while the tropomyosin complex moves back over the binding site. The enzyme Na^+-K^+ ATPase utilizes ATP to actively pump Na^+ out of the cell while K^+ is actively pumped back into the cell.

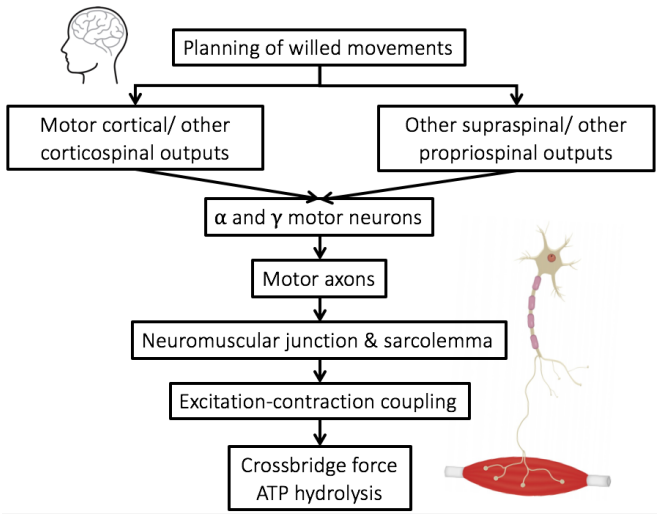

Figure 2.2. A simplified schematic diagram redrawn from Gandevia, 2001. The chain of events required for voluntary contraction from the brain to the muscle.

2.2.2. Physiological characteristics of muscle fibre types

Skeletal muscle is composed of three different muscle fibre phenotypes, with relative proportions within each muscle group dependent on its function (Johnson et al., 1973; Tesch et al., 1984). Accordingly, these fibre types are characterized by their structural, contractile, and biochemical properties (Bottinelli et al. 2000; Table 2.1). In sequential order of activation, the three classifications are: type I (slow-twitch, oxidative) fibres, type IIa (fast-twitch, oxidative) fibres, and type IIx (fast-twitch, glycolytic) fibres. Importantly, the muscle fibre types are not completely distinct given that a spectrum of contractile and metabolic properties exist within each specific fibre type, consequently leading to 'hybrid' fibres within muscle groups. However, in general, type I muscle fibres have greater mitochondrial density, oxidative enzyme activity and capillary density, as well as a lower peak power and lower shortening velocity compared to type IIx fibres. Accordingly, type I muscle fibres have greater fatigue resistance and efficiency (Coyle et al., 1992; Willis & Jackman, 1994; Szentesi et al., 2001). In contrast, type IIx muscle fibres have greater fatigability but are well suited to high power and high velocity contractions. Type IIa muscle fibres have an intermediate contractile force and velocity in relation to type I and IIx muscle fibres. The continuum of muscle fibre types is an elegant physiological design which permits skeletal muscle to respond to dynamic metabolic demands.

The order of muscle fibre activation is related to the size of the motor neuron, known as the size principle (Henneman et al., 1965) and to factors such as the rate of muscle contraction and exercise intensity. For example, type I fibres are recruited first at the onset of low force contractions given that these fibres have the smallest motor neurons and therefore have a lower relative power output (Coyle et al., 1992). However, with increased contraction duration, additional progressive recruitment of type II fibres may be required to sustain force output as type I muscle fibres fatigue (Krustrup et al., 2004; Shinohara & Moritani, 1992). During high-intensity exercise whereby high force outputs are required, a greater relative proportion of type II fibres are recruited at the onset of exercise (Casey et al., 1996; Gollnick et al., 1974; Greenhaff et al., 1994; Soderlund et al., 1992).

Table 2.1. Skeletal muscle fiber type characteristics.

	Type I	Type IIa	Type IIx
Structural			
Motor neuron size	Small	Medium	Large
Myosin ATPase	Slow	Fast	Fastest
Mitochondrial/capillary density	High	Medium	Low
Myoglobin content/colour	High (dark red)	Medium (red)	Low (white)
Contraction			
Recruitment order	1st	2nd	3rd
Oxidative capacity/fatigue resistance	High	Medium	Low
Biochemical			
Metabolism	Oxidative	Oxidative/glycolytic	Glycolytic
Glycogen stores	Low	Medium	High

2.3. Physiological factors affecting skeletal muscle contraction

2.3.1. Metabolic changes

It is well established that during prolonged repeated contractions, the reduction in finite intramuscular glycogen reserves leads to impaired excitation-contraction coupling and fatigue (Bergström & Hultman, 1966; Bergström et al., 1967; Hermansen et al., 1967). By the same token, carbohydrate ingestion has been shown to enhance exercise tolerance (Christensen & Hansen, 1939; Greenhaff et al., 1987; Karlsson & Saltin, 1971; Maughan & Poole, 1981). Muscle glycogen stores play a significant role in muscle function as low muscle glycogen stores are associated with decreased SR Ca^{2+} release rate (Ahlborg et al., 1967; Black et al., 2017; Chin & Allen, 1997; Duhamel et al., 2006a; Duhamel et al., 2006b; Nielsen et al., 2009; Ørtenblad et al., 2011). Although the mechanistic basis is currently unclear, it is purported that SR function is dependent on muscle glycogen due to glycolytic intermediates and/or glycolysis contributing to the maintenance of coupling between Ca^{2+} release proteins, such as RyR (Ørtenblad et al., 2011) or for the Krebs cycle (Helander et al., 2002**;** Sahlin et al., 1990). Alternatively, or in addition, carbohydrate status could influence the pH balance of muscle and buffer metabolic acidosis to preserve muscle function (Maughan et al., 1997).

The attainment of critically low levels of intramuscular PCr (<10 mM) and/or the accumulation of P_i contributes to impaired exercise tolerance (Allen et al., 2008; Cooke et al., 1988; Dahlstedt et al., 2001; Hogan et al., 1999; Westerblad & Allen, 2003; Vanhatalo et al., 2010b). During prolonged or intense repeated contractions, the

accumulation of P_i reduces myofibrillar and Ca^{2+} sensitivity (Danieli-Betto et al., 2000; Martyn & Gordon, 1992; Millar & Homsher, 1990) as well as SR Ca^{2+} reuptake (Hill et al., 2001; Leppik et al., 2004; Li et al., 2002) which facilitate the development of fatigue (Westerblad et al., 2002). In rodents that did not express CK, force production was depressed, crossbridge kinetics were impaired and Ca^{2+} sensitivity decreased compared to wild type rodents, indicating the role of accumulated P_i in contraction (Dahlstedt et al., 2001). Interventions that reduce the PCr slow component (Rossiter et al., 2001; 2002) were associated to a reduction in metabolic perturbation and improved exercise tolerance (Jones et al., 2007; Vanhatalo et al., 2010b). Moreover, preserved muscle [PCr] and concomitant reductions to P_i accumulation have been reported to improve severe-intensity exercise tolerance (Bailey et al., 2010).

During exercise which require prolonged and/or intense sustained contractions, mitochondrial efficiency may decline over time. The decline in mitochondrial efficiency may be due to increased muscle temperature (Brooks et al., 1971; Willis & Jackman, 1994) or mitochondrial uncoupling actions (Brand et al., 1994) such as proton leak (Echtay et al., 2002; Rolfe et al., 1994), or redox slip reactions (Groen et al., 1990), or changes in substrate (Hopker, O'Grady & Pageaux, 2017).

2.3.2. Motor unit recruitment

In addition to metabolic changes, alterations to muscle fibre recruitment patterns may influence excitation-contraction coupling. The central nervous system activates different muscle fibre types according to the work load (Bigland-Ritche et al., 1986; Enoka & Stuart, 1992). For example, over prolonged repeated contractions, initially active muscle fibres may 'drop out', and/or a progressive recruitment of 'fresh' type II fibres could be required to sustain the power output (Gollnick et al., 1974; Krustrup et al., 2004), causing a higher ATP cost of contraction (Crow & Kushmerick, 1982c; Reggiani et al., 1997; Willis & Jackman, 1994), greater glycogen depletion (Casey et al., 1996; Soderlund et al., 1992) and slower O_2 uptake kinetics (Wüst et al., 2013). Consequently, this could contribute to an increased O_2 cost of contraction given that type II fibres have a relatively greater fatigability (Barclay, 1996; Cannon et al., 2014; Murgatroyd & Wylde, 2011; Woledge, 1998). Moreover, prolonged contractions decrease overall motor unit firing rates (Bigland-Ritchie et al., 1983) and an increased

number of action potentials are required to initiate firing compared to the initial contractions as the originally activated fibres fatigue (Garland et al., 1994).

2.3.3. Muscle excitability

For muscle contraction, action potentials propagate the transverse tubule system to excite the muscle enough to stimulate voltage-sensor activation of DHPR and RyR for Ca^{2+} release. However, changes to muscle excitability may have deleterious effects on force production (Allen et al., 2008). Muscle excitability is influenced by the ionic exchange of Na^+ and K^+ during muscle membrane depolarization. During prolonged or intense repeated skeletal muscle contractions, an accumulation of extracellular K^+ may increase from resting values of ~4 mM to ~10 mM (Nielsen et al., 2004; Sejersted & Sjøgaard, 2000) due to an increased number of action potentials (Clausen, 2003; Hodgkin & Horowicz, 1959; Juel et al., 2000; Sjøgaard et al., 1985). Moreover, maximal Na^+-K^+ ATPase activity declines over prolonged exercise (Fraser et al., 2002; Leppik et al., 2004; Sandiford et al., 2004), impairing membrane excitability (Fowles et al., 2002) and contributes to impaired Na^+ and K^+ homeostasis. The mechanistic bases of a progressively declining maximal Na^+-K^+ ATPase activity during prolonged exercise is speculated to be caused by the accumulation of Ca^{2+} and free radicals in damaging associated proteins (Kourie, 1998; Kukreja et al., 1990; Sen et al., 1995b; Sulova et al., 1998). Substantial changes to the K^+ electrochemical gradient and extracellular K^+ accumulation may result in failed excitation (Gong et al., 2003; Yensen et al., 2002) and thus reduction in force production (Balog & Fitts, 1996; Burton & Smith, 1997; Clausen et al., 1996; Sejersted & Sjøgaard, 2000). Moreover, prolonged contractions may decrease the action potential propagation speed (Juel, 1988). Interestingly, training has been evidenced to improve sarcolemmal excitability and force production through increased Na^+-K^+ ATPase protein content (Clausen, 2003; Iaia & Bangsbo, 2011) and decreased K^+ efflux (Nielsen et al., 2004).

2.3.4. Redox interactions: free radicals

Free radicals are defined as atoms or molecules which contain one or more unpaired electrons (Halliwell & Gutteridge, 2007). Two main free radical cascades are produced from O_2 which are superoxide ($O_2^{\bullet -}$) and nitric oxide (NO) (Stamler et al., 1992). Both cascades are interdependent, react with thiols (i.e. sulfhydryl moieties) and metals, have a role in activating cellular signalling pathways, and are critical to contractile

function (Balon & Nadler, 1994; Halliwell & Gutteridge, 2007; Power & Jackson, 2008; Stamler & Meissner, 2001; Fig. 2.3). Skeletal muscles produce low levels of free radicals (Commoner et al., 1954; Koren et al., 1980) such as reactive oxygen species (ROS) (Diaz et al., 1993; Reid et al., 1992), reactive nitrogen species (RNS) (Balon & Nadler, 1994; Patwell et al., 2004) and its derivatives at rest which are required for force production (Reid et al., 1993; Regnier et al., 1992). In contrast, force production may be impaired during situations of increased muscular activity (Davies et al., 1982), increased physiological temperature (Zuo et al., 2000), and muscle acidosis (Arbogast & Reid, 2004), as these conditions markedly enhance ROS and RNS production. Transiently elevated ROS and RNS production causes oxidative and nitrosative stress, respectively (Smith & Reid, 2006), and consequently, contribute to the development of skeletal muscle fatigue by impairing key contractile processes (Alessio et al., 1988; Allen et al., 2008; Davies et al., 1982; Diaz et al., 1993; Dillard et al., 1978; Reid et al., 1992; 1993). The equilibrium between ROS/RNS and the endogenous antioxidant buffering system (i.e. redox homeostasis) and any imbalances are important in influencing contractile function.

2.3.5. Redox interactions: reactive oxygen species

When ROS production exceeds endogenous antioxidant buffering systems, oxidative stress occurs (e.g. reflected by lipid peroxidation, protein carbonylation, glutathione depletion) causing cellular damage (Dalle-Donne et al., 2003) through oxidation (Sen et al., 2000). Muscle dysfunction may occur in both locomotor (Matuszczak et al., 2005) and respiratory muscle (Zergeroglu et al., 2003) due to oxidative damage to Ca^{2+} regulation (Brotto & Nosek, 1996; Moonpanar & Allen, 2005) and the associated proteins regulating Ca^{2+} handling such as SERCA, RyR, Na^{+}-K^{+} ATPase (Abramson & Salama, 1989; Brotto & Nosek, 1996; Grover et al., 1997; Gutierrez-Martin et al., 2004; McKenna et al., 2006; Pessah & Feng, 2000; Scherer & Deamer, 1986; Viner et al., 1996) as well as proteins regulating myofilament function such as troponin, tropomyosin, actin, and myosin (Andrade et al., 2001; Brooke & Kaiser, 1970; Callahan et al., 2001; Canton et al., 2004; Crowder & Cooke, 1984; Hinshaw et al., 1991; Konczol et al., 1998; Putkey et al., 1993; Sweeney et al., 1993; Williams & Swenson, 1982; Yamada et al., 2006). Therefore, excessive ROS is implicated in depressed contractile function (Fig. 2.3). Interestingly, elevating ROS by low to moderate amounts with supplementation has been shown to enhance force production

(Andrade et al., 1998; Oba et al., 1996; Reid et al., 1993). These data highlight the complexity and importance of the balance of ROS levels in skeletal muscle in regulating muscle contraction.

2.3.6. Redox interactions: nitric oxide

In addition to ROS, skeletal muscle generates NO (see section: *Nitric Oxide Biochemistry and Metabolism*) which also influences contractile function. NO has three general molecular actions: 1) interactions with ROS; 2) reactions with transition metals; and 3) reactions with protein thiols (Stamler & Meissner, 2001). NO can react with O_2 and ROS, such as $O_2^{\bullet-}$, to form NO derivatives that can induce redox reactions (i.e. transfer of electrons) (Stamler et al., 1992). In addition, NO can exert an antioxidant effect through the reaction between NO and $O_2^{\bullet-}$ which forms peroxynitrite ($ONOO^-$), consequently reducing the bioavailability of $O_2^{\bullet-}$ (Halliwell, 1994; Kanner et al., 2001; Moylan & Reid, 2007) and prevents further ROS from forming (Wink et al., 1993). The second general action of NO is its ability to interact with transition metals. NO can induce cGMP signalling to depress force production by interacting with haem iron or iron-sulpher centres (Abraham et al., 1998; Kobzik et al., 1994). Lastly, NO can target thiols (i.e. moieties with sulphydryl groups, RSH or RS^-) to form RSNO groups in a reversible process called S-nitrosylation which alters protein structure and function (Stamler, 1994). Contractile proteins such as SERCA, RyR, and DHPR are likely the main targets for S-nitrosylation and their activity may be increased (Aghdasi et al., 1997; Stoyanovsky et al., 1997) or decreased (Ishii et al., 1998; Mezaros et al., 1996; Suko et al., 1999). Moreover, NO may influence contractile force production through slowing down myosin cycling kinetics (Evangelista et al., 2010; Galler et al., 1997; Heunks et al., 2001) and may induce reversible inhibition of CK activity which decreases ATP synthesis (Gross et al., 1996).

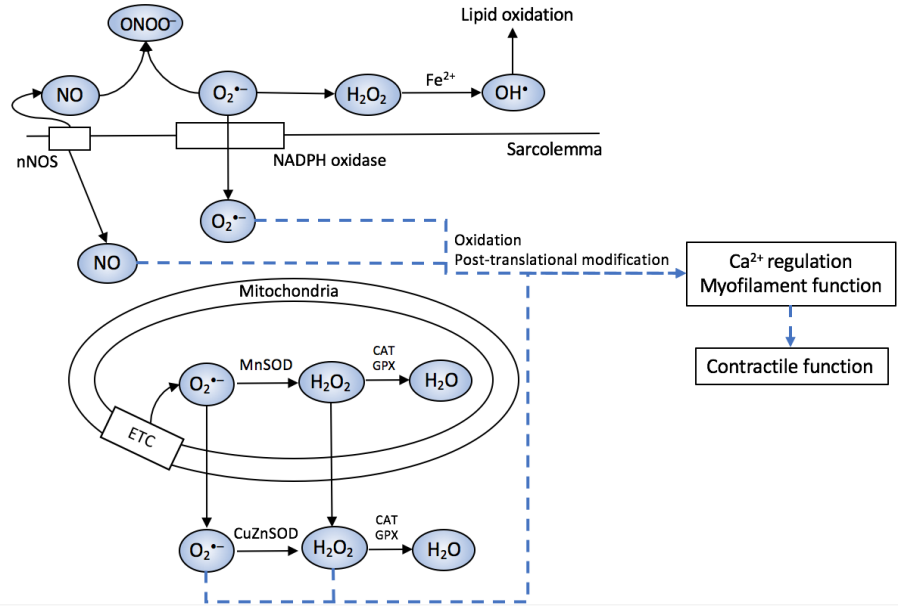

Figure 2.3. A simplified schematic diagram redrawn from Smith & Reid, 2006. Main reactive oxygen species cascade in the skeletal muscle (adapted from Smith & Reid, 2006). Superoxide ($O_2^{\bullet-}$) is mainly produced from the mitochondria in complex I and III of the ETC (Barja, 1999; Li et al.,1999; Turrens, 2003) at a rate of ~1-2% of O_2 consumption (St Pierre et al., 2002) as well as by nicotinamide dinucleotide phosphate (NADPH) oxidase located in the transverse tubules and SR (Hidalgo et al., 2006). Other purported sites of $O_2^{\bullet-}$ production include xanthine oxidase (Gomez-Cabrera et al., 2005), and lipoxygenase (Zuo et al., 2000) although it should be noted that xanthine oxidase may shift to producing NO in hypoxic conditions (Webb et al., 2004). $O_2^{\bullet-}$ is dismutated spontaneously or by superoxide dismutases (SOD) to form hydrogen peroxide (H_2O_2) with the copper-zinc isoform existing in the cytosol (CnZnSOD) and the manganese isoform existing in the mitochondria (MnSOD) (Halliwell & Gutteridge, 2007; Smith & Reid, 2006). H_2O_2 is an important signalling molecule given that it can freely diffuse across cell membranes and has a relatively long half-life (Veal et al., 2007). H_2O_2 is broken down through enzymatic reactions involving catalase (CAT) and glutathione peroxidase (GPX), with the latter pathway requiring glutathione (Powers & Jackson, 2008). Alternatively, H_2O_2 may be broken down into the noxious hydroxyl radical ($OH^{\bullet}$) through reactions with free transition metals such as ferrous iron (Fe^{2+}) (Powers & Jackson, 2008). Both nitric oxide (NO) and ROS act as important signalling molecules (Halliwell & Gutteridge, 1998; Powers et al., 2010; Smith & Reid, 2006; Stamler & Meissner, 2001) and interact with each other. For example, NO reacts with $O_2^{\bullet-}$ to form reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) (Halliwell, 1994; Reid & Durham, 2002). Excessive ROS and RNS production may impair contractile function by altering Ca^{2+} handling and myofilament function (Reid, 2016a).

2.3.7. ROS-mediated activation of group III and IV muscle afferent feedback

Muscle afferents are sensory fibres that inhibit or facilitate motor unit recruitment of active contracting muscle, as well as adjust cardiovascular and ventilatory responses by modifying the central motor drive (Amann & Dempsey, 2008; Gandevia, 2001;

Kennedy et al., 2013; 2014) in response to mechanical and intramuscular metabolite changes (Mitchell et al., 1983; Rotto & Kaufman, 1988). Specifically, group III and IV muscle afferents are active during exercise (Adreani et al., 1997; Amann et al. 2010) and are recognized to contribute to impaired excitation-contraction coupling (Amann & Dempsey, 2008; Laurin et al., 2015). Group III muscle afferents are stimulated by force production (Hayes et al., 2009) while group IV muscle afferents are stimulated by muscle metabolites (Darques et al., 1998; Haouzi et al., 1999; Rotto & Kaufman, 1988) and ROS (Arbogast et al., 2000; Delliaux et al., 2009). Thus, it is possible for heavy-intensity and/or prolonged exercise to activate group III and IV muscle afferents from metabolite and/or ROS accumulation such that voluntary muscle contraction declines (Smith & Reid, 2006; Supinski et al., 1997) and a muscle metaboreflex phenomenon redirects blood flow away from skeletal muscle (Dempsey et al., 2006; Harms et al., 1997; Sheel et al., 2001).

2.4. Oxygen uptake and exercise efficiency

At the onset of exercise, $\dot{V}O_2$ increases and reflects the integrative ability of the body to increase gas exchange, cardiac output, blood flow, muscle O_2 extraction, and muscle O_2 utilization to meet ATP demands. Accordingly, exercise efficiency may be reflected in the O_2 cost of exercise for a given work rate since the expected average O_2 cost of exercise is ~10 $mL \cdot min^{-1} \cdot W^{-1}$ below the gas exchange threshold (GET; Jones & Poole, 2005). An increased muscle metabolic rate during exercise or a decline in muscle efficiency and function could therefore be reflected as progressive increases to the O_2 cost of exercise (Barstow et al., 1990; Hopker, O'Grady & Pageaux, 2017; Koga et al., 2005). The pulmonary $\dot{V}O_2$ response, measured at the mouth using automated gas analyzers, is an indirect measure of muscle oxidative phosphorylation and is less invasive and therefore more suitable for routine laboratory testing compared to direct measures (Barstow et al., 1990). It is well established that the $\dot{V}O_2$ response during constant work-rate exercise is distinct within the three exercise-intensity domains of moderate-, heavy-, and severe-intensity exercise (Jones & Poole, 2005; Whipp et al., 1994) which are described below and are illustrated in Fig 2.4.

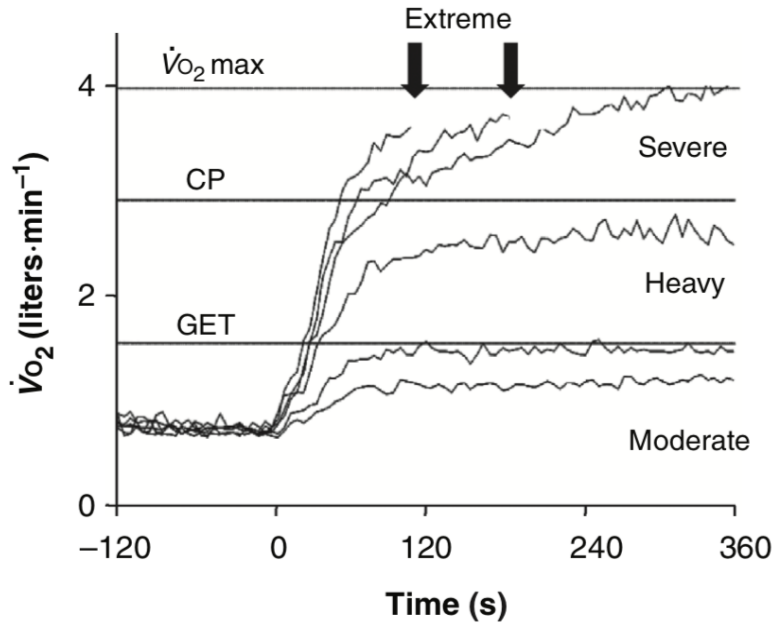

Figure 2.4. The pulmonary $\dot{V}O_2$ response following the onset of constant work-rate exercise in the moderate-, heavy-, and severe-intensity domains. Abbreviations: GET (gas exchange threshold), CP (critical power), $\dot{V}O_{2max}$ (maximal aerobic capacity). Adapted from Wilkerson et al. (2004).

2.4.1. Moderate-intensity exercise domain

Moderate-intensity exercise consists of all work rates below the GET. At the onset of exercise, an O_2 deficit occurs representing a temporary mismatch between the energy requirement and the energy being produced via oxidative ATP resynthesis (Krogh & Lindhard, 1913). However, a $\dot{V}O_2$ steady-state (i.e. a matching between ATP supply from oxidative metabolism and ATP utilization) is achieved within 2 to 3 min in most healthy young people whereas it may be considerably longer in individuals who are sedentary, ageing, and/or with chronic diseases (Whipp & Wasserman, 1972). The tight coupling between $\dot{V}O_2$ and ATP resynthesis in the moderate-intensity exercise domain preserves homeostasis of the metabolic environment such that metabolites do not disrupt contractile processes. Accordingly, exercise in this domain may be sustained for several hours; however, muscle inefficiency manifests with longer exercise durations. Impairments to Ca^{2+} release rates are a contributing factor to the decline in contractile function (Allen et al., 2008) and the depletion of muscle glycogen is an important determinant of muscle function and excitability within the moderate-intensity exercise domain (Black et al., 2017). Consequently, a decline in muscle

efficiency is represented by an upward drift in $\dot{V}O_2$ over time when performing moderate-intensity exercise for prolonged durations ≥ 60 min (Coyle et al., 1991; Hagberg et al., 1978; Hagan et al., 1992; Kalis et al., 1988).

2.4.2. Heavy-intensity exercise domain

Heavy-intensity exercise consists of all work rates between the GET and the critical power (CP), where the CP represents the highest sustainable rate of oxidative metabolism without progressive energy contribution from anaerobic pathways (Chidnok et al., 2013; Jones et al., 2008; Poole et al., 1988; Vanhatalo et al., 2016). In this exercise domain, a $\dot{V}O_2$ slow component ($\dot{V}O_{2sc}$) manifests shortly after the onset of exercise (90 to 120 seconds), reflecting a loss of linearity in the relationship between the work rate and $\dot{V}O_2$ (Burnley & Jones, 2007). The $\dot{V}O_{2sc}$ differs to the O_2 drift phenomenon that occurs during prolonged moderate-intensity exercise. The $\dot{V}O_{2sc}$ occurs during heavy- and severe-intensity exercise exhibited by a large magnitude of increased $\dot{V}O_2$ compared to the gradual increase by < ~200 mL during the O_2 drift (Gaesser & Poole, 1996). Mechanistically, the $\dot{V}O_{2sc}$ is underpinned by several factors including reduced contractile efficiency (Rossiter et al., 2002; Tonkonogi et al., 1999), slow $\dot{V}O_2$ kinetics of initially recruited type II fibres (Barclay et al., 1996; Crow & Kushmerick, 1982b), the O_2 cost of recovery processes in fatigued fibres (Hepple et al., 2010), and the progressive activation of type II fibres (Krustrup et al., 2004; Jones et al., 2011). However, progressive muscle fibre recruitment has been shown to not always be requisite and may be related to the decline in efficiency over time in already recruited fibres (Cannon et al., 2014; Rossiter et al., 2002; Vanhatalo et al., 2011b; Zoladz et al., 2008). The main functional consequence of the $\dot{V}O_{2sc}$ for exercise tolerance and fatigue in this domain is the reduction in work efficiency, which is also linked to fatigue. Despite the development of the $\dot{V}O_{2sc}$, exercise within the heavy-intensity domain may be sustained for several hours given that metabolites and $\dot{V}O_2$ eventually reaches an elevated steady-state which can take up to 15 to 20 minutes (Poole et al., 1988). However, during prolonged heavy-intensity continuous exercise, a decline in muscle efficiency (and thus, an increased upward drift in $\dot{V}O_2$ in the steady-state $\dot{V}O_2$ response) may occur over time (Hopker et al., 2017; Passfield & Doust, 2000). A decline in muscle efficiency during prolonged heavy-intensity exercise is multifaceted and relates to changes in muscle excitability, muscle glycogen stores,

intramuscular metabolite accumulation (i.e. metabolic perturbation), progressive type II muscle fibre recruitment, and the accumulation of ROS (Allen et al., 2008; Black et al., 2017; Krustrup et al., 2004; Powers & Jackson, 2008).

2.4.3. Severe-intensity exercise domain

Severe-intensity exercise consists of all work rates between the CP and the maximal aerobic capacity ($\dot{V}O_{2max}$) determined from an incremental exercise test to exhaustion (Poole et al., 1988). Exercise in this domain can be sustained for ~10 to 20 min before exhaustion occurs (Jones & Poole, 2005) and is characterized by a hyperbolic relationship, known as the power-duration relationship (Jones et al., 2008). The CP occurs at the asymptote of the power-duration relationship, and the finite quantity of work above the CP is termed the W' (Fukuba et al., 2003; Poole et al., 1988). Within this exercise intensity domain, the $\dot{V}O_{2sc}$ drives the pulmonary $\dot{V}O_2$ response to reach $\dot{V}O_{2max}$. Concomitantly, the attainment of critical levels of low [PCr] ([] denotes concentration) and pH, as well as high [P_i] induces fatigue in this exercise intensity domain (Allen et al., 2008; Fitts, 1994; Fuchs et al., 1970; MacDonald & Stephenson, 2001; Meissner et al., 1986; Vanhatalo et al., 2010b; 2016).

2.5. Nitric oxide biochemistry and metabolism

The physiological responses to exercise are partly regulated by NO, a ubiquitous signalling molecule that regulates a plethora of physiological functions (Furchgott & Zawadzki, 1980; Ignarro et al., 1987; Murad et al., 1978; Stamler & Meissner, 2001). The production of NO occurs at rest and dramatically increases during exercise (Balon & Nadler, 1994). In addition, NO bioavailability may be dependent on muscle fibre type recruitment patterns given that NO production is facilitated by low pH (Modin et al., 2001) and hypoxia (Castello et al., 2006), and thus, NO production is influenced by exercise intensity and duration (see section: *One step reduction of NO_2^- to NO*; Castello et al., 2006; Modin et al., 2001; Richardson et al., 1995). NO has a role in regulating vasodilation (Joyner & Tschakovsky, 2003; Moncada & Higgs, 1993), skeletal muscle glucose uptake (Merry et al., 2010), mitochondrial cellular respiration (Brown & Cooper, 1994; Shiva et al., 2007), neurotransmission (Garthwaite, 2008), skeletal muscle contraction (Reid, 1998; Stamler & Meissner, 2001; Viner et al., 2000), and fatigue development (Percival et al., 2010). Depending on the intramuscular

conditions, the half-life of NO is < 2 milliseconds in blood (Liu et al., 1998) to seconds (Crawford et al., 2006; Kelm, 1999). Therefore, continual NO production and recycling is crucial for physiologic function. Two known pathways exist which produce NO and are discussed below (Fig. 2.5).

2.5.1. O_2 dependent–nitric oxide synthase (NOS) pathway

The classical pathway by which NO is produced was discovered in the 1980s (Moncada et al., 1989) and is through the conversion of the amino acid L-arginine into NO by a group of nitric oxide synthase (NOS) enzymes (Bredt, 1999; Moncada & Higgs, 1993). These isoforms include: neuronal NOS (nNOS, NOS-1; Nakane et al., 1993) located in myocytes (Brenman et al., 1996; Kobzik et al., 1994), inducible NOS (iNOS, NOS-2, Williams et al., 1994) located primarily in macrophages (Park et al., 1996; Williams et al., 1994), that are responsive to inflammatory processes (Reid, 1998), and endothelial NOS (eNOS, NOS-3, Förstermann et al., 1991) located in vascular endothelial cells (Kobzik et al., 1994). For NO production to occur, the reaction between L-arginine and O_2 requires the presence and action of several cofactors (Moncada & Higgs, 1993). Specifically, the binding of calmodulin/Ca^{2+} is required to facilitate the donation of electrons from NADPH to FAD and subsequently to flavin mononucleotide (FMN) (Alderton et al., 2001). The electron flow continues to react with haem iron and 5,6,7,8-tetrahydrobiopterin (BH_4), to catalyse the reaction between L-arginine and O_2 (Alderton et al., 2001). The end products of this reaction are the formation of L-citrulline and NO (Alderton et al., 2001). The production of NO through this pathway would be compromised if O_2 or any of the cofactors were limited by poor bioavailability. The importance of NO production is highlighted by impairments to NOS that have been associated to pathophysiology such as sarcopenia (Hall et al., 2011), type 2 diabetes (Wu & Meininger, 2009), cardiovascular disease (Förstermann, 2010), and chronic obstructive pulmonary disease (Barnes & Kharitonov, 1996).

2.5.2. NO_3^--NO_2^--NO pathway

Another route for NO production in humans is the NO_3^-–NO_2^-–NO pathway (Benjamin et al., 1994; Lundberg et al., 1994; Speigelhalder et al., 1976). The discovery of this pathway was surprising given that traditionally, circulating NO_3^- and NO_2^- were thought to be biologically inactive and stable end-product molecules of NO metabolism (Moncada & Higgs, 1993). However, it is now widely accepted that NO_3^- and NO_2^-

represent storage pools of NOS-derived NO (Lundberg et al., 2008). Therefore, two major sources of NO_3^- are from NOS-derived NO oxidation and exogenous dietary NO_3^- sources. The existence of the NO_3^-–NO_2^-–NO pathway highlights the importance of NO recycling given that both endogenous NOS-derived NO_3^- and exogenous NO_3^- and NO_2^- (i.e. from dietary sources) will enter the cycle to ensure NO generation at all times (Lundberg et al., 2008).

In vivo NO_3^- production occurs through the oxidation of excess NOS-derived NO (Doyle & Hoekstra, 1981) and is formed when NO reacts with oxy-heme proteins (i.e. oxyhaem, oxymyoglobin, and other still unknown heme proteins; Keszler et al., 2008). NO_2^- production occurs through the oxidation of NO (Ignarro et al., 1993), a reaction catalysed by multi-copper oxidase, and NO oxidase ceruloplasmin (Shiva et al., 2006). Endogenous and exogenous NO_3^- and NO_2^- can be serially reduced back into NO and other bioactive nitrogen oxides such as RSNO in the blood and other tissues such as the skeletal muscle (Piknova et al., 2015), heart (Zweier et al., 1995), and liver (Gilliard et al., 2018). The reduction process is facilitated under hypoxic conditions, during which the NO_3^-–NO_2^-–NO pathway gradually becomes more activated as the O_2-dependent L-arginine pathway becomes dysfunctional (Lundberg & Weitzberg, 2009; Fig. 2.6). Therefore, NO_3^-, NO_2^-, and RSNO can be used as markers for NO bioavailability (Ignarro, 2002; Moncada & Higgs, 1993) and are recognized to also represent reservoirs for maintaining NO homeostasis and bioactivity in tissues during metabolic stress (Lundberg & Weitzberg, 2009). Interestingly, the NO_2^- anion (Lundberg et al., 2008) and RSNO (Jia et al., 1996) may also function as intracellular signalling molecules on their own, inducing important physiological responses such as rapid vasodilation (Bryan et al., 2005; Dejam et al., 2007) or post translational protein modifications (Biswas et al., 2006; Hogg, 2002).

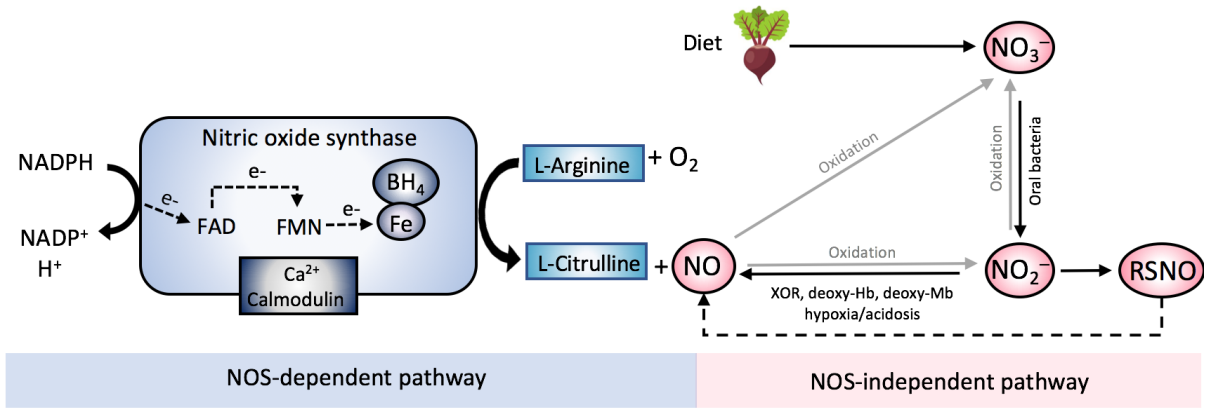

Figure 2.5. A schematic diagram of the pathways for nitric oxide (NO) production. In the NOS-dependent pathway, nitric oxide synthase (NOS) enzymes catalyse the production of NO using substrates L-arginine and oxygen (O_2), along with cofactors calmodulin/calcium (Ca^{2+}), nicotinamide dinucleotide phosphate (NADPH), flavin adenine nucleotide (FAD), flavin mononucleotide (FMN), haem iron (Fe) and 5,6,7,8-tetrahydrobiopterin (BH_4). In the NOS-independent pathway, NOS-derived and exogenous sources of nitrate (NO_3^-) undergoes reduction to form nitrite (NO_2^-), a reaction catalysed by anaerobic facultative bacteria in the oral cavity. NO_2^- is further metabolized in a process facilitated by xanthine oxidase (XOR), deoxyhaemoglobin (deoxy-Hb), deoxymyoglobin (deoxy-Mb) and mitochondrial respiration complexes into NO and S-nitrosothiols (RSNO) which can induce signalling and post-translational protein modifications. This reduction process is potentiated by hypoxic and acidic environments. In addition, RSNO may decompose and release NO. Endogenous and exogenous (i.e. dietary derived) NO will recycle to form higher nitrogen oxides NO_2^- and NO_3^-. Through the NO_3^--NO_2^--NO pathway, ingestion of NO_3^--rich foods may increase NO_2^- and NO production.

2.5.3. The enterosalivary circulation

Following ingestion of NO_3^- rich foods, NO_3^- is absorbed by the gastrointestinal tract and enters the systemic circulation within 1 h (Lundberg et al., 2008). In addition, NO_3^- is also absorbed into other tissues such as the skeletal muscle (Piknova et al., 2015) and the liver (Gilliard et al., 2018), which are tissues that also participate in NO_3^- metabolism. Around 25% of the NO_3^- is actively transported by the NO_3^- transporter protein sialin (Qin et al., 2012) located in the salivary glands, causing NO_3^- to enter the enterosalivary circulation (Petersson et al., 2015; Qin et al., 2012). Following entering the enterosalivary circulation, NO_3^- in the saliva is subsequently concentrated up to ~20-fold (Govoni et al., 2008). Once in the oral cavity, anaerobic facultative

bacteria on the dorsal surface of the tongue reduce ~20% of absorbed NO_3^- into NO_2^- via bacterial respiration whereby NO_3^- reductases use NO_3^- as a terminal electron acceptor (Duncan et al., 1995; Spiegelhalder et al., 1976). A portion of the NO_2^- reduces into NO in the stomach (Benjamin et al., 1994) but some of the NO_2^- enters the systemic circulation, as evidenced by increased plasma [NO_2^-] (Webb et al., 2008). Although the half-life of NO_3^- in the blood is 5 to 8 h, the half-life of NO_2^- in the blood is 1 to 5 min (Lundberg et al., 2008). The last step of reducing NO_2^- to NO is ubiquitous and occurs through enzymatic and non-enzymatic pathways, discussed in the next section.

2.5.4. One step reduction of NO_2^- to NO

The final one step reduction into NO (and other nitrogen intermediates such as RSNO) may occur in the blood stream and in other tissues and is facilitated by enzymatic reactions catalyzed by cytochrome P450 (Kozlov et al., 2003), aldehyde oxidase (Li et al., 2008), xanthine oxidase (Zhang et al., 1997), deoxyhaemoglobin (Cosby et al., 2003; Nagababu et al., 2003), deoxymyoglobin (Rassaf et al., 2007a; Shiva et al., 2007), and mitochondrial respiration complexes (Kozlov et al., 1999), as well as non-enzymatic reactions with protons facilitated by ascorbate (Carlsson et al., 2001) and polyphenols (Gago et al., 2007; Peri et al., 2005). As previously mentioned, this pathway is potentiated in hypoxic (i.e. low O_2 tension, PO_2; Castello et al., 2006; Millar et al., 1998), and acidic environments (i.e. low pH; Modin et al., 2001) which is in stark contrast to the L-arginine pathway. Therefore, the NO_3^--NO_2^--NO pathway acts as an alternative process to yield NO in conditions otherwise unfavourable to the classical NOS-dependent pathway and is facilitated where NO is most required (i.e. tissue undergoing ischemia and hypoxia) (Giraldez et al., 1997; Lundberg et al., 2008; Oestergaard et al., 2007).

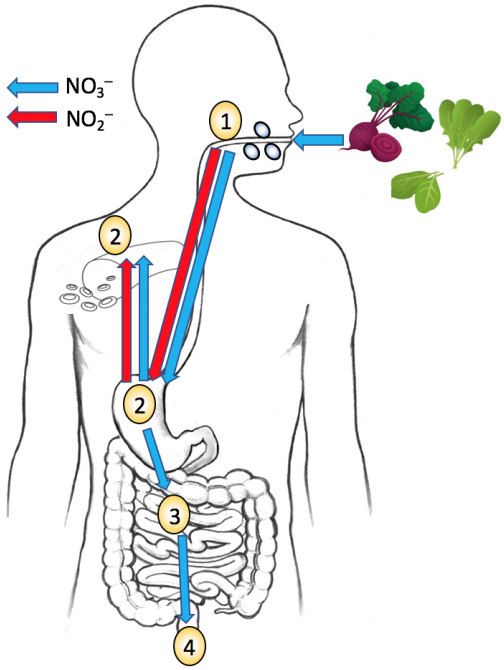

Figure 2.6. A simplified schematic diagram redrawn from Gilchrist et al., 2011. The enterosalivary circulation of NO_3^- from dietary sources in the NO_3^-–NO_2^-–NO pathway: 1) endogenous NOS-derived NO_3^- mixed with exogenous NO_3^- from the diet is converted into NO_2^- by facultative anaerobic bacteria in the oral cavity and is swallowed; 2) a portion of the NO_2^- is reduced into NO in the stomach (McKnight et al., 1997) by acid solutions and a portion of NO_2^- enters systemic circulation undergoes a single electron reduction to form NO in reactions catalysed by NO_2^- reductases (see text for details); 3) rest of NO_3^- is absorbed by the kidneys; and 4) NO_3^- is excreted in the urine.

2.5.5. Dietary NO_3^- and NO_2^-

It is estimated that ~80% of NO_3^- is consumed in the diet particularly from leafy green vegetables such as rocket, kale, beetroot and spinach, containing ~250 mg or ~4 mmol of NO_3^- (62 mg per mmol) for 100 g of fresh weight produce (Gangolli et al., 1994; Hord et al., 2009; Lidder et al., 2013). In Europe, 31 to 185 $mg \cdot d^{-1}$ of dietary NO_3^- is consumed on average (Mensinga et al., 2003) whilst in the United States, ~40 to 100 $mg \cdot d^{-1}$ of dietary NO_3^- is consumed on average (Gangolli et al., 1994). Importantly, some individuals will consume far more or less than the reported intake due to habitual dietary choices. Total dietary NO_3^- intake is influenced by the NO_3^- content of the soil, H_2O, type of vegetable, and quantity of vegetables consumed (Pennington, 1998). In contrast, minimal amounts of NO_2^- are ingested from the diet ranging from 0 to 20 $mg \cdot d^{-1}$ (Khatri et al., 2017; Pennington, 1998) primarily found as colourant, preservatives, and flavour enhancers within cured meats (Cassens, 1995). The

discovery of the NO_3^--NO_2^--NO pathway enables increased NO bioavailability through consumption of dietary NO_3^- sources (Ysart et al., 1999) in addition to circulating endogenous NO_3^- and NO_2^- produced from NO oxidation (Doyle & Hoekstra, 1981; Ignarro et al., 1993; Keszler et al., 2008). Therefore, this pathway provides potential for novel dietary interventions to elevate NO bioavailability to evoke positive physiological effects (Hord et al., 2009; Lundberg et al., 2008; Moncada & Higgs, 1993).

2.5.6. Factors affecting NO_3^- and NO_2^- bioavailability

The oral microbiome is becoming recognized as crucial in NO_3^--NO_2^--NO pathway (Hyde et al., 2014) and several factors may be related to the reduction of NO_3^-. Firstly, the type and relative proportion of bacterial colonies in the oral cavity may be important, such as the *Vionella* (Doel et al., 2005), as well as *Neisseria* and *Rothia* (Hyde et al., 2014; Velmurugan et al., 2016). Secondly, interventions that have interfered with the oral microbiome, such as the use of antiseptic and antibacterial mouthwash (Govoni et al., 2008; McDonagh et al., 2015; Woessner et al., 2016) or the prevention of swallowing saliva (Webb et al., 2008) have been reported to attenuate elevations in NO_2^- following NO_3^- ingestion. Lastly, co-ingestion of NO_3^- with other compounds which compete for the same transporter, such as thiocyanate (i.e. cruciferous vegetables; Olea & Parras, 1992) and iodide (i.e. seafood and dairy products; Edwards et al., 1954; Leung & Braverman, 2012), may attenuate elevations in NO_2^- (Bailey et al., 2016; 2017; Dewhurst-Trigg et al., 2018).

2.6. The effects of dietary NO_3^- supplementation on exercise efficiency

The pulmonary $\dot{V}O_2$ response is reflective of muscle O_2 uptake, and thus the muscle metabolic rate during exercise (Barstow et al., 1990; Koga et al., 2005; Grassi et al., 1996). Therefore, the absolute O_2 cost of exercise relative to an individuals' $\dot{V}O_{2max}$ is an indication of exercise efficiency. Exercise efficiency can be defined as the ratio of mechanical energy output to the metabolic energy input (Grassi et al., 2015). An improved exercise efficiency (i.e. lowered O_2 cost of constant work rate exercise) would be expected to enable a greater amount of work to be completed at the same given force output, and thus could positively impact exercise performance. By the

same token, a decline to muscle efficiency is reflected in an increased O_2 cost of exercise.

Dietary NO_3^- supplementation is the first dietary supplement shown to lower the O_2 cost of exercise (Larsen et al., 2007). Given that the expected increase in pulmonary $\dot{V}O_2$ during moderate-intensity exercise is normally ~9 to 11 $ml \cdot min^{-1} \cdot W^{-1}$, and is invariant to factors such as age, fitness level, and health status (Jones & Poole, 2005; Moseley et al., 2004), the ability for dietary NO_3^- supplementation to lower the O_2 cost of exercise is fascinating. Larsen et al. (2007) were the first group who reported that 3 days of sodium NO_3 ($NaNO_3$, 0.1 mmol $kg^{-1}day^{-1}$ of NO_3^-) supplementation induced a reduction in $\dot{V}O_2$ in recreationally active men. Participants cycled in 5 min bouts at submaximal intensities ranging from 40-85% of $\dot{V}O_{2max}$, and the O_2 cost of exercise was lowered by 5% over the first four stages from 2.98 $\pm$ 0.57 $L \cdot min^{-1}$ to 2.82 $\pm$ 0.58 $L \cdot min^{-1}$ (Larsen et al., 2007). Interestingly, the dosage was equivalent to ~150-250 g of NO_3^--rich food, an amount easily accessible to the general population (Hord et al., 2009; Larsen et al., 2007). Importantly, the lack of change in lactate was indicative that there was no compensatory change in glycolytic ATP production and that the metabolic efficiency was improved (Larsen et al., 2007). Following this, Bailey et al. (2009) provided a natural dietary source of NO_3^-, using non-concentrated beetroot (BR) juice (0.5 $L \cdot day^{-1}$ containing 5.6 mmol of NO_3^-), to healthy recreationally active males for 4-6 days, and observed that $\dot{V}O_2$ was reduced by 5% during constant work rate moderate-intensity cycling (Fig 2.7a). In addition, the improvement in exercise efficiency, as reflected by the lowering of $\dot{V}O_2$, translated into a 16% improved time-to-exhaustion (TTE) during severe-intensity cycling (Bailey et al., 2009; Fig 2.7b). In line with findings from Larsen et al. (2007), lactate and respiratory exchange ratio were unchanged by BR juice supplementation, suggesting a lack of compensatory change in anaerobic energy production (Bailey et al., 2009).

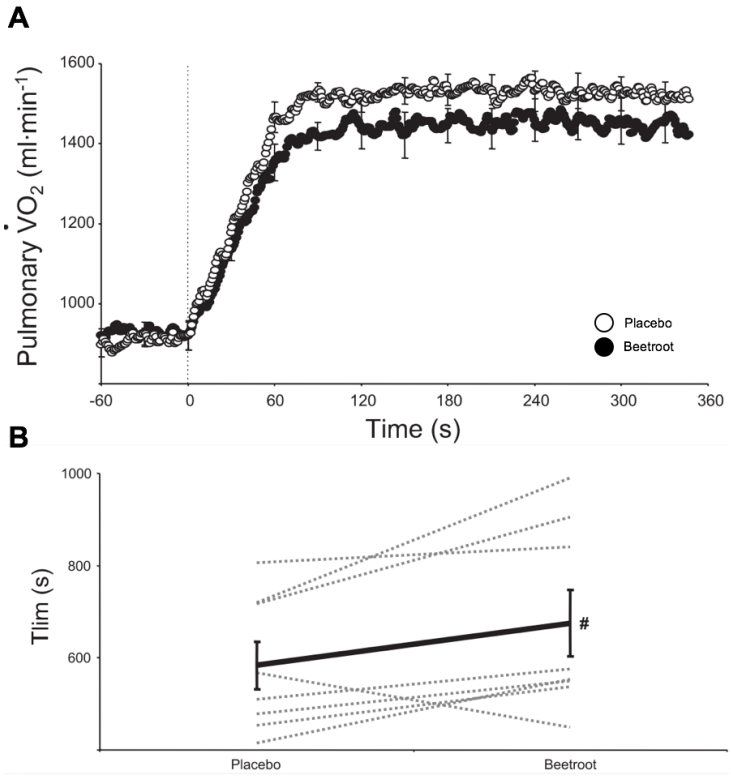

Figure 2.7. Mean pulmonary $\dot{V}O_2$ response during moderate-intensity cycling exercise (panel A) and individual changes in exercise tolerance (dotted lines) and the group mean change (solid line; panel B) following ingestion of non-concentrated NO_3^--rich beetroot juice and blackcurrant cordial (adapted from Bailey et al., 2009).

Since these earlier studies, there has been a surge in publications investigating the effect of dietary NO_3^- supplementation on exercise efficiency in healthy individuals that have yielded equivocal results (Table 2.2). To date, dietary NO_3^- supplementation has been reported to improve exercise efficiency in healthy humans over a range of exercise modalities, such as cycling (Cermak et al., 2012a; Flueck et al., 2016; Larsen et al., 2010; 2011; Muggeridge et al., 2014; Porcelli et al., 2016; Thompson et al., 2017; 2018; Vanhatalo et al., 2010a; Whitfield et al., 2016; Wylie et al., 2013a; 2016), walking (Kuennen et al., 2015; Lansley et al., 2011b), running (Lansley et al., 2011b), kayaking (Muggeridge et al., 2013; Peeling et al., 2015) and knee extension exercise (Bailey et al., 2010). However, it is important to note that there are also studies which have not observed any significant influence of NO_3^- supplementation on exercise efficiency (Balsalobre-Fernández et al., 2018; Bescós et al., 2011; Betteridge et al., 2016; Boorsma et al., 2014; Breese et al., 2013; Christensen et al., 2013; 2017; Ghiarone et al., 2017; Kelly et al., 2014; Kocoloski et al., 2018; Mosher et al., 2019;

Muggeridge et al., 2015; Nyakayiru et al., 2017c; Oskarsson et al., 2018; Peacock et al., 2012; Sandbakk et al., 2015; Thompson et al., 2014; Wickham et al., 2019; Wilkerson et al., 2012).

There is some evidence suggesting that the discrepancy between studies may be in part due to subject training status (Carriker et al., 2016; Porcelli et al., 2015; Shannon et al., 2016). Carriker et al. (2016) reported a significant correlation between the change in $\dot{V}O_2$ during submaximal exercise following 4 days of BR juice supplementation (6.2 mmol of NO_3^- per day) and the individual $\dot{V}O_{2peak}$ such that subjects with higher aerobic fitness levels exhibited less of a reduction in $\dot{V}O_2$. Moreover, Porcelli et al. (2015) observed a correlation between the change in $\dot{V}O_2$ during constant work-rate moderate-intensity exercise following 6 days of NO_3^- supplementation and the $\dot{V}O_{2peak}$ of subjects such that low-aerobically fit subjects exhibited the greatest reduction in $\dot{V}O_2$ following NO_3^- supplementation (Fig. 2.8). Together, these studies suggest that individuals with higher aerobic fitness are less likely to respond to NO_3^- supplementation. The influence of training status may be underpinned by exercise-induced physiological adaptations such as increased capillary density (Andersen & Henriksson, 1977), enhanced oxidative capacity (Hopker et al., 2009; Tonkonogi & Sahlin, 2002) and increased NO production (Delp, 1995; Tsukiyama et al., 2017). In support of this interpretation, at the onset of this thesis, 21 studies had reported non-significant results, and 13 of these studies used trained subjects (Table 2.2) highlighting that training status may partly account for the disparate results. However, subject training status may not be the only factor to influence the efficacy of NO_3^- supplementation on exercise efficiency given that the remaining 8 investigations that observed no significant effect used untrained subjects (Table 2.2). The only systematic review and meta-analysis that has examined the influence of dietary NO_3^- supplementation on exercise efficiency concluded that the O_2 cost of exercise was likely to be lowered during moderate- and heavy-intensity exercise, but was unable to evaluate the impact of training status (Pawlak-Chaoach et al., 2016). Thus, other contributing factors to the lack of effect on the O_2 cost of exercise following NO_3^- ingestion are currently unresolved.

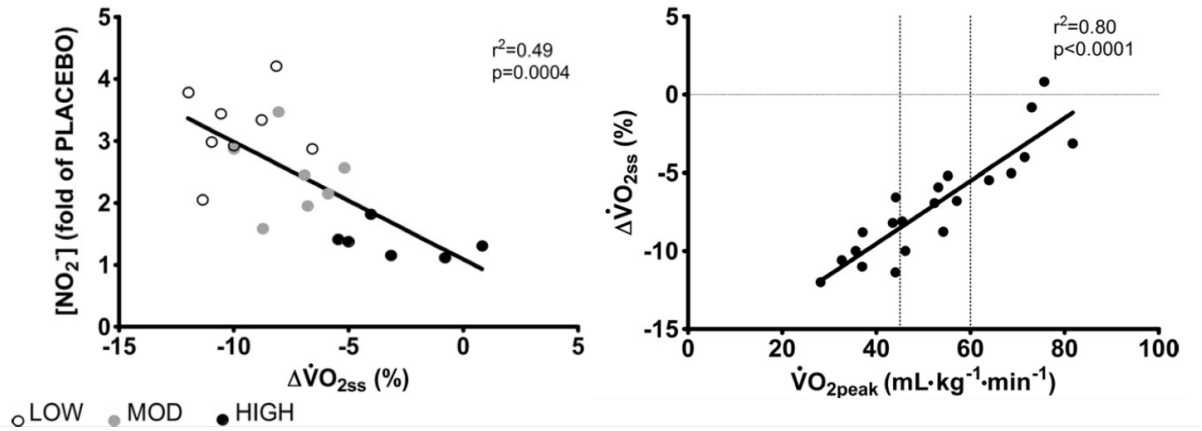

Figure 2.8. Individual change of plasma [NO_2^-] (expressed as fold of placebo) compared to individual percentage change of $\dot{V}O_2$ during moderate-intensity exercise (panel A) and % change in $\dot{V}O_2$ vs individual $\dot{V}O_{2peak}$ (panel B) following NO_3^- supplementation (adapted from Porcelli et al., 2015).

Dietary NO_3^- supplementation is commonly administered as concentrated NO_3^--rich BR juice and is compared to a placebo beverage of concentrated NO_3^--depleted BR juice that is identical in colour, taste, and smell (Lansley et al., 2011b). Importantly, bioactive phytochemicals are present in both NO_3^--rich and NO_3^--depleted BR juice such as flavonoids, betalains, and ascorbic acid (Shepherd et al., 2015; Wootton-Beard & Ryan, 2012). Although speculative, the presence of these antioxidant constituents potentially contributes to the elevation of NO bioavailability given that polyphenols facilitate the reduction of NO_2^- to NO (Gago et al., 2007; Peri et al., 2005; Rocha et al., 2009). Moreover, it could be possible that NO_3^- synergistically reacts with the polyphenolic compounds to induce physiological modulations. This has important implications for understanding the factors that influence the efficacy of dietary NO_3^- supplementation on exercise efficiency given that polyphenol-induced elevations to NO following ingestion of the NO_3^--depleted BR juice (i.e. placebo) could potentially obscure results. Indeed, Flueck et al. (2016) investigated the influence of 3.0, 6.0, and 12.0 mmol of NO_3^- administered as BR juice or NO_3^- salts on the O_2 cost of moderate- and severe-intensity cycling exercise in healthy untrained individuals. The administration of 6.0 mmol of NO_3^- in the BR juice form significantly lowered severe-intensity exercise $\dot{V}O_2$ by 4.2% compared to a water control but there was no significant effect following ingestion of 6.0 mmol of NO_3^- in the form of NO_3^- salts (Flueck et al., 2016). The results of this study indicate that the vehicle of administration

of NO_3^- (i.e. juice vs. salts) may be a factor in the influence of NO_3^- supplementation which could be due to differences in antioxidant constituents between BR juice and NO_3^- salts. Specifically, if NO bioavailability is influenced by NO_3^--depleted BR juice, this could potential obscure results and partly explain the divergent results in the literature. Further research is required to identify the role of polyphenolic compounds in NO_3^--rich and NO_3^--depleted BR juice on physiological modulations during exercise.

Table 2.2. Description of studies that have investigated the effect of dietary NO_3^- supplementation on the O_2 cost of exercise in humans.

Author	Participants	$\dot{V}O_{2peak}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	Supplementation	Exercise mode	Protocol	NO indices	$\dot{V}O_2$ response during submaximal exercise
Larsen et al., (2007)	9 TR males	55 ± 4	3 d $NaNO_3$ supplementation (0.l $mmol \cdot kg^{-1} \cdot d^{-1}$)	Cycling	5 min bouts at work rate at 45, 60, 70, 80, 85 and 100% $\dot{V}O_{2peak}$	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during exercise at 45, 60, 70, and 80% $\dot{V}O_{2peak}$
Bailey et al., (2009)	8 UT males	49 ± 5	6 d of NO_3^- rich BR juice supplementation (~5.6 mmol $NO_3^- \cdot d^{-1}$)	Cycling	4 moderate-intensity bouts at 80%GET on days 4 to 6 of supplementation	↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during moderate-intensity exercise
Bailey et al., (2010)	7 UT males	N/A	6 d of NO_3^- rich BR supplementation (~5.1 mmol $NO_3^- \cdot d^{-1}$)	Two-legged knee-extension	6 low-intensity bouts at 15% MVC iEMG signal on days 4 to 6 of supplementation	↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during low-intensity exercise
Larsen et al., (2010)	7 UT males/2 UT females	N/A	1h prior to exercise ingestion of $NaNO_3$ (0.1 $mmol \cdot kg^{-1}$)	Cycling	5 min low-intensity bout	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during low-intensity exercise
Vanhatalo et al., (2010a)	8 UT males/females	47 ± 8	2.5h prior to exercise, 5 d, and 15 d of NO_3^- rich BR juice supplementation (~5.2 mmol $NO_3^- \cdot d^{-1}$)	Cycling	2 x moderate-intensity bouts at 90%GET after 2.5h, 5 d, and 15 d of supplementation	↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during moderate-intensity exercise
Bescós et al., (2011)	11 TR male cyclists/triathletes	65 ± 6	3h prior to exercise ingestion of $NaNO_3$ (10 $mg \cdot kg^{-1}$)	Cycling	6 min bouts at work rates equivalent to 2, 2.5, 3, and 3.5 $W \cdot kg^{-1}$	↑ Plasma $[NO_2^-]$	↔ $\dot{V}O_2$ at all exercise bouts
Lansley et al., (2011b)	9 UT male	55 ± 7	6 d of NO_3^- rich BR juice supplementation (~5.1 mmol $NO_3^- \cdot d^{-1}$)	Walking/running	2 x 6 min bouts walking at 4 km·h-1 4 moderate-intensity running bouts at 80%GET on days 4 and 5 of supplementation	↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during walking and running
Larsen et al., (2011)	13 UT males/females	N/A	3 d $NaNO_3$ supplementation (0.l $mmol \cdot kg^{-1} \cdot d^{-1}$)	Cycling	Bout at work rate equivalent to 50%$\dot{V}O_{2peak}$	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↓ $\dot{V}O_2$ during exercise
Cermak et al., (2012a)	13 TR male cyclists/triathletes	58 ± 2	6 d of NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^- \cdot d^{-1}$)	Cycling	30 min bout at 45% and 65% PPO	↑ Plasma $[NO_3^-]$	↓ $\dot{V}O_2$ during exercise at both intensities
Peacock et al., (2012)	10 TR male skiers	70 ± 5	2.5h prior to exercise ingestion of KNO_3 (9.9 mmol NO_3^-)	Running	5 min bouts at 10 and 14 $km \cdot h^{-1}$	↑ Plasma $[NO_2^-]$	↔ $\dot{V}O_2$ at all exercise bouts
Wilkerson et al., (2012)	8 TR male cyclists	63 ± 8	2.5h prior to exercise ingestion of NO_3^- rich BR juice (~6.2 mmol NO_3^-)	Cycling	50 km TT	↑ Plasma $[NO_2^-]$	↔ $\dot{V}O_2$
Breese et al., (2013)	9 UT males/females	M: 48 ± 6 F: 46 ± 9	6 d NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^- \cdot d^{-1}$)	Cycling	3 x 4 min moderate-intensity bouts at 80%GET on day 3 to 6 of supplementation	↑ Plasma $[NO_2^-]$	↔ $\dot{V}O_2$ at all exercise bouts

Christensen et al., (2013)	10 TR male cyclists	72 ± 4	6 d NO_3^- rich BR juice supplementation (8.0 mmol NO_3^-·d^{-1})	Cycling	3 x 6 min bouts at 70% PPO	↑ Plasma [NOx]	↔ $\dot{V}O_2$ at all exercise bouts
Muggeridge et al., (2013)	8 TR male kayakers	49 ± 6	2.5h prior to exercise ingestion of NO_3^- rich BR juice (4.2 mmol NO_3^-)	Kayaking	15 min bout at 60%HR_{max}	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during exercise
Wylie et al., (2013a)	10 UT males	N/A	2.5h prior to exercise ingestion of NO_3^- rich BR juice (4.2, 8.4, 16.8 mmol NO_3^-)	Cycling	2 x 5 min bouts at 80%GET	Dose-dependent ↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during last 30 s of moderate-intensity exercise bout
Boorsma et al., (2014)	8 TR male 1500m runners	80 ± 5	2.5h prior to exercise ingestion of NO_3^- rich BR (19.5 mmol NO_3^-), 8 d BR juice supplementation where day 1 and 8 subjects consumed 19.5 mmol NO_3^-·d^{-1} and days 2 to 7 subjects consumed 13 mmol NO_3^-·d^{-1})	Running	5 to 7 min bout at 50, 65, and 80%WR_{peak}	↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ at all exercise bouts
Kelly et al., (2014)	12 UT males	58 ± 6	6 d NO_3^- rich BR juice supplementation (8.4 mmol NO_3^-·d^{-1})	Cycling	2 x 5 min moderate-intensity bouts at 80%GET on day 3 to 6 of supplementation	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ at all exercise bouts
Muggeridge et al. (2014)	9 TR male cyclists	52 ± 6	3h prior to exercise ingestion of NO_3^- rich BR juice (~5 mmol NO_3^-)	Cycling	15 min at 60%WR_{peak} at 2500 m simulated altitude	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$
Thompson et al., (2014)	16 UT males	47 ± 6	90 min prior to exercise ingestion of NO_3^- rich BR juice (5.0 mmol NO_3^-)	Cycling	2 x 20 min bouts at 50% and 70%$\dot{V}O_{2peak}$	↑ Plasma [NO_2^-]	Trend ↓ $\dot{V}O_2$ during exercise at 50%$\dot{V}O_{2peak}$ (*P*=0.110)
Kuennen et al., (2015)	9 UT males	51 ± 2	6 d NO_3^- rich BR juice supplementation (8.4 mmol NO_3^-·d^{-1})	Desert marching	45 min treadmill walking (1.5% incline at 4.83 km·h^{-1}) dressed in a military ensemble	↑ Plasma [NO_3^-]	↓ $\dot{V}O_2$
Muggeridge et al., (2015)	9 TR male cyclists	53 ± 4	2.5h prior to exercise ingestion of NO_3^- rich gel (~8.1 mmol NO_3^-)	Cycling	10 min bout at 60%WR_{max}	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	Trend ↓ $\dot{V}O_2$ during exercise (*P*=0.086)
Peeling et al., (2015)	Study 1: 6 TR male kayakers	N/A	Study 1: 2.5h prior to exercise ingestion of NO_3– rich BR juice (4.8 mmol NO_3^-)	Kayaking	Study 1: 4 min max lab kayak ergometer TT	↑ Plasma [NO_2^-]	Study 1: ↓ $\dot{V}O_2$
Porcelli et al., (2015)	8 UT males 7 moderately-TR males 6 TR males	28-44 46-57 64-82	6 d $NaNO_3$ supplementation (5.5 mmol NO_3^-·d^{-1})	Running	4 x 6 min moderate-intensity bouts at 80%GET	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-] for all groups, dependent on fitness	UT and moderately-TR: ↓ $\dot{V}O_2$ during all exercise bouts TR: ↔ $\dot{V}O_2$ during all exercise bouts

Rienks et al., (2015)	10 UT females	37 ± 5	2.5 prior to exercise ingestion of NO_3^- rich BR juice (~13 mmol NO_3^-)	Cycling	2 x 20 min TT followed by 5 min at 75W	N/A	↔ $\dot{V}O_2$ at 20 min bout ↓ $\dot{V}O_2$ during 5 min at 75 W
Sandbakk et al., (2015)	9 TR male cross-country skiers	69 ± 6	2.5h prior to exercise ingestion of KNO_3 (9.9 mmol NO_3^-)	Running	2 x 5 min bouts at 10 km·h^{-1} and 14 km·h^{-1}	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ at all exercise bouts
Betteridge et al., (2016)	8 UT males	46 ± 3	2.5h prior to exercise ingestion of NO_3^- rich BR juice (8.0 mmol NO_3^-)	Cycling	60 min bout at 65%$\dot{V}O_{2peak}$	↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ during exercise
Carriker et al., (2016)	5 UT males 6 TR males	UT: 42 ± 3 TR: 60 ± 5	4 d NO_3^- rich BR juice supplementation (6.2 mmol NO_3^-·d^{-1})	Running	5 min bouts at 45, 60, 70, 80, and 85%$\dot{V}O_{2peak}$	↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during exercise at 45 and 60%$\dot{V}O_{2peak}$ in UT subjects only
Flueck et al., (2016)	12 UT males	59	3h prior to exercise ingestion of NO_3^- rich BR juice or $NaNO_3$ (3.0, 6.0, 12.0 mmol NO_3^-)	Cycling	5 min at 50%$\dot{V}O_{2peak}$ and 8 min at 80%$\dot{V}O_{2peak}$	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during severe-intensity exercise only in BR but not $NaNO_3$
Porcelli et al., (2016)	7 UT males	41 ± 5	6 d NO_3^- rich diet (~8.2 mmol NO_3^-·d^{-1})	Cycling	2 x 6 min moderate-intensity bouts at 50%WR_{peak}	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during exercise bout
Shannon et al., (2016)	6 UT males 6 TR male triathletes/1500 m runners	47 to 77	3h prior to exercise ingestion of NO_3^- rich BR juice supplementation (15.2 mmol NO_3^-·d^{-1})	Running	15 min bouts at 45 and 60%$\dot{V}O_{2peak}$ at 2500 m stimulated altitude	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-] ↑ exhaled NO	↓ $\dot{V}O_2$ during exercise at 45 and 60%$\dot{V}O_{2peak}$ in UT only
Whitfield et al., (2016)	10 UT male	50 ± 1	7 d NO_3^- rich BR juice supplementation (26.0 mmol NO_3^-·d^{-1})	Cycling	10 min bout at 50% and 70%$\dot{V}O_{2peak}$	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ at 50%$\dot{V}O_{2peak}$ ↓ $\dot{V}O_2$ during exercise at 70%$\dot{V}O_{2peak}$
Wylie et al., (2016)	34 UT males/females	N/A	2h prior to exercise, 7 d, 28 d, 30 d NO_3^- rich dry BR extract supplementation ~3.0 mmol NO_3^-·d^{-1} and 6.0 mmol NO_3^-·d^{-1}	Cycling	2 x 5 min moderate-intensity bouts at 80%GET	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-] Greater increase following 6.0 mmol	↔ $\dot{V}O_2$ during exercise following supplementation with 3.0 mmol NO_3^-·d^{-1} ↓ $\dot{V}O_2$ during exercise following 7 d & 28 d and ↔ $\dot{V}O_2$ during exercise 2h post-ingestion of 6.0 mmol NO_3^-·d^{-1}
Christensen et al., (2017)	9 TR male cyclists 8 UT males	TR: 64 ± 3 UT: 46 3	2.5h prior to exercise ingestion of NO_3^- rich BR juice supplementation (9 mmol NO_3^-·d^{-1})	Incremental leg cycling and arm cranking	N/A	N/A	↔ $\dot{V}O_2$ in both exercise protocols
Ghiarone et al., (2017)	11 UT males	39 ± 10	2.5h prior to exercise ingestion of $NaNO_3$ (~8.8 mmol NO_3^-·d^{-1})	Cycling	10 min moderate-intensity bout at 90%GET 15 min heavy-intensity at 40%Δ	N/A	↔ $\dot{V}O_2$ at all exercise bouts
Nyakayiru et al., (2017c)	17 TR male cyclists	65 ± 4	1 & 6 d $NaNO_3$ supplementation (~12.9 mmol NO_3^-·d^{-1})	Cycling	30 min bouts at 45% and 65%WR_{peak}	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ at all exercise bouts

Thompson et al., (2017)	36 UT males/females	M: 50 ± 11 F: 40 ± 6	28 d NO_3^- rich BR juice supplementation (13.0 mmol $NO_3^- \cdot d^{-1}$) with and without sprint training	Cycling	5 min moderate-intensity bout at 80%GET	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during exercise with BR regardless of sprint training
Balsalobre-Fernández et al. (2018)	12 TR runners	72 ± 5	15 d NO_3^- rich BR juice supplementation (~800 mg $NO_3^- \cdot d^{-1}$)	Running	TTE	N/A	↔ $\dot{V}O_2$
Oskarsson et al., (2018)	7 UT males 2 UT females	M: 59 ± 3 F: 53 ± 11	2.5h prior to exercise ingestion of NO_3^- rich BR juice supplementation (7.3 mmol $NO_3^- \cdot d^{-1}$)	Running	2 x 5 min bouts at 70 and 80%$\dot{V}O_{2peak}$	N/A	↔ $\dot{V}O_2$ at all exercise bouts
Kocoloski et al., (2018)	8 UT males	34 ± 4	2h prior to exercise ingestion of NO_3^- rich BR (0.4g $NO_3^- \cdot d^{-1}$)	Cycling	45 min at 38%WR_{peak}	N/A	↔ $\dot{V}O_2$ at all exercise bouts
Thompson et al., (2018)	18 UT males 12 UT females	M: 46 ± 8 F: 40 ± 4	28 d KNO_3 and BR juice supplementation (both 6.4 mmol $NO_3^- \cdot d^{-1}$) with sprint interval training	Cycling	5 min moderate-intensity bout at 80%GET	↑ Plasma [NO_2^-]	↓ $\dot{V}O_2$ during exercise with BR and KNO_3
Mosher et al., (2019)	11 TR cyclists	61 ± 7	3 d BR juice supplementation (12.8 mmol $NO_3^- \cdot d^{-1}$)	Cycling	40 km TT	N/A	↔ $\dot{V}O_2$
Wickham et al., (2019)	12 UT females	N/A	2.5h prior to exercise and 8 d BR juice supplementation (26 mmol NO_3^-)	Cycling	10 min at 50% and 70%$\dot{V}O_{2peak}$	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ $\dot{V}O_2$ at all exercise bouts

[ ] = concentration; ↑ = significant increase; ↓ = significant decrease; ↔ = no difference; BR = beetroot; d = days; Δ = difference between power output at GET and $\dot{V}O_{2peak}$ plus the power output at GET; GET = gas exchange threshold; HR_{max} = maximal heart rate; KNO_3 = potassium nitrate; $NaNO_3$ = sodium nitrate; NO_3^- = nitrate; NO_2^- = nitrite; NOx = nitrate and nitrite; NO = nitric oxide; TR = trained; UT = untrained; $\dot{V}O_2$ = oxygen uptake; $\dot{V}O_{2peak}$ = peak aerobic capacity; WR_{peak} = maximum work rate

2.7. *Mechanisms for improved exercise efficiency following dietary NO_3^- supplementation*

The potential candidate mechanisms underpinning the lowering of the O_2 cost of exercise following dietary NO_3^- supplementation include the independent or combined improvements to mitochondrial efficiency and/or contractile efficiency (Fig. 2.9).

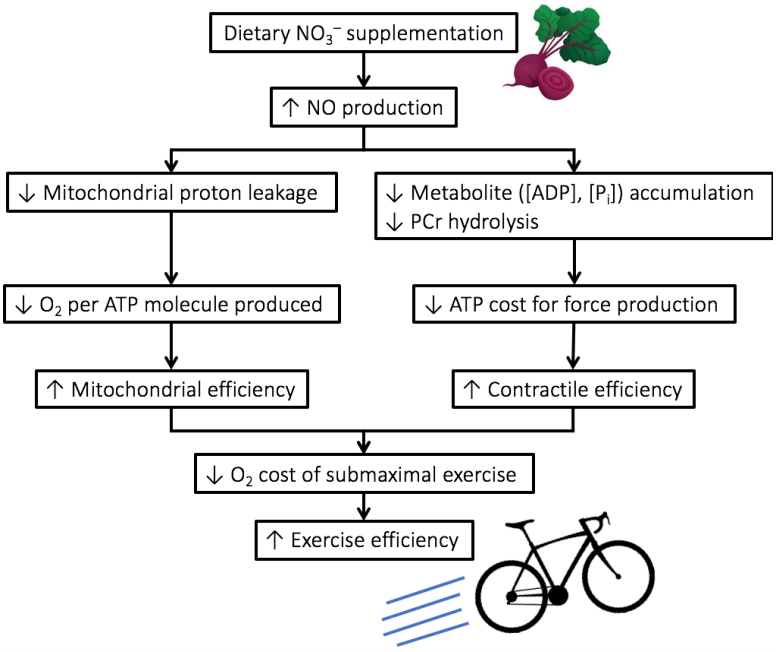

Figure 2.9. Schematic diagram on the mechanistic bases of dietary NO_3^- supplementation in improving exercise efficiency.

2.7.1. *Mitochondrial efficiency*

It has been postulated that dietary NO_3^- supplementation increases the ATP yield per O_2 molecule (mitochondrial P/O ratio) by reducing the ATP lost in uncoupling processes such as mitochondrial proton pump leakage (Brand, 2005; Echtay et al., 2002). In addition, NO can act as an alternative terminal electron acceptor in the electron transport chain, thereby competing with O_2 for ATP production (Basu et al., 2008; Brown & Cooper, 1994; Cleeter et al., 1994). Larsen et al. (2011) sought to investigate the potential influence of NO_3^- supplementation on mitochondrial efficiency by having healthy humans undergo 3 days of $NaNO_3$ supplementation (0.1 mmol of NO_3^- per kg per day) then have muscle biopsies taken from the *vastus lateralis* for the assessment of mitochondrial P/O ratio. $NaNO_3$ supplementation improved indices of mitochondrial efficiency by increasing the P/O ratio, maximal rate of ATP production, and improved the ratio between coupled (i.e. state 3) and uncoupled (i.e. state 4)

respiration (Larsen et al., 2011, Fig. 2.10). Specifically, uncoupled respiration was reduced by 48% which was attributed to a reduction in ANT protein expression although UCP3 was unchanged (Larsen et al., 2011). The reduction in ANT could be expected to contribute to an improved P/O ratio given that ANT is a protein located on the inner mitochondrial membrane and contributes to uncoupling activity via proton leakage (Larsen et al., 2011; Parker et al., 2008). Importantly, the change in P/O ratio was correlated to whole body steady-state exercise $\dot{V}O_2$ (Larsen et al., 2011). Therefore, these data indicate that NO_3^- supplementation lowers the O_2 cost of exercise by improving mitochondrial efficiency through the lowering of uncoupling protein expression (Larsen et al., 2011).

Despite the elegant study by Larsen et al. (2011) who demonstrated that a lower O_2 cost following NO_3^- supplementation could be attributed to improved mitochondrial efficiency, recent findings challenge this mechanism (Whitfield et al., 2016). Whitfield et al. (2016) observed a lowered $\dot{V}O_2$ during whole-body exercise following 7 days of BR juice supplementation (~26 mmol of NO_3^- per day) but without any modulations to uncoupling proteins ANT and UCP3 or changes to the P/O ratio. The authors speculated that changes to redox status may have underpinned the effect of a lowered $\dot{V}O_2$ during exercise given that they observed an elevated emission of H_2O_2 levels (Andrade et al., 1998; Whitfield et al., 2016). ROS have an important role in skeletal muscle force production (Andrade et al., 1998; Lawler et al., 1997; Oba et al., 1996; Reid et al., 1993) and interact with NO in a complex fashion to induce cell signalling (Powers & Jackson, 2008; Smith & Reid, 2006). It is possible that ROS-signalling has a role in the favourable effects observed with NO_3^- supplementation (Reid, 1998); however, the influence of redox status on the efficacy of NO_3^- remains to be investigated. Therefore, this equivocal literature suggests that changes to mitochondrial efficiency are not the only explanation for enhanced exercise efficiency following NO_3^- supplementation.

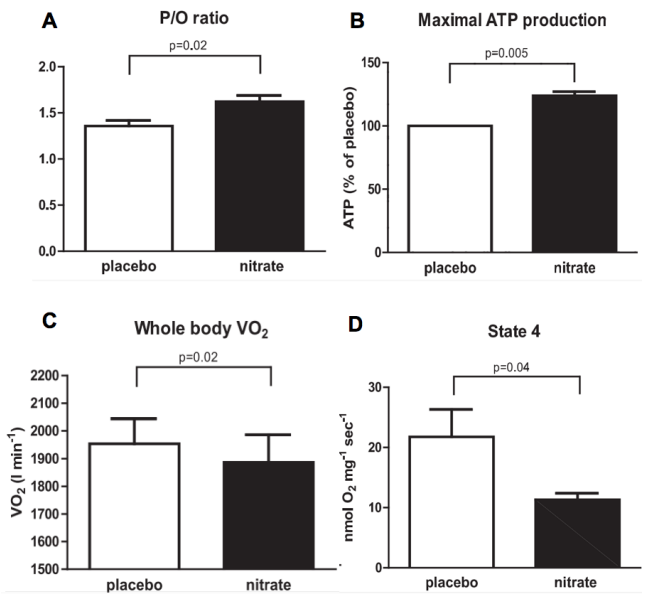

Figure 2.10. Oxidative phosphorylation efficiency (panel A), maximal ATP production (panel B), O_2 cost of exercise (panel C), state 4 respiration (panel D) was improved following NO_3^- supplementation compared to placebo (adapted from Larsen et al., 2011).

2.7.2. Contractile efficiency

Using ^{31}P magnetic resonance spectroscopy (^{31}P-MRS) for non-invasive *in vivo* assessment of intramuscular metabolites, Bailey et al. (2010) was the first to report that a lowering in the O_2 cost of exercise following 6 days of NO_3^- supplementation (0.5 L of BR juice containing 5.1 mmol NO_3^- per day) was accompanied by a reduced ATP cost of force production. Specifically, ATP turnover from PCr hydrolysis and oxidative phosphorylation were lower following NO_3^- supplementation which consequently indicated that the total ATP cost of force production was lower for the same given force output (Bailey et al., 2010, Fig. 2.11). In addition, the changes in intramuscular metabolites (i.e. PCr depletion and accumulation of ADP and P_i) were attenuated following NO_3^- which provides a mechanistic basis for the lowering of $\dot{V}O_2$ (Bailey et al., 2010) given that there would be less metabolites to stimulate oxidative phosphorylation (Chance & Williams, 1955; Mahler, 1985). Mechanistically, NO potentially influenced crossbridge kinetics (Gross et al., 1996) or contractile protein function (Reid et al., 1998). It is also possible that a reduction in fatigue-associated metabolites could also be related in part to NO-mediated improvements to blood flow (Stamler & Meissner, 2001). For example, NO may reversibly inhibit O_2 from binding to cytochrome c oxidase, enabling greater diffusion of O_2 from the capillaries to distal

muscle fibres (Hagen et al., 2003) which could improve matching local O_2 delivery to the O_2 requirement. Thus, the result is that working skeletal muscle have better homogeneity in muscle perfusion and oxygenation, thereby offsetting fatigue-related metabolite production (Bailey et al., 2010). Together, these results provide evidence indicating that improvements in mitochondrial efficiency (e.g. Larsen et al., 2011) are not required to explain the reduced O_2 cost of exercise following NO_3^- supplementation. Therefore, the data from Bailey et al. (2010) indicate that this improvement may not be related to changes to the P/O ratio and could be due to changes to improved ATP hydrolysis and muscle force production coupling.

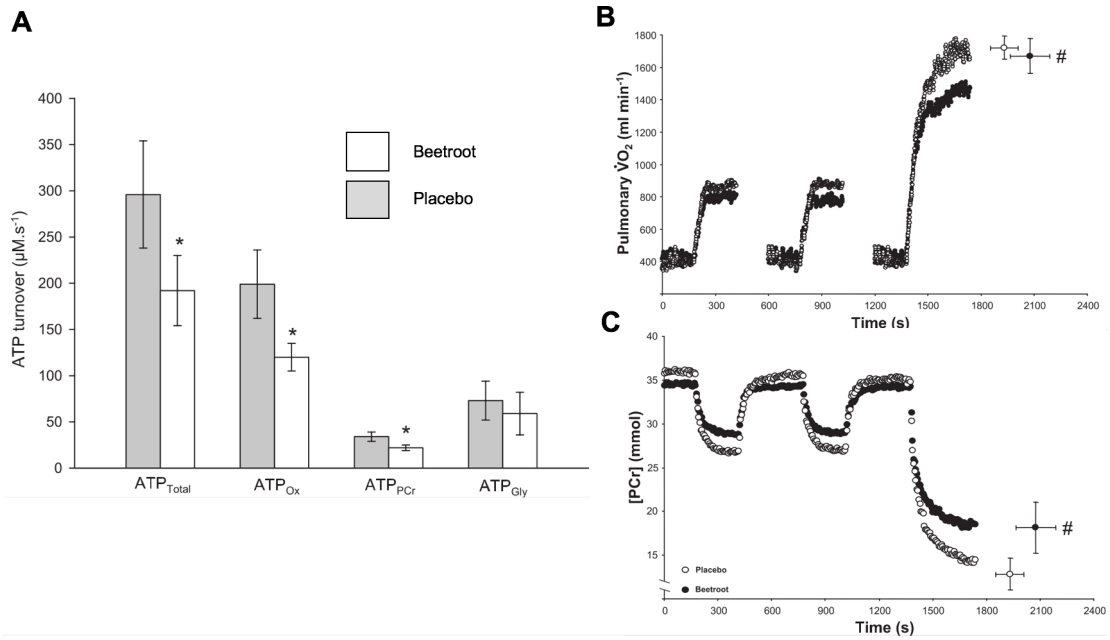

Figure 2.11. ATP resynthesis averaged over the entire low-intensity knee-extension exercise (panel A) as well as the pulmonary $\dot{V}O_2$ response (panel B) and intramuscular [PCr] response (panel C) to high- and low-intensity knee-extension exercise. Abbreviations: ATP_{total} = total ATP turnover; ATP_{Ox} = ATP derived from oxidative phosphorylation; ATP_{PCr} = ATP derived from PCr hydrolysis; ATP_{Gly} = ATP derived from glycolysis (adapted from Bailey et al., 2010).

In summary, NO-mediated improvements to both contractile efficiency (Bailey et al., 2010) and/or mitochondrial efficiency (Larsen et al., 2011) may contribute, either independently or in combination, to the reduction in $\dot{V}O_2$ during exercise following dietary NO_3^- supplementation. In addition, recent advances suggest that changes to redox status could also contribute to lowering the O_2 cost of exercise given that NO_3^- supplementation elevated ROS levels (Whitfield et al., 2016) and that ROS can influence force production (Andrade et al., 1998) and/or signalling (Reid et al., 1998).

Collectively, these physiological effects following dietary NO_3^- supplementation have important implications for exercise performance and remain to be established.

2.8. The effects of dietary NO_3^- supplementation on exercise performance

Given that dietary NO_3^- supplementation has been shown to improve exercise efficiency (Bailey et al., 2009; 2010; Larsen et al., 2011) and that exercise efficiency is an important determinant of exercise performance (Bassett & Howley, 2000), it could be expected that dietary NO_3^- supplementation would elicit an ergogenic effect. Recently published reviews support NO_3^- supplementation as an ergogenic aid (Jones et al., 2014; 2018; Maughan et al., 2018; McMahon et al., 2017; Van de Walle et al., 2018). In addition, dietary NO_3^- supplementation was recently classified by the International Olympic Committee as one of only five dietary supplements with strong evidence for enhancing performance (Maughan et al., 2018). However, despite the conclusions of these publications, it is important to note that caution should be used when interpreting the conclusions and language used within these literature reviews. For example, Maughan et al. (2018) has classified NO_3^- supplementation as having 'good evidence' supporting it as an ergogenic aid and introduces NO_3^- under headings such as 'supplements that directly improve sports performance' (Maughan et al., 2018). Although Maughan et al. (2018) provides suggestions on how athletes could decide on whether to use supplements or not, language and phrases, such as the aforementioned examples, may be misinterpreted (i.e. that NO_3^- always works). The importance of critcal and balanced analysis is further highlighted as at the onset of this thesis, 68 published studies have investigated the potential ergogenic effect of dietary NO_3^- supplementation but have yielded equivocal results in both tests of exercise capacity and exercise performance (Table 2.3). Therefore, to understand when and who should take part in NO_3^- supplementation, it is important to remain critical of all available literature, and be cautious when interpreting the results of literature reviews.

2.8.1. Exercise capacity

At the onset of this thesis, 29 investigations have examined the ergogenic potential of dietary NO_3^- supplementation using tests of exercise capacity (i.e. time to exhaustion or total work performed) with 22 studies that have reported an ergogenic effect (Table 2.3). Exercise capacity has been reported to be enhanced in multiple modes of exercise, including severe-intensity cycling (+16%, Bailey et al., 2009; +22% during

115 rpm only, Bailey et al., 2015; +22%, Breese et al., 2013; +12 to 17%, Kelly et al., 2013; +16%, Thompson et al., 2014;; +12%, Wylie et al., 2013a), running (+8%, Balsalobre-Fernández et al., 2018; +15%; Lansley et al., 2011b), knee extensions (+25%, Bailey et al., 2010; +37%, Husmann et al., 2019; +41%, Porcelli et al., 2016; +21%, Vanhatalo et al., 2011a), severe-intensity cycling following sprint interval training combined with NO_3^- supplementation (SIT+BR) compared to SIT alone (+~9%, Muggeridge et al., 2017; +14% Thompson et al., 2017) severe-intensity cycling following SIT+BR compared to SIT combined NO_3^- salt supplementation (+24%, Thompson et al., 2018), intermittent sprint tests (+19%, Aucouturier et al., 2015; +3%, Nyakayiru et al., 2017a; +3%, Thompson et al., 2015; +4%, Thompson et al., 2016; +4%, Wylie et al., 2013b), as well as incremental exercise (+4%, Lansley et al., 2011b; +5%, Masschelein et al., 2012; +3%, Vanhatalo et al., 2010a). In contrast, 7 studies have reported no significant effect of NO_3^- supplementation on exercise capacity in both trained (Arnold et al., 2015; Bescós et al., 2011; Handzlik & Gleeson, 2013) and untrained individuals (Buck et al., 2015; Ghiarone et al., 2017; Kelly et al., 2014; Larsen et al., 2010).

2.8.2. Exercise performance

Although the ergogenic effect of dietary NO_3^- supplementation during tests of exercise capacity range have been reported to be large, particularly during open-ended cycling tests (12% to 25%, Bailey et al., 2009; Breese et al., 2013), a 15% improvement to exercise capacity would only be expected to enhance a test of exercise performance such as time trials (i.e. completing a set distance in the shortest amount of time) by ~1-2% (Hopkins et al., 1999). This is supported by Lansley et al. (2011a) who was the first to report that acute NO_3^- supplementation (0.5 L of BR juice, ~6.2 mmol of NO_3^- per day) enhanced 4 km and 16.1 km cycling TT performance by ~2.7% and ~2.8%, respectively, in untrained individuals. Since then, NO_3^- supplementation has been reported to enhance performance of rowing TT (Bond et al., 2012; Hoon et al., 2014b), 8 km cycling TT (Jo et al., 2017), 10 km cycling TT (Cermak et al., 2012a; Rokkedal-Lausch et al., 2019), 16 km cycling TT at altitude (Muggeridge et al., 2014), kayaking TT (Peeling et al., 2015), 3 km running TT (Porcelli et al., 2015), 5 km running TT (Murphy et al., 2012), 1500 m running TT (Shannon et al., 2016a), as well as 1500 m running TT in altitude (Shannon et al., 2016b). However, while 12 studies have observed a positive influence of dietary NO_3^- supplementation on TT performance, 27

studies have not found significant performance effects (Table 2.3). Several factors may contribute to the divergent evidence regarding the ergogenic effect of dietary NO_3^- supplementation such as the supplementation strategy, subject training status, the mode of exercise.

2.8.3. The potential influence of supplementation strategy

One of the factors that could account for disparate results could be the dosing supplementation strategy. Indeed, there are many studies that have employed acute NO_3^- ingestion which have not observed performance enhancements (Boorsma et al., 2014; Cermak et al., 2012b; de Castro et al., 2019; Glaister et al., 2015; Hoon et al., 2014a; Lane et al., 2014; Lowings et al., 2017; MacLeod et al., 2015; Muggeridge et al., 2015; Nybäck et al., 2017; Oskarsson et al., 2018; Peacock et al., 2012; Sandbakk et al., 2015; Wickham et al., 2019; Wilkerson et al., 2012) although short-term (3 to 7 days) NO_3^- supplementation has also been shown to be ineffective in some studies (Bescós et al., 2012; Bourdillon et al., 2015; Callahan et al., 2017; Christensen et al., 2013; Esen et al., 2018; Fan et al., 2018; Jonvik et al., 2018; Kent et al., 2018; McQuillan et al., 2017a; 2018; Mosher et al., 2019; Nyakayiru et al., 2017b).

The magnitude of increase in plasma $[NO_2^-]$ following NO_3^- supplementation has been shown to be associated with performance (Dreissigacker et al., 2010; Porcelli et al., 2015; Wilkerson et al., 2012, Fig. 2.12). In addition, Wylie et al. (2013a) observed a dose-response relationship between the magnitude of increase in plasma $[NO_2^-]$ and exercise performance. Specifically, healthy untrained individuals consumed 70 ml, 140 ml, and 280 ml of concentrated NO_3^--rich BR juice (i.e. 4.2 mmol, 8.4 mmol, and 16.8 mmol of NO_3^-, respectively) 2.5 h prior to exercise and subsequent severe-intensity cycling TTE was enhanced by 12% and 14% following 8.4 mmol and 16.8 mmol of BR juice, but not following 4.2 mmol (Wylie et al., 2013a). Together, these data suggest a role for the dose of the bolus ingested and the relative magnitude of change in plasma $[NO_2^-]$. However, Boorsma et al. (2014) demonstrated that a high acute dosage of NO_3^- (19.5 mmol of NO_3^-) had no effect on 1500 m running TT performance, suggesting that administering a NO_3^- bolus with a high NO_3^- concentration does not necessarily provide a greater magnitude of effect. In contrast, prolonging the total supplementation period could be a contributing factor as 15 days of NO_3^- supplementation (~5.2 mmol of NO_3^- per day) tended to increase exercise tolerance

but acute ingestion 2.5 h prior to the exercise test did not induce any ergogenic effect (Vanhatalo et al., 2010). It is possible that long-term NO_3^- supplementation (i.e. ≥ 7 days) could evoke protein modifications beneficial to exercise performance (e.g. modulations to Ca^{2+} handling proteins; Hernández et al., 2012) whereas short-term NO_3^- supplementation (i.e. ≤ 3 days) may induce physiological modulations through cGMP signalling (Maréchel & Gailly, 1999; Stamler & Meissner, 2001). In summary, these data indicate that the ergogenic potential of NO_3^- supplementation might be more likely to occur if the supplementation period is extended rather than administered as an acute bolus.

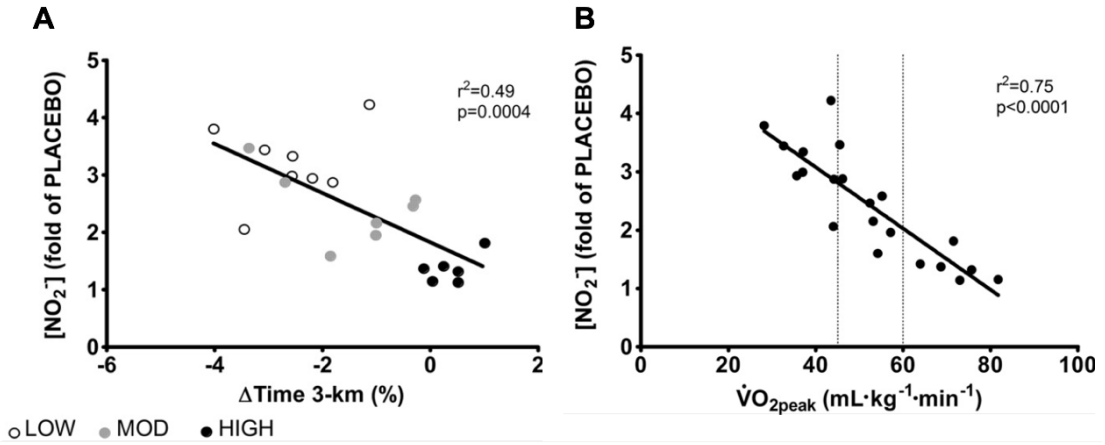

Figure 2.12. Individual change in plasma [NO_2^-] (expressed as fold of placebo) compared to time percentage change in time to complete 3 km TT (panel A) and changes to plasma [NO_2^-] (expressed as fold of placebo) in low-, moderate-, and high-aerobic fitness groups after NO_3^- supplementation vs. individual $\dot{V}O_{2peak}$ values (panel B). Dotted lines define the low-, moderate-, and high-aerobic fitness groups (adapted from Porcelli et al., 2015).

2.8.4. The potential influence of subject training status

Of the 27 studies that have reported no effect of NO_3^- supplementation on TT performance, 19 studies were performed in trained individuals ($\dot{V}O_{2peak}$ ≥ 60 ml·min^{-1}·kg^{-1}) which cumulatively suggests that training status may be an important determinant in the efficacy of NO_3^- supplementation on exercise performance (Table 2.3). As previously discussed (see section: *The effects of dietary NO_3^- supplementation on exercise efficiency*), highly trained populations have a higher baseline endogenous NO production due to greater NOS expression (McConnell et

al., 2007) and consequently exhibit higher baseline plasma [NO_2^-] compared to subjects with low- to moderate- aerobic fitness (Bescós et al., 2012; Christensen et al., 2017; Jungersten et al., 1997; Porcelli et al., 2015). In addition, NO_3^- ingestion has been reported to preferentially augment type II muscle fibres in terms of contractile function (Coggan et al., 2015; Hernández et al., 2012) and blood flow distribution (Ferguson et al., 2013; Jones et al., 2016). Given that a higher $\dot{V}O_{2peak}$ is correlated with a greater proportion of type I muscle fibres, and that the reduction of NO_2^- to NO occurs in hypoxic environments (Castello et al., 2006) such as in type II muscle fibres (Behnke et al., 2003), the ergogenic potential of NO_3^- may be reduced in elite athletes due to differences in fibre type composition (Ivy et al., 1980). Lastly, recent evidence indicates that redox signalling could be important in the efficacy of NO_3^- supplementation (Whitfield et al., 2016). Therefore, training adaptations in elite athletes (e.g. increased antioxidant enzyme content and activity) would render their endogenous antioxidant buffering system to be superior compared to their untrained counterparts, and potentially contribute to the lack of effect of NO_3^- by quenching ROS and any ROS-induced favourable effects on contractile function (Andrade et al., 1998; Powers & Jackson, 2008).

2.8.5. The potential influence of exercise modality

Recent advances suggest that NO_3^- supplementation induces effects in an fibre specific manner (see section: *Mechanisms for improved exercise performance following dietary NO_3^- supplementation*) by potentiating blood flow (Ferguson et al., 2013) and contractile protein modulations (Hernández et al., 2012) in muscle groups predominantly comprised of type II muscle fibres. Therefore, it could be reasoned that the mode of exercise could be an important determinant in the efficacy of NO_3^- supplementation on exercise performance. For example, several investigations have reported enhanced TT performance in exercise modalities requiring a greater proportion of type II muscle fibres such as upper body sports (Polgar et al., 1973) in kayaking (Hoon et al., 2014; Peeling et al., 2015) and rowing (Bond et al., 2012) whereas TT performance during cycling is equivocal (Cermak et al., 2012a; 2012b; Lansley et al., 2011b; Christensen et al., 2013). Therefore, the mode of exercise should be considered when administering dietary NO_3^- supplementation.

Collectively, current literature suggests that the ergogenic potential of dietary NO_3^- supplementation depends on several factors which include supplementation strategy, training status, and mode of exercise. In agreement, several recent meta-analyses have indicated that dietary NO_3^- supplementation is more likely (small to moderate significant effect) to improve performance tasks assessing exercise capacity (Hoon et al., 2013; McMahon et al., 2017; Van de Walle et al., 2018) in untrained individuals (Campos et al., 2018). However, further research is required to understand the determinants to the efficacy of NO_3^- supplementation to guide its application. Future studies should consider that the test sensitivity (i.e. reliability) of exercise performance protocols is critical (Jeukendrup & Currell, 2005). Measurement error, assessed by the coefficient of variation (CV), is reported to be markedly less using TT (~1 to 3%) compared to TTE (~10 to 30%) for assessing exercise performance (Jeukendrup et al., 1996; Jeukendrup & Currell, 2005; McLellan et al., 1995). However, it is possible that improvements to performance are magnified in TTE tasks which may compensate for the larger CV (Hinckson et al., 2005; Hopkins et al., 2001). Other authors suggest that assessing performance is similar between the TT and TTE (Amann et al., 2008). Importantly, TTE tasks are effective at assessing endurance capacity, whereas TT tasks are effective at assessing exercise performance (Jeukendrup & Currell, 2005). This difference should be considered when creating the experimental design.

Table 2.3. Description of studies that have investigated the effect of dietary NO_3^- supplementation on exercise performance protocols in humans.

Author	Participants	$\dot{V}O_{2peak}$ ($ml \cdot min^{-1} \cdot kg^{-1}$)	Supplementation	Exercise mode	Protocol	NO indices	Performance change
Bailey et al., (2009)	8 UT males	49 ± 5	6 d of NO_3^- rich BR juice supplementation (~5.6 mmol $NO_3^- \cdot d^{-1}$)	Cycling	TTE at severe-intensity 70%Δ on day 4 of supplementation	↑ Plasma [NO_2^-]	↑ exercise tolerance
Bailey et al., (2010)	7 UT males	N/A	6 d of NO_3^- rich BR juice supplementation (~5.1 mmol $NO_3^- \cdot d^{-1}$)	Two-legged knee-extension	TTE at high-intensity (30% MVC iEMG signal) on day 4 of supplementation	↑ Plasma [NO_2^-]	↑ exercise tolerance
Larsen et al., (2010)	9 UT males/females	N/A	2 d $NaNO_3$ supplementation (0.1 mmol·$kg^{-1} \cdot d^{-1}$)	Leg/arm cycling	Incremental test to exhaustion	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	Trend ↑ exercise tolerance (P=0.13)
Vanhatalo et al., (2010a)	8 UT males	47 ± 8	2.5h prior to exercise, 5 d, and 15 d of NO_3^- rich BR juice supplementation (~5.2 mmol $NO_3^- \cdot d^{-1}$)	Cycling	Incremental test to exhaustion following 2.5h, 5 d, and 15 d of supplementation	↑ Plasma [NO_2^-]	↑ PPO after 15 d but not 2.5h or 5 d of supplementation
Bescós et al., (2011)	11 TR male cyclists/triathletes	65 ± 6	3h prior to exercise ingestion of $NaNO_3$ (10 mg·kg^{-1})	Cycling	Incremental test to exhaustion	↑ Plasma [NO_2^-]	↔ exercise tolerance
Lansley et al., (2011a)	9 TR male cyclists	56 ± 6	2 to 2.5h prior to exercise ingestion of NO_3^- rich BR juice (~6.2 mmol NO_3^-)	Cycling	4 km and 16.1 km TT	↑ Plasma [NO_2^-]	↑ 4 km and 16.1 km TT performance
Lansley et al., (2011b)	9 UT males	55 ± 7	6 d of NO_3^- rich BR juice supplementation (~5.1 mmol $NO_3^- \cdot d^{-1}$)	Running/single-legged knee-extension	TTE at severe-intensity 75%Δ and TTE incremental single-legged knee-extension on days 4 to 6 of supplementation	↑ Plasma [NO_2^-]	↑ exercise tolerance on both tests
Vanhatalo et al., (2011a)	7 UT males 2 UT females	N/A	24h prior to exercise ingestion of NO_3^- rich BR (0.75 L of ~9.3 mmol NO_3^-)	Single-legged knee extension	TTE at 14.5% FiO_2	↑ Plasma [NO_2^-]	↑ exercise tolerance
Bescós et al., (2012)	13 TR male cyclists/triathletes	60 ± 7	3 d $NaNO_3$ supplementation (10 mmol·$kg^{-1} \cdot d^{-1}$)	Cycling	40 min TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance
Bond et al., (2012)	14 TR male rowers	N/A	6 d of NO_3^- rich BR juice supplementation (5.5 mmol $NO_3^- \cdot d^{-1}$)	Ergometer rowing	6 x 500 m TT	↔ Plasma [NO_2^-]	↑ performance
Cermak et al., (2012a)	13 trained M cyclists/triathletes	58 ± 2	6 d of NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^- \cdot d^{-1}$)	Cycling	10 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ performance
Cermak et al., (2012b)	20 TR male cyclists	60 ± 1	2.5h prior to exercise ingestion of NO_3^- rich BR juice (8.7 mmol NO_3^-)	Cycling	1 h TT	↑ Plasma [NO_3^-]	↔ performance
Masschelein et al., (2012)	15 UT males	62 ± 2	6 d of NO_3^- rich BR juice supplementation (0.07 mmol $NO_3^- \cdot kg^{-1}$BW)	Cycling	TTE at 5000m	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise tolerance
Murphy et al., (2012)	5 UT males 6 UT females	N/A	75 min prior to exercise of baked beetroot (~500 mg or 8 mmol of NO_3^-)	Running	5 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance
Peacock et al., (2012)	10 trained M skiers	70 ± 5	2.5h prior to exercise ingestion of KNO_3 (9.9 mmol NO_3^-)	Running	5 km TT	↑ Plasma [NO_2^-]	↔ performance
Wilkerson et al., (2012)	8 TR male cyclists	63 ± 8	2.5h prior to exercise ingestion of NO_3^- rich BR juice (~6.2 mmol NO_3^-)	Cycling	50 km TT	↑ Plasma [NO_2^-]	↔ performance
Breese et al., (2013)	9 UT males/females	M: 48 ± 6	6 d NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^- \cdot d^{-1}$)	Cycling	TTE at severe-intensity 70%Δ following moderate-	↑ Plasma [NO_2^-]	↑ exercise tolerance

		F: 46 ± 9			intensity bout on day 6 of supplementation		
Christensen et al., (2013)	10 TR male cyclists	72 ± 4	6 d NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	400 kcal TT following pre-load 120 min	↑ Plasma NO_x	↔ performance
Handzlik & Gleeson, (2013)	14 TR males	63 ± 10	~2.5h prior to exercise ingestion of NO_3^- rich BR juice (~8.0 mmol NO_3^-)	Cycling	TTE at 80%$VO2_{max}$	↑ Salivary [NO_3^-] ↑ Salivary [NO_2^-]	↔ exercise tolerance
Kelly et al., (2013)	9 UT males	55 ± 8	7 to 12 d of NO_3^- rich BR juice supplementation (8.2 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	TTE at severe-intensity 60, 70, 80%Δ and 100%PPO	↑ Plasma [NO_2^-]	↑ exercise tolerance at 60, 70, and 80%Δ ↔ exercise tolerance at 100%PPO
Wylie et al., (2013a)	10 UT males	N/A	2.5h prior to exercise ingestion of NO_3^- rich BR juice (4.2, 8.4, 16.8 mmol NO_3^-)	Cycling	70%Δ TTE	Dose-dependent ↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise tolerance with 8.4 and 16.8 mmol of NO_3^-
Wylie et al., (2013b)	14 UT males	52 ± 7	1 d NO_3^- rich BR juice supplementation (16.4 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	Yo-Yo IR1 intermittent recovery test	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise tolerance
Boorsma et al., (2014)	8 TR male 1500m runners	80 ± 5	2.5h prior to exercise ingestion of NO_3^- rich BR (19.5 mmol NO_3^-), 8 d BR supplementation where day 1 and 8 subjects consumed 19.5 mmol $NO_3^-{\cdot}d^{-1}$ and days 2 to 7 subjects consumed 13 mmol $NO_3^-{\cdot}d^{-1}$)	Running	1500 m TT	↑ Plasma [NO_3^-]	↔ performance
Hoon et al., (2014a)	26 TR cyclists	N/A	150 min or 75 min prior to exercise ingestion of NO_3^- rich BR juice (~6.2 mmol NO_3^-) and 35 ml top up dose	Cycling	2 x 4 min TT separated by 75 min rest	↑ Plasma [NO_2^-]	↔ performance
Hoon et al., (2014b)	10 TR male rowers	N/A	3h prior to exercise ingestion of NO_3^- rich BR juice (~4.2 or 8.4 mmol NO_3^-)	Rowing	2000m TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance for 4.2 mmol dose ↑ performance for 8.4 mmol dose
Kelly et al., (2014)	12 UT males	58 ± 6	6 d NO_3^- rich BR juice supplementation (8.4 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	TTE at severe-intensity 70%Δ	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ exercise tolerance
Lane et al., (2014)	12 TR male cyclists 12 TR female cyclists	M: 72 ± 5 F: 60 ± 5	1 d NO_3^- rich BR juice supplementation (8.4 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	M: 43.83 km TT F: 29.35 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance for M & F
Muggeridge et al. (2014)	9 TR male cyclists	52 ± 6	3h prior to exercise ingestion of NO_3^- rich BR juice (~5 mmol NO_3^-)	Cycling	16.1 km TT at 2500 m simulated altitude	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ performance
Thompson et al., (2014)	16 UT males	47 ± 6	90 min prior to exercise ingestion of NO_3– rich BR juice (5 mmol NO_3^-)	Cycling	TTE at severe-intensity 90%$\dot{V}O_{2peak}$	↑ Plasma [NO_2^-]	↑ exercise tolerance
Arnold et al., (2015)	10 TR male runners	66 ± 7	2.5h prior to exercise of NO_3^- rich BR juice (~7 mmol NO_3^-)	Running	TTE and 10 km TT at simulated 2500 m	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance and exercise tolerance
Aucouturier et al., (2015)	12 UT males	47 ± 3	3 d NO_3^- rich BR juice supplementation (680 mg $NO_3^-{\cdot}L^{-1}$)	Cycling	Repeated sprints	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise tolerance

Bailey et al., (2015)	7 UT males	N/A	9 d NO_3^- rich BR juice supplementation (8.4 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	TTE at severe-intensity 80%Δ at 35 rpm and 115 rpm	↑ Plasma [NO_2^-]	↑ exercise tolerance at 115 rpm
Bourdillon et al., (2015)	12 TR male cyclists	N/A	3 d $NaNO_3$ supplementation (0.1 mmol $NO_3^-{\cdot}kg^{-1}{\cdot}d^{-1}$)	Cycling	15 km TT	↑ Expired NO	↔ performance
Buck et al., (2015)	13 UT females	N/A	3h prior to exercise ingestion of NO_3– rich BR juice (6 mmol NO_3^-)	Running	Repeated sprints	↑ Plasma [NO_3^-]	↔ performance
Glaister et al., (2015)	14 TR female cyclists	N/A	3h prior to exercise ingestion of NO_3– rich BR juice (~7.3 mmol NO_3^-)	Cycling	20 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance
MacLeod et al., (2015)	11 TR males	68 ± 6	2h prior to exercise ingestion of NO_3– rich BR juice (6.5 mmol NO_3^-)	Cycling	10 km TT at normoxia and 2500 m simulated altitude	↑ Plasma [NO_3^-] ↑exhaled NO	↔ performance for both TT
Muggeridge et al., (2015)	9 TR male cyclists	53 ± 4	2.5h prior to exercise ingestion of NO_3^- rich gel (~8.1 mmol NO_3^-)	Cycling	16.1 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance
Peeling et al., (2015)	Study 1: 6 TR male kayakers Study 2: 5 TR female kayakers	N/A	Study 1: 2.5h prior to exercise ingestion of NO_3– rich BR juice (4.8 mmol NO_3^-) Study 2: 2.5h prior to exercise ingestion of NO_3– rich BR juice (9.6 mmol NO_3^-)	Kayaking	Study 1: 4 min max lab kayak ergometer TT Study 2: 500 m on water kayak TT	↑ Plasma [NO_2^-]	Study 1: ↔ performance Study 2: ↑ performance
Porcelli et al., (2015)	8 recreationally active M 7 moderately-trained M 6 trained M	28-44 46-57 64-82	6 d $NaNO_3$ supplementation (5.5 mmol $NO_3^-{\cdot}d^{-1}$)	Running	3 km TT	↑ Plasma [NO_3^-]/ [NO_2^-] for all groups dependent on fitness	UT: ↑ performance Mod-TR: ↑ performance TR: ↑ performance
Sandbakk et al., (2015)	9 TR male cross-country skiers	69 ± 6	2.5h prior to exercise ingestion of KNO_3 (9.9 mmol $NO3^-$)	Running	5 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance
Thompson et al. (2015)	16 UT males	50 ± 7	7 d NO_3^- rich BR juice supplementation (12.8 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	Repeated sprints	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise capacity
Porcelli et al., (2016)	7 UT males	41 ± 5	6 d NO_3^- rich diet (~8.2 mmol $NO_3^-{\cdot}d^{-1}$)	Knee-extensions/repeated sprint test	Fatiguing submaximal knee-extension TTE/Repeated sprints	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise tolerance/↑ performance
Shannon et al., (2016a)	8 TR male runners/triathletes	62 ± 8	3h prior to exercise ingestion of NO_3^- rich BR juice supplementation (~12.0 mmol NO_3^-)	Running	1500 m & 10 000 m TT	↑ Plasma [NO_2^-]	↑ performance during 1500 m TT ↔ performance during 10 000m TT
Shannon et al., (2016b)	6 UT males 6 TR male triathletes/1500 m runners	47 to 77	3h prior to exercise ingestion of NO_3^- rich BR juice supplementation (15.2 mmol $NO_3^-{\cdot}d^{-1}$)	Running	1500 m TT at 2500 m simulated altitude	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-] ↑ exhaled NO	↑ performance
Thompson et al., (2016)	36 UT males	N/A	5 d NO_3^- rich BR juice supplementation (6.4 mmol $NO_3^-{\cdot}d^{-1}$)	Running	20 m sprints & Yo-Yo IR1 intermittent recovery test	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ exercise capacity
Callahan et al., (2017)	8 TR males	65 ± 4	3 d NO_3^- rich crystal supplementation (15 g $NO_3^-{\cdot}d^{-1}$)	Cycling	4 km TT	↔ Plasma [NO_2^-]	↔ performance

Ghiarone et al., (2017)	11 UT males	39 ± 10	2.5h prior to exercise ingestion of $NaNO_3$ (~8.8 mmol $NO_3^-·d^{-1}$)	Cycling	TTE at severe-intensity 80%Δ	N/A	↔ exercise capacity
Jo et al., (2017)	29 UT males/females	N/A	2h prior to exercise ingestion and 14 d of NO_3^- rich supplementation (~8.0 mmol $NO_3^-·d^{-1}$)	Cycling	8 km TT	N/A	↑ performance following 14 d of NO_3^- rich supplementation only
Lowings et al., (2017)	10 TR male/female swimmers	N/A	3h prior to exercise ingestion of NO_3^- rich BR juice supplementation (~12.5 mmol NO_3^-)	Swimming	168 m TT	↑ exhaled NO	↔ performance
McQuillan et al., (2017a)	9 TR male cyclists	68 ± 3	7 d NO_3^- rich BR juice supplementation (8.0 mmol $NO_3^-·d^{-1}$)	Cycling	1 and 4 km TT	↔ Plasma $[NO_2^-]$	↔ performance
Muggeridge et al., (2017)	27 UT males	42 ± 7	2.5h prior to exercise ingestion of NO_3^- rich gel (8.0 mmol NO_3^-)	Cycling	Repeated sprints	↑ Plasma $[NO_2^-]$	↑ performance
Nyakayiru et al., (2017a)	32 TR male soccer players	N/A	6 d NO_3^- rich BR juice supplementation (800 mg $NO_3^-·d^{-1}$)	Running	Yo-Yo IR1 intermittent recovery test	↑ Plasma & salivary $[NO_3^-]$/ $[NO_2^-]$	↑ exercise capacity
Nyakayiru et al., (2017b)	17 TR male cyclists	65 ± 4	1 & 6 d $NaNO_3$ supplementation (~12.9 mmol $NO_3^-·d^{-1}$)	Cycling	10 km TT	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↔ performance
Nybäck et al., (2017)	5 TR male skiers 4 TR female skiers	M: 72 ± 5 F: 58 ± 3	2.5h prior to exercise ingestion of NO_3^- rich BR juice supplementation (13.0 mmol NO_3^-)	Roller ski treadmill	1000 TT	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↔ performance
Thompson et al., (2017)	36 UT males/females	M: 50 ± 11 F: 40 ± 6	28 d NO_3^- rich BR juice supplementation (13.0 mmol $NO_3^-·d^{-1}$) with and without sprint training	Cycling	TTE at severe-intensity 80%Δ/incremental test	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↔ exercise tolerance/ ↑ performance during incremental test
Balsalobre-Fernández et al. (2018)	12 TR runners	72 ± 5	15 d NO_3^- rich BR juice supplementation (~800 mg $NO_3^-·d^{-1}$)	Running	TTE	N/A	↑ performance
Esen et al., (2018)	10 moderately TR swimmers	N/A	3 d NO_3^- rich BR juice supplementation (~800 mg $NO_3^-·d^{-1}$)	Swimming	100 m and 200 m TT	↑ Plasma $[NO_2^-]$	↔ performance
Fan et al., (2018)	12 UT male cyclists	N/A	3 d $NaNO_3$ supplementation (0.1 mmol $NO_3^-·kg^{-1}·d^{-1}$)	Cycling	15 km TT	↑ Expired NO	↔ performance
Jonvik et al. (2018)	20 UT cyclists 22 moderately-TR 10 TR	N/A	6 d NO_3^- rich BR juice supplementation (~800 mg $NO_3^-·d^{-1}$)	Cycling	Repeated sprints	↑ Plasma $[NO_3^-]$ ↑ Plasma $[NO_2^-]$	↑ performance in all fitness levels
Kent et al., (2018)	12 TR male cyclists	66 ± 6	3 d NO_3^- rich BR juice supplementation (6.5 mmol $NO_3^-·d^{-1}$)	Cycling	TT	↑ Salivary $[NO_3^-]$/ $[NO_2^-]$	↔ performance
McQuillan et al., (2018)	8 TR male cyclists	64 ± 5	3 d NO_3^- rich BR juice supplementation (~8.0 mmol $NO_3^-·d^{-1}$)	Cycling	4 km TT in the heat	↑ Serum $[NO_2^-]$	↔ performance
Oskarsson et al., (2018)	7 UT males 2 UT females	M: 59 ± 3	2.5h prior to exercise ingestion of NO_3^- rich BR juice supplementation (7.3 mmol $NO_3^-·d^{-1}$)	Cycling	1 km TT	N/A	↔ performance

		F: 53 ± 11					
Thompson et al., (2018)	18 UT males 12 UT females	M: 46 ± 8 F: 40 ± 4	28 d KNO_3 and BR juice supplementation (both 6.4 mmol $NO_3^-{\cdot}d^{-1}$) with sprint interval training	Cycling	TTE at severe-intensity 85%Δ	↑ Plasma [NO_2^-]	↑ exercise tolerance in all conditions but with the greatest improvement following BR and sprint training
de Castro et al., (2019)	14 UT males	45 ± 6	2 h prior to exercise and 3 d BR juice supplementation (8.4 mmol $NO_3^-{\cdot}d^{-1}$)	Running	10 km TT	N/A	↔ performance
Husmann et al., (2019)	12 UT males	N/A	2 h prior to exercise and 5 d BR juice supplementation (6.5 mmol $NO_3^-{\cdot}d^{-1}$)	Single-legged knee extension	TTE	N/A	↑ exercise tolerance
Mosher et al., (2019)	11 TR cyclists	61 ± 7	3 d BR juice supplementation (12.8 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	40 km TT	N/A	↔ performance
Rokkedal-Lausch et al., (2019)	12 TR cyclists	N/A	7 d BR juice supplementation (12.4 mmol $NO_3^-{\cdot}d^{-1}$)	Cycling	10 km TT	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↑ performance
Wickham et al., (2019)	12 UT females	N/A	2.5h prior to exercise and 8 d BR juice supplementation (26 mmol NO_3^-)	Cycling	TT (4 kJ·kg body mass^{-1})	↑ Plasma [NO_3^-] ↑ Plasma [NO_2^-]	↔ performance

[ ] = concentration; ↑ = significant increase; ↓ = significant decrease; ↔ = no difference; BR = beetroot; d = days; Δ = difference between power output at GET and $\dot{V}O_{2peak}$ plus the power output at GET; GET = gas exchange threshold; kcal = kilocalories; KNO_3 = potassium nitrate; $NaNO_3$ = sodium nitrate; NO_3^- = nitrate; NO_2^- = nitrite; NOx = nitrate and nitrite; NO = nitric oxide; PPO = peak power output; rpm = revolutions per minute; TR = trained; UT = untrained; $\dot{V}O_2$ = oxygen uptake; $\dot{V}O_{2peak}$ = peak aerobic capacity; WR_{peak} = maximum work rate

2.9. Mechanisms for improved exercise performance following dietary NO_3^- supplementation

The potential ergogenic effect of dietary NO_3^- supplementation may be underpinned by NO-mediated improvements to skeletal muscle contractile function (Coggan et al., 2015; Haider & Folland, 2014; Hernández et al., 2012; Whitfield et al., 2017) and fibre-type specific augmentations to type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012; Fig. 2.13).

In murine models, Hernández et al. (2012) demonstrated that 7 days of supplementation with $NaNO_3$ enriched water (~3.75 µmol of NO_3^- per day) increased the rate of force development at 100 Hz by 35% and force production at 50 Hz compared to age-matched controls. The increase in contractile function was attributed to increased expression of Ca^{2+} handling proteins calsequestrin 1 (CASQ1) and DHPR (Hernández et al., 2012). Interestingly, these physiological changes occurred in the *extensor digitorum longus*, a muscle group predominantly comprised of type II muscle fibres, but not the *soleus* which is comprised mainly of type I muscle fibres (Hernández et al., 2012). Moreover, crossbridge sensitivity may have been improved given that myoplasmic free Ca^{2+} was not different between controls and NO_3^--fed mice despite an increased force production in NO_3^--fed mice only (Hernández et al., 2012). These data indicate that NO_3^- supplementation can enhance contractile force production at low stimulation frequencies and may specifically target type II muscle fibres. Interestingly, several other reports also observed that NO_3^- supplementation increased force production at low stimulation frequencies (Haider & Folland, 2014; Whitfield et al., 2017). However, Whitfield et al. (2017) had healthy individuals supplement with NO_3^- for 7 days (26 mmol of NO_3^- per day) and observed increased peak force during evoked isometric twitches and accelerated rate of force production following NO_3^- supplementation, but this was not accompanied by changes to Ca^{2+} handling proteins SERCA, CASQ1 or DHPR, nor were there changes to muscle redox status. The reason for the divergent responses between humans and rodent models is unclear and whether NO_3^- supplementation induces changes to protein expression in human skeletal muscle requires further investigation.

Dietary NO_3^- supplementation may also induce fibre-type specific effects to blood flow distribution (Ferguson et al., 2013; 2015). Ferguson et al. (2013) reported that in

rodents, 5 days of NO_3^- supplementation (1 mmol of NO_3^- per kg per day) increased blood flow by 38% in hind limb muscles with a higher proportion of type II muscle fibres. The same research group demonstrated that NO_3^- supplementation enhanced metabolic control by observing that 5 days of NO_3^- supplementation (1 mmol of NO_3^- per kg per day) in Sprague-Dawley rats had greater blood-to-myocyte O_2 driving force compared to placebo, particularly in the type II muscle fibres (Ferguson et al., 2015). Given that the reduction of NO_2^- into NO is facilitated by low microvascular O_2 pressure (Castello et al., 2006) and low pH environments (Modin et al., 2001), such as in muscle groups comprised predominantly of type II fibres during exercise (Richardson et al., 1995), it could be speculated that NO-mediated mechanisms preferentially augment type II muscle fibres by improving muscle oxygenation and force production which contribute to its ergogenic effect.

To investigate the potential fibre-type specific influence of NO_3^- supplementation in humans, several studies employed interventions designed to modify muscle fibre recruitment patterns by manipulating movement frequency (i.e. cycling pedalling cadence; Bailey et al., 2015), altering muscle contraction velocity (Coggan et al., 2015) and incorporating work rate transitions from low to higher work rates (Breese et al., 2013). These interventions provide insight to NO-mediated fibre-type specific effects given that type II muscle fibres are likely to provide a greater proportional contribution at higher work rates (Henneman, 1965) and higher velocities of contraction (Sargeant, 1999). Breese et al. (2013) reported that in humans, 4 to 6 days of NO_3^- supplementation (~8 mmol of NO_3^- per day) improved O_2 utilization, assessed by muscle deoxyhaemoglobin kinetics, during the transition from moderate- to severe-intensity exercise (where type II fibres are likely to be recruited), but not during the transition from low- to moderate-intensity exercise. In addition, Bailey et al. (2015) reported that in humans, 9 days of NO_3^- supplementation (~8.4 mmol of NO_3^- per day) increased muscle oxygenation of the *vastus lateralis* using near-infrared spectroscopy system and accelerated the adjustment in metabolism during faster pedalling rates (115 rpm), wherein a greater proportion of type II fibres activation would occur, but not at slower pedalling rates (35 rpm). Moreover, Coggan et al. (2015) observed that acute NO_3^- ingestion (11.2 mmol of NO_3^-) enhanced voluntary maximal force and velocity of isometric knee extensor exercise performed at an angular velocity that is expected to activate a greater proportion of type II fibres (Coyle et al., 1979). In all, these data

indicate that dietary NO_3^- supplementation may augment the physiological responses during exercise protocols requiring a greater proportional contribution of type II fibres and provide insight to the mechanistic bases underpinning enhanced NO-mediated contractile function.

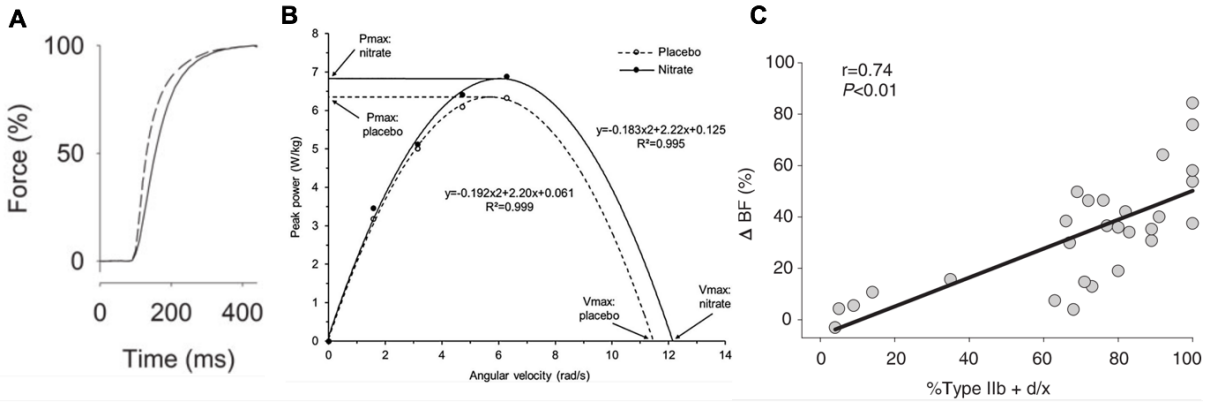

Figure 2.13. Putative mechanisms by which dietary NO_3^- supplementation enhances exercise performance in type II muscle fibres: improved rate of force development (panel A); improved peak power and peak velocity; increased blood flow (BF) towards muscle comprised of a greater proportion of type II fibres (panel C). (Adapted from Coggan et al., 2015; Ferguson et al., 2013; Hernández et al., 2012).

2.10. The potential effects of dietary NO_3^- supplementation in prolonged exercise lasting > 1 h

At the onset of this thesis, dietary NO_3^- supplementation has been reported to enhance exercise efficiency and performance by improving contractile function (Bailey et al., 2010), mitochondrial function (Larsen et al., 2011; cf. Whitfield et al., 2016), and tissue perfusion (Ferguson et al., 2013). Moreover, dietary NO_3^- supplementation has been evidenced to induce fibre-type specific effects on fatigue-sensitive type II fibres (Ferguson et al., 2013; 2015; Hernández et al., 2012). Collectively, these data provide a rationale to explore the efficacy of NO_3^- supplementation in exercise modalities expected to require a greater proportional contribution from type II muscle fibres. Accordingly, many recent publications have focused on the effects of dietary NO_3^- supplementation in high-intensity intermittent type exercise (Jonvik et al., 2018; Muggeridge et al., 2017; Nyakayiru et al., 2017a; Thompson et al., 2015; Wylie et al., 2013b) whereby a relatively greater proportion of type II muscle fibres are expected to be recruited (Krustrup et al., 2004). However, it can also be reasoned that dietary NO_3^- supplementation may influence the physiological responses during prolonged type

exercise given that a progressive decline in physiologic function may occur over time (Hermansen et al., 1967; Hopker et al., 2017). Specifically, during long duration constant work rate exercise, contractile efficiency (i.e. ATP cost of force production) and/or mitochondrial efficiency (i.e. the O_2 cost of ATP resynthesis) could decline over time (Hopker et al., 2017). In addition, muscle fibre activation could change over time such that a progressive recruitment of type II muscle fibres are recruited to replace or work alongside fatigued fibres (Vøllestad et al., 1984). Thus, in light of recent advances in understanding the mechanistic bases of NO_3^- supplementation, NO_3^- supplementation could potentially attenuate the decline in physiological function during prolonged type exercise. Despite this, there are few published reports that have employed long duration exercise ≥ 1 h (Betteridge et al., 2016). In addition, recent advances suggest that NO bioavailability is important in the efficacy of dietary NO_3^- supplementation (Dreissigacker et al., 2010; Porcelli et al., 2015; Wilkerson et al., 2012). Given that NO bioavailability is represented by the elevation in plasma [NO_2^-] (Webb et al., 2008; Wylie et al., 2013a), and that the decline in plasma [NO_2^-] during exercise is presumed to reflect a reduction of NO_2^- to NO (Kelly et al., 2014; Thompson et al., 2015; Wylie et al., 2013b), preserving NO bioavailability during prolonged type exercise may be important. Accordingly, supplementation strategies which aim to enhance or preserve NO bioavailability during prolonged exercise may further improve or maintain potential physiological augmentations following dietary NO_3^- supplementation. Therefore, the current thesis aims to explore the influence of dietary NO_3^- supplementation on physiological responses in prolonged endurance-type exercise and supplementation strategies to enhance NO bioavailability.

2.10.1. The potential fibre-type specific effects of dietary NO_3^- supplementation in prolonged exercise

It is well established that the decline in force-generating capacity is associated with fatigue in type II muscle fibres (Greenhaff et al., 1994; Soderlund et al., 1992) and that they are less efficient compared to their type I counterparts (Behnke et al., 2003; Crow & Kushmerick, 1982a; Reggiani et al., 1997; Willis & Jackman, 1994). Type II muscle fibre recruitment contributes to loss of skeletal muscle force production, which are in part due to a greater utilization of glycogen and PCr in these fibres compared to their type I muscle fibres (Greenhaff et al. 1994; Casey et al. 1996). Furthermore, progressive activation and recruitment of type II muscle fibres further increase the

metabolic demand given their greater reliance on anaerobic energy systems (Krustrup et al., 2004). Consequently, the increased metabolic cost of the additional type II fibres is in addition to fatigued fibres that are still contributing to force production and have an elevated O_2 cost (Jones et al., 2011; Krustrup et al., 2004). It could be possible that increased type II muscle fibre activation during prolonged exercise requires progressively increased ATP production from PCr hydrolysis, which would be detrimental for subsequent exercise performance (Casey et al., 1996). Dietary NO_3^- supplementation has been evidenced to enhance contractile function by reducing the PCr cost of force production (Bailey et al., 2010; Fulford et al., 2013), attenuating the accumulation of [ADP] and [P_i] (Bailey et al., 2010), and promoting blood flow and oxygenation towards type II fibres (Ferguson et al., 2013). Indeed, type II fibres have a lower microvascular oxygen pressure compared to type I fibres which may facilitate the reduction of NO_2^- to NO (Behnke et al., 2003; Castello et al., 2006). Therefore, given that the progressive recruitment of type II fibres could contribute to a progressive decline in contractile function over prolonged durations, it could be anticipated that NO_3^- ingestion may be particularly effective *during* prolonged continuous exercise in preserving skeletal muscle function. Therefore, an overarching theme of this thesis is to investigate whether NO_3^- supplementation improves or attenuates the decline in physiological responses in prolonged type exercise.

2.10.2. The potential effects of dietary NO_3^- supplementation on muscle glycogen stores in prolonged exercise

Skeletal muscle glycogen depletion during prolonged exercise is associated with impaired SR Ca^{2+} release (Hermansen et al., 1967; Nielsen et al., 2011; Ørtenblad et al., 2011) and Na^+-K^+ pump activity (Nielsen et al., 2009). Consequently, low intramuscular glycogen stores lead to impaired excitation-contraction coupling (Allen et al., 2008). Therefore, muscle glycogen depletion is an important factor in contractile efficiency particularly during prolonged moderate- and heavy-intensity exercise (Black et al., 2017; Ørtenblad et al., 2011). Indeed, one of the many functions of NO is in the regulation of skeletal muscle glucose uptake (McConell, Rattigan, Lee-Young, Wadley & Merry, 2012). Earlier studies showed that NOS inhibition attenuated glucose uptake in men (Bradley et al., 1999) and in rodents (Ross et al., 2007). These data suggest that NO-mediated uptake of extramyocyte glucose may spare intramyocyte reserves, preserve contractile function and reduce additional motor unit recruitment (Ørtenblad

et al., 2011).

Only a few studies to date have observed changes to carbohydrate metabolism following NO_3^- supplementation. Two studies have reported that the respiratory exchange ratio (RER) was increased following NO_3^- supplementation during submaximal exercise following $NaNO_3$ supplementation (Larsen et al., 2011) and concentrated BR juice supplementation (Wylie et al., 2013a). These results indicate that energy production was being produced relatively more by carbohydrate stores. In addition, Wylie et al. (2013b) reported that blood [glucose] was lower following NO_3^- supplementation during exhaustive intermittent exercise suggesting an increase in glucose uptake by skeletal muscle. A potential mechanism by which NO_3^- supplementation influences carbohydrate metabolism during exercise is through increased glucose transporter protein translocation (Jiang et al., 2014). However, a recent investigation observed that an acute dose of BR juice (8.0 mmol of NO_3^-) prior to a 1 h cycle at 65%$\dot{V}O_{2peak}$ did not significantly influence $\dot{V}O_2$, blood glucose, blood lactate, or muscle glycogen (Betteridge et al., 2016). It is possible that the single bolus of NO_3^- administered was not sufficient to induce physiological modulations (Betteridge et al., 2016) given that extended periods of supplementation may induce changes to contractile protein expression (Hernández et al., 2012). Further research is required to elucidate the potential influence of NO_3^- supplementation on indices of carbohydrate metabolism during prolonged exercise.

2.10.3. The potential effects of ROS production and dietary NO_3^- supplementation in prolonged exercise

During prolonged or heavy-intensity exercise bouts, endogenous ROS may accumulate to levels that impair skeletal muscle contractile function (Allen et al., 2008; Bruton et al., 2008; Ferreira & Reid, 2008). The accumulation of ROS could be detrimental to the efficacy of dietary NO_3^- supplementation given that ROS can inhibit eNOS activity (Jaimes et al., 2001) and spontaneously react with NOS and/or NO_2^- derived NO to form $ONOO^-$ (Halliwell, 1994; Pattwell et al., 2004), thus potentially reducing NO bioavailability. These detrimental effects could be expected to occur during the latter stages of prolonged exercise given that ROS production increases as exercise duration and intensity increases (Powers & Jackson, 2008). Moreover, type

II muscle fibres produce ROS relatively more than type I muscle fibres, which could further exacerbate disrupted contractile function during prolonged exercise as type II fibres become progressively activated (Anderson & Neufer, 2006). Therefore, it could be anticipated that environments facilitating excessive ROS production, such as prolonged and/or heavy-intensity exercise (Alessio et al., 1988; Davies et al., 1982; Dillard et al., 1978; Duthie et al., 1990; Reid et al., 1992), also exhibit a greater decline in NO bioavailability as ROS increases. Dietary NO_3^- supplementation could preserve contractile function in the face of ROS accumulation given that NO can induce a protective effect by reacting with ROS to form less damaging ROS and by undergoing reversible post-translational redox modifications of proteins (Morrison et al., 1996; Wink et al., 1993). Contractile function, blood flow and oxygenation are increased in type II fibres following NO_3^- supplementation (Bailey et al., 2015; Breese et al., 2013; Ferguson et al., 2013; Hernández et al., 2012), which may also contribute to preserving skeletal muscle function and offsetting oxidative damage to contractile proteins by ROS. Taken together, it could be reasoned that dietary NO_3^- supplementation may improve the physiological responses during prolonged exercise; however, this has yet to be explored.

2.10.4. The potential effects of dietary NO_3^- supplementation on neuromuscular function in prolonged exercise

The accumulation of ROS activates group IV muscle afferents which inhibit motorneurons (Delliaux et al., 2009) consequently reducing skeletal muscle force production (Enoka & Duchateau, 2007; Lepers et al., 2002). Group III and IV muscle afferent feedback provides input from the contracting skeletal muscle to the central nervous system to maintain homeostasis of the metabolic milieu (Mitchell et al., 1983; Rotto & Kaufman, 1988). Motor units are activated to elicit a given force output and to control the activation of muscle groups but several factors may cause a decline in firing rates (Enoka & Heckman, 2012). These include a decrease in muscle excitability over time (Taylor et al., 2016), increased group III and IV muscle afferent activation which decrease motorneuron firing (Darques et al., 1997; 1998), and/or interactions between group III and IV muscle afferents and the cardiorespiratory system that regulates blood flow and O_2 distribution to exercising muscle (Taylor et al., 2016). The potential favourable effects of NO_3^- ingestion on neuromuscular function may arise from improved K^+ handling (Wylie et al., 2013b) given that NO has a role in regulating Na^+-

K^+ ATPase (Pirkmajer et al., 2016). In addition, NO_3^- supplementation has been shown to reduce the PCr cost of force production and intramuscular metabolite accumulation (Bailey et al., 2010), preserve skeletal muscle force production (Tillin et al., 2018), enhance the rate of force development (Coggan et al., 2015; Haider & Folland, 2014; Whitfield et al., 2017) and improve both vascular and metabolic control (Ferguson et al., 2013; 2015). Therefore, given that dietary NO_3^- supplementation may attenuate the accumulation of metabolites, it could be anticipated that group III and IV muscle afferent firing would be reduced and muscle excitability would be preserved as a consequence. Moreover, it could be anticipated that dietary NO_3^- supplementation offsets the decline in skeletal muscle contractile function and enhance excitation-contraction coupling processes. Accordingly, the present thesis aims to address the potential effect of dietary NO_3^- supplementation on neuromuscular function over prolonged continuous exercise.

2.10.5. The potential effects of dietary NO_3^- supplementation on respiratory muscle fatigue in prolonged exercise

Respiratory muscle fatigue has been demonstrated to negatively affect whole body exercise performance (Mado & Acevedo, 1991; Romer & Polkey, 2008; Taylor et al., 2006) due to a reduction in blood flow to locomotor working limbs via the muscle metaboreflex phenomenon (Dempsey et al., 2006; Harms et al., 1997; Ichinose et al., 2008; Smith et al., 2014; Stark-Leyva et al., 2004). Respiratory muscle fatigue is induced with glycogen depletion as well as the accumulation of H^+ and ROS (Barreiro et al., 2006; Davies et al., 1982; Reid et al., 1994). This is exacerbated during prolonged and intense exercise given that ROS production at the skeletal (Powers & Jackson, 2008) and respiratory muscles (Shindoh et al., 1990; Supinski et al., 1997) may activate group III and IV muscle afferent feedback to stimulate redirection of blood flow away from locomotor muscle and towards respiratory muscles instead (Romer & Polkey, 2008). Decreasing ROS through administering antioxidant supplementation has been shown to attenuate exercise-induced respiratory muscle fatigue (Kelly et al., 2009; Smith et al., 2014); however, there are no published reports investigating the potential influence of dietary NO_3^- supplementation on respiratory muscle fatigue. In addition to its potential antioxidant effects, dietary NO_3^- supplementation has been reported to attenuate the accumulation of intramuscular metabolites (Bailey et al., 2010) which could contribute to reducing the group III and IV muscle afferent feedback

and thus reduce the redirection of blood flow. Moreover, NO_3^- supplementation could attenuate fatigue in locomotor muscle groups by increasing blood flow to more fatigable type II muscle fibres (Ferguson et al., 2013) as well as enhance contractile force production (Fulford et al., 2013; Hernández et al., 2012; Tillin et al., 2018) which could contribute to offsetting the metaboreflex. Given that increased ROS and muscle metabolite accumulation may occur during prolonged exercise, the present thesis aims to explore the potential influence of dietary NO_3^- supplementation on respiratory muscle fatigue during prolonged exercise.

2.10.6. The potential effects of combining dietary NO_3^- and antioxidant supplementation in prolonged exercise

During prolonged and intense exercise, increasing ROS production could offset benefits induced by NO_3^- ingestion given that ROS facilitate damage to key contractile proteins (Debold, 2015; Gutierrez-Martin et al., 2004; McKenna et al., 2006; Moopanar & Allen, 2005; Scherer & Deamer, 1986) and may quench NO (Powers & Jackson, 2008). Therefore, co-ingestion of NO_3^- and an antioxidant supplement could potentially increase NO bioavailability compared to NO_3^- ingestion alone. However, there are currently no studies that have combined dietary NO_3^- supplementation and a supplement with antioxidant properties to optimize NO bioavailability.

The most important non-enzymatic antioxidant to buffer oxidative damage caused by ROS accumulation is the thiol glutathione (Leeuwenburgh et al., 1997; Meister & Anderson, 1983). Glutathione is the most abundant thiol and has numerous roles, including quenching H_2O_2 through the enzymatic action of glutathione peroxidase (Meister & Anderson, 1983). Glutathione synthesis may be augmented with N-Acetylcysteine (NAC) administration. NAC is a reduced thiol donor providing cysteine, which is the rate-limiting step to glutathione generation (Sen, 1999). In addition to increasing glutathione synthesis, NAC itself has nonspecific antioxidant properties (Ferriera & Reid, 2008). To date, NAC is the only antioxidant supplement reported to delay muscle fatigue (Goldfarb, 1999; Powers et al., 2004; Sen, 1995a) and enhance exercise performance (Cobley et al., 2011; Medved et al., 2004; McKenna et al., 2006). Given the importance of an elevated NO bioavailability for the efficacy of NO_3^- ingestion (Porcelli et al., 2015), it could be possible that NAC ingestion would increase glutathione and thus increase the endogenous antioxidant buffering system to reduce

oxidative damage and preserve NO bioavailability.

Interestingly, both NO and ROS are critical redox signalling molecules for normal physiological function as well as physiological responses during exercise and therefore are highly interrelated (Ferreira & Reid, 2008; Gómez-Cabrera et al., 2015; Mason et al., 2016; Merry & McConnell, 2010; Reid, 2016b; Smith & Reid, 2006; Stamler & Meissner, 2001). Increasing NO bioavailability through NO_3^- supplementation during prolonged exercise could stimulate NO-ROS interactions (i.e. redox signalling) such as reversible post-translational redox modifications of cysteine thiols on key contractile proteins associated to exercise (Biswas et al., 2006; Jackson, 2009; Lo Conte et al., 2013; Powers et al., 2011). Such processes include S-nitrosylation (i.e. NO attachment to a cysteine thiol; Marozkina et al., 2012), and S-glutathionylation (i.e. glutathione attachment with cysteine sulfhydryls of proteins; Dalle-Donne et al., 2009) which increase or decrease protein activity in response to changing NO and ROS levels (Biswas et al., 2006; Dalle-Donne et al., 2009). In addition, formation of $ONOO^-$ from NO reacting with $O_2^{\bullet-}$ could induce $ONOO^-$ signalling (Merry & McConnell, 2010). The derivatives of NO and ROS are involved in complex redox signalling and thus involved in the regulation of proteins integral to exercise-induced responses (Mason et al., 2016). In summary, combining NO_3^- with a supplement with antioxidant properties holds the potential to induce synergistic effects compared to NO_3^- ingestion alone; however, this has yet to be investigated.

2.10.7. The ergogenic potential of dietary NO_3^- supplementation ingested during prolonged exercise

Lastly, central to the efficacy of dietary NO_3^- supplementation is the elevation of plasma $[NO_2^-]$ as NO_2^- could reflect a substrate pool for NO production (Cosby et al., 2003). Indeed, plasma $[NO_2^-]$ has been evidenced to be correlated to exercise performance (Dreissigacker et al. 2010; Porcelli et al., 2015; Wilkerson et al., 2012). In addition, plasma $[NO_2^-]$ has been reported to decline during short duration constant work rate exercise (Kelly et al., 2014) and high-intensity sprint interval exercise (Thompson et al., 2015; 2016; Wylie et al., 2013b). However, with longer duration exercise bouts, it could be possible that plasma $[NO_2^-]$ substantially declines such that the associated positive physiological effects from NO_3^- supplementation are no longer elicited during the latter stages of exercise. It is possible that ingestion of an additional

supplement, or a 'top-up' dose, *during* prolonged exercise could preserve the elevation of plasma [NO_2^-] and maintain any beneficial physiological effects throughout the exercise bout, subsequently enhancing exercise performance. Whether NO_3^- ingestion during exercise can modulate physiological function remains to be investigated and has important implications for supplementation strategies in endurance athletes.

2.11. Summary

In summary, NO is a molecule that is fundamental for physiological function. Supplementing with dietary NO_3^- has been shown to elevate NO bioavailability, as reflected by increased plasma [NO_2^-]. Improvements to exercise efficiency and exercise performance following NO_3^- supplementation may be underpinned by the independent or a combined effect of enhanced mitochondrial efficiency, contractile efficiency, and/or augmentation of blood flow and O_2 delivery in type II muscle fibres. These recent advances in our understanding of the mechanistic bases of NO_3^- supplementation provided a rationale to explore exercise modalities by which NO_3^- could be expected to be efficacious (i.e. exercise protocols recruiting a greater proportion of type II muscle fibres and where a decline in skeletal muscle efficiency occurs). In light of these findings, the majority of recent studies have focused on the effects of dietary NO_3^- supplementation during intense intermittent type exercise. However, the decline in muscle efficiency and potential for progressive type II muscle fibre activation during prolonged exercise may be another type of exercise in which NO_3^- is particularly effective, although limited data are available. Moreover, given that the elevation of NO bioavailability is thought to be important in the efficacy of NO_3^- supplementation, and that there is potential for NO bioavailability to decline over time during exercise, preserving NO bioavailability during prolonged exercise may be critical. Therefore, the current thesis aims to further extend the current knowledge firstly by elucidating the potential influence of dietary NO_3^- supplementation on the physiological responses during prolonged exercise, and secondly by exploring potential supplementation strategies to enhance NO bioavailability.

2.12. Aims and hypotheses

The aim of the current thesis is to provide novel insight to the effects of dietary NO_3^- supplementation on physiological responses during prolonged exercise and to

investigate potential supplementation strategies to optimize NO bioavailability. This thesis will address the following research questions in recreationally active participants:

1) What is the effect of NO_3^--depleted beetroot juice compared to nitrate-rich beetroot juice and a control intervention on NO bioavailability and $\dot{V}O_2$ and what is the effect of the number of transitions on the pulmonary $\dot{V}O_2$ responses during moderate-intensity exercise following NO_3^--rich beetroot juice supplementation?

It was hypothesized that NO_3^--depleted beetroot juice supplementation would not elevate NO biomarkers or influence the O_2 cost of exercise compared to a negative control (i.e. water) and that NO_3^--rich beetroot juice supplementation would lower $\dot{V}O_2$ during moderate-intensity exercise when four step-transitions were averaged but not with when fewer than four step-transitions were averaged.

2) What are the effects of dietary NO_3^- supplementation *before* and *during* moderate-intensity prolonged exercise on physiological responses and exercise performance?

It was hypothesized that an additional dose of dietary NO_3^- supplementation consumed *during* a prolonged exercise bout, in addition to pre-exercise NO_3^- supplementation, would maintain the elevation of plasma [NO_3^-] and [NO_2^-], attenuate the decline in muscle [glycogen] and the O_2 cost of prolonged exercise which would enable enhanced subsequent exercise performance of a 4 km TT.

3) How does contractile and mitochondrial efficiency change over a prolonged exercise bout and how does pre-exercise dietary NO_3^- supplementation influence these physiological responses?

It was hypothesized that contractile and mitochondrial efficiency would decline over time during prolonged exercise, and that pre-exercise dietary NO_3^- supplementation would attenuate the decline in contractile and mitochondrial efficiency.

4) What are the physiological and performance effects of co-ingesting pre-exercise dietary NO_3^- and NAC supplementation during prolonged exercise?

It was hypothesized that combining dietary NO_3^- and NAC supplementation would increase indices of NO and thiols while concomitantly attenuating the elevation in biomarkers of oxidative stress, attenuating the decline in neuromuscular function, lowering the O_2 cost of exercise, attenuating the increase in respiratory muscle fatigue, and subsequently enabling greater exercise capacity compared to independent ingestion of dietary NO_3^- and NAC.

Chapter 3: General Methods

The four experimental studies in this thesis (Chapters 4 to 7) that comprise this thesis required 291 exercise tests, totalling 1,180 hours in duration. All exercise tests took place in an air-conditioned exercise physiology laboratory (Chapter 4, 6 and 7) or MRI suite (Chapter 5) at sea level with an ambient temperature of 18-22ºC.

3.1. Ethics approval and informed consent

The procedures employed in each of these experimental Chapters were approved by the University Ethics Committee prior to the commencement of data collection (See Appendix IV). Participants were provided with a Participant Information Sheet, an explanation of the potential risks and benefits of participation, data storage safety, publication, and anonymity by a certified informed consent personnel prior to providing their written informed consent (See Appendix I and II).

3.2. Health and Safety

All procedures during the experimental testing followed the health and safety guidelines established by the School of Sport and Health Sciences at the University of Exeter to ensure safety of the human subjects. This included sterilization of ergometers, work surfaces, and floors using dilute Virkon disinfectant following each test. Routine safety checks on all testing areas and equipment were also conducted by members of the technical staff. Muscle biopsies were taken using Bergström needles that had undergone sterilisation procedures and confirmed as sterile prior to sampling. Researchers involved in the collection of muscle biopsies wore disposable sterile gloves during sampling and when dressing the wound. Participants who took part in the muscle biopsy procedure were provided with a Post-Procedure Care for your Biopsy Site information sheet (See Appendix V). All sharps and biohazardous waste materials were disposed of immediately and later incinerated.

3.3. Subjects

The subjects who participated for all experiments were recruited from the student University population and from the local community of Exeter. All volunteers were

screened prior to recruitment to ensure they were non-smokers, free from disease, did not have contraindications to exercise and not using dietary supplements (See Appendix III, VI and XI). The subjects were recreationally active at the point of recruitment, which included regular structured exercise and competitive sport, though none were elite-level athletes. Subjects were instructed to arrive to the laboratory for exercise testing having avoided strenuous exercise and alcohol in the 24 h preceding, and caffeine 8 h preceding each experimental visit. Subjects recorded in a dietary and exercise log 24 h prior to experimental testing and was repeated prior to each visit (See Appendix IX and X). Subjects recorded in a supplementation log during each experimental condition (See Appendix VIII). Subjects refrained from using antibacterial mouthwash and other dietary supplements throughout the entire duration of the experimental testing (See Appendix VII). Subjects underwent experimental procedures at the same time of day (± 2 h). All subjects were familiarised to the exercise protocols and procedures prior to the commencement of the experimental testing.

3.4. Supplementation

For all experimental Chapters, subjects ingested inorganic NO_3^- using concentrated NO_3^--rich BR juice (Beet it, James White Drinks, Ipswich, UK) and concentrated NO_3^--depleted BR juice (PL). Subjects were allocated in a double-blind, randomized crossover design, with a minimum of a 5-day washout period between each condition. NO_3^- ions were removed by having the passage of juice through a column with an ion-exchange resin. The result is a product identical in appearance, taste, smell, and texture (Lansley et al. 2011b).

In Chapters 4-7, the supplements were NO_3^--rich BR (2 x 70 mL/day providing ~12.4 mmol of NO_3^-/day) and/or NO_3^--depleted PL (2 x 70 mL/day providing ~0.04 mmol of NO_3^-/day). For these investigations, subjects consumed 1 x 70 mL of supplement each morning and evening for the duration of the supplementation period. On experimental days, subjects consumed 2 x 70 mL of their supplement 2.5 h prior to the commencement of the exercise test. In Chapter 4, an additional supplement (1 x 70 mL) was consumed half-way during the 2 h exercise test.

Subjects were informed of potential beeturia (red urine) and red stool due to supplementation. No adverse effects were reported for Chapters 4 to 7.

3.5. Measurements

Prior to the commencement for all experimental testing, descriptive data were measured and recorded of each subject including height, weight, and age. Cycle ergometry was employed for Chapter 4, 6 and 7, in which peak power output, $\dot{V}O_{2peak}$, were recorded, as well as the $\dot{V}O_2$ and power output at GET (see below).

3.5.1. Blood sampling

For Chapters 4, 5 and 6, venous blood samples were obtained at rest and during exercise from an antecubital vein using venous cannulation. At the start of all experimental visits, a cannula (20g; BD Venflon, Becton Dickinson, Helsingborg, Sweden) was inserted into the antecubital vein and kept patent with an infusion of 0.9 saline at 10 ml/h. For Chapter 7, venous blood samples were obtained at rest from using venipuncture (21G, Becton-Dickinson, NJ, USA). All blood samples were collected into 6 mL lithium-heparin tubes (Vacutainer, Becton-Dickinson, NJ, USA). The blood collected in Chapter 4 was immediately analysed for blood [glucose] and [lactate]. The blood collected in Chapter 6 was immediately derivatized for later analysis of plasma [cysteine] and [glutathione]. All blood samples in Chapters 4-7 were centrifuged within 1 min of collection at 4000 rpm at 4ºC for 8 minutes and were immediately extracted and frozen at -80ºC. These plasma samples were used for later analysis of plasma [NO_3^-] and [NO_2^-] in Chapters 4-7, as well as plasma [MDA] analysis in Chapter 6.

3.5.2. Measurement of plasma nitrate and nitrite concentrations

For all investigations, plasma samples were analysed for plasma [NO_3^-] and [NO_2^-] via gas phase chemiluminescence. This procedure causes NO_3^- and NO_2^- to reduce into NO. Subsequently, the NO gas reacts with ozone to yield nitrogen dioxide, causing an infrared light to be emitted. This light is quantified by a red-sensitive photomultiplier tube and amplified to produce an analogue millivolt output signal. These millivolt signals are compared to calibration plot values obtained from standard concentrations of 25 nM to 1 μM of sodium NO_2^- and from

250 nM to 50 μM of sodium NO_3^-. The coefficient of variation for duplicate samples of [NO_3^-] and [NO_2^-] were ~2% and ~6%, respectively.

All glassware, utensils, and surfaces used for the following procedures were regularly rinsed with fresh deionized water to remove residual NO_2^-. All plasma samples were deproteinized using cold ethanol precipitation. Thawed samples were centrifuged at 14 000 G for 10 min, before 200 μL of sample was added to 400 μL of ice-cold ethanol and incubated on ice for 30 min. Subsequently, samples were centrifuged at 14 000 G for 10 min again, and the supernatant was removed for NO_2^- analysis. For NO_2^- reduction, this supernatant was injected (100 μL) into the gas sealed purge vessel containing 4 mL glacial acetic acid and 1 mL sodium iodide (NaI; 4% w/v) at 35ºC. Before the determination of plasma [NO_3^-], the deproteinized supernatant was diluted by adding 100 μL of supernatant to 400 μL of deionized water. For NO_3^- reduction, an additional gas bubbler containing 1 M sodium hydroxide was used to prevent acid vapor from the assay in damaging the NO analyser (Sievers NOA 280i, Analytix Ltd, Durham, UK). The diluted supernatant was injected into the gas sealed purge vessel containing 0.8% (w/v) vanadium trichloride in 1 M HCl at 95ºC.

3.6. Exercise testing procedures

3.6.1. Cycle ergometry

All cycling exercise tests in Chapters 4, 6 and 7 were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Ramp incremental tests to volitional exhaustion were performed during baseline visits to the laboratory for the determination of peak work rate, $\dot{V}O_{2peak}$, and gas exchange threshold (GET). Individualized work rates were subsequently determined.

3.6.2. Ramp incremental test

In Chapters 4, 6 and 7, ramp incremental tests were employed by having a 3 min baseline period where subjects pedalled at 20 W at a self-selected cadence between 75 and 90 rpm. Following this, the work rate linearly increased at a rate of 30 $W \cdot min^{-1}$ until task failure. Task failure occurred when the pedal rate fell by

>10 rpm below the target cadence. The self-selected cadence, seat height and handle bar configuration were recorded and reproduced on subsequent visits.

3.6.3. Pulmonary gas exchange and the determination of $\dot{V}O_{2peak}$ and GET

For all investigations, breath-by-breath pulmonary gas exchange data were collected continuously during all exercise tests and averaged over 10 s periods. Gas analysis was measured using a metabolic cart system of a bidirectional TripleV digital transducer and differential paramagnetic (O_2) and infrared absorption (CO_2) (Jaeger Oxycon Pro, Hoechberg, Germany). Gases with known concentrations were used to calibrate the gas analyser. Volume sensor calibration was conducted using a 3-L syringe (Hans Rudolph, Kansas City, MO). Subjects wore a nose clip and breathed through a low resistance, turbine volume transducer (Triple V, Jaeger, The Netherlands). Gas was continuously drawn down a capillary line which sampled for $\dot{V}O_2$, carbon dioxide ($\dot{V}CO_2$), and minute ventilation ($\dot{V}E$). Raw breath-by-breath gas exchange and ventilation data were exported for later analysis. The $\dot{V}O_{2peak}$ was taken as the highest 30 s average value attained before the subject's volitional exhaustion. The GET was determined by identifying the first disproportionate increase in $\dot{V}CO_2$ from visual inspection of plots of $\dot{V}CO_2$ compared to $\dot{V}O_2$ (V-slope method; Beaver et al. 1986) and an increase in $\dot{V}E/\dot{V}O_2$ with no increase in $\dot{V}E/\dot{V}CO_2$ with account taken of the mean response time for $\dot{V}O_2$ during ramp exercise (i.e., two-thirds of the ramp rate was deducted from the power output at GET and peak; Whipp et al., 1981).

3.6.4. Constant work rate calculation

In Chapters 4, 6 and 7, constant work-rate exercise was employed to assess individual physiological responses following supplementation. Individualized work rates for these experimental visits were calculated from the peak work rate and GET determined from the initial ramp incremental tests. In addition, work rates were adjusted for the initial $\dot{V}O_2$ 'lag' time or mean $\dot{V}O_2$ response (Whipp et al. 1981). Therefore, the peak work rate and GET used were 20 W less than the work rates that coincide with the appearance of the GET and $\dot{V}O_{2peak}$.

In Chapter 4 and 7, the moderate-intensity work rates used were calculated as 80% of the GET. For Chapter 4, the resistance for the 100 kJ time trial (TT) was set by using the linear mode such that the subject would attain the power output associated with GET plus 65% of the difference between GET and peak power output (65%Δ) on reaching a pedal cadence of 90 rpm. In Chapter 6, the heavy- and severe-intensity work rates used were calculated as 20%Δ and 70%Δ, respectively.

The reliability of measuring steady-state pulmonary gas exchange during moderate-intensity exercise in Chapter 4 was determined by repeat assessments on two separate days in four individuals, yielding a coefficient of variation of 2.9%. The reliability of measuring pulmonary gas exchange during heavy-intensity exercise in Chapter 6 was determined by repeat assessments on two separate days in five individuals, yielding a coefficient of variation of 1.7%. The reliability of measuring pulmonary gas exchange during moderate-intensity exercise in Chapter 7 was determined in a pilot experiment from repeat assessments on two separate days in five individuals, yielding a coefficient of variation of 1.6%.

3.6.5. Reliability of exercise performance/tolerance measurements

In Chapter 4, exercise performance was assessed as the time to complete a 100 kJ TT during severe-intensity cycling exercise. The reliability of this measure was determined by repeat assessment on two separate days in four individuals, yielding a coefficient of variation of 2.9%.

In Chapter 6, exercise tolerance was assessed as the time-to-exhaustion (TTE) during severe-intensity constant work-rate cycling exercise. The reliability of this measure was determined by repeat assessment on two separate days in five individuals, yielding a coefficient of variation of 3.6%.

3.7. Statistical analyses

For all investigations, statistical analyses were performed using the Statistical Package for Social Sciences (SPSS v.22). The statistical procedures specific to each chapter are outlined in the according sections below. All data are presented

as mean ± SD unless otherwise stated. Statistical significance was accepted at $P \leq 0.05$.

Dietary nitrate supplementation and the oxygen cost of exercise: the influence of nitrate-depleted beetroot juice and the number of transitions

The first aim of the present thesis was to investigate the potential effects of nitrate-depleted beetroot juice (i.e. placebo juice) on nitric oxide bioavailability and oxygen uptake parameters given that nitrate-depleted beetroot juice contains compounds other than nitrate such as bioactive constituents with antioxidant-like properties. Understanding the potential influence of nitrate-depleted beetroot juice could provide insight into whether previous literature may have yielded equivocal results in part due to potential effects of nitrate-depleted beetroot juice in obscuring any results of nitrate-rich beetroot juice.

Introduction

Dietary nitrate (NO_3^-) supplementation, from pharmacological (i.e. NO_3^- salts) and vegetable juice sources (i.e. beetroot juice), is the only nutritional aid shown to improve exercise economy by lowering steady-state pulmonary O_2 uptake ($\dot{V}O_2$) during submaximal exercise (Bailey et al., 2009; Cermak et al., 2012; Larsen et al., 2007; Whitfield et al., 2016;) although not all studies have observed this effect (Betteridge et al., 2016; Boorsma et al., 2014; Oskarsson et al., 2018; Sandbakk et al., 2015). Improvements following inorganic NO_3^- supplementation have been largely ascribed to the elevation of nitric oxide (NO), nitrite (NO_2^-), and/or S-nitrosothiol bioavailability and subsequent nitroso signalling (Bryan et al., 2005; Ignarro et al., 1981; Lundberg et al., 2008; Stamler, 1994). The candidate mechanisms responsible include the independent or combined improvements to mitochondrial efficiency (i.e. O_2 cost per ATP production; Larsen et al., 2011; cf. Whitfield et al., 2016) and/or contractile efficiency (i.e. ATP cost of contraction; Bailey et al., 2010).

To date, the plethora of investigations that have administered NO_3^--rich beetroot juice products to investigate the influence of NO_3^- supplementation either provided commercially available blackcurrant juice cordial (Bailey et al., 2009; Carriker et al., 2016; Haider & Folland, 2014; Vanhatalo et al., 2010), or concentrated NO_3^--depleted beetroot juice (Kelly et al., 2013; Lansley et al.,

2011; Wylie et al., 2013a; 2016) to serve as the placebo. However, in addition to NO_3^-, beetroot (*Beta vulgaris*) contains an array of phytochemicals (Clifford et al., 2017; Wootton-Beard & Ryan, 2012) such as flavonoids and betalains (Shepherd et al., 2015). These bioactive polyphenolic compounds are found in both NO_3^--rich and NO_3^--depleted beetroot juice (e.g. commonly used as placebo), and could exert protective antioxidant properties (Davies et al., 1982; Mason et al., 2016; Reid et al., 1992) as well as facilitate the NO_3^--NO_2^--NO pathway to elevate NO bioavailability (Gago et al., 2007; Lundberg et al., 2008; Peri et al., 2005). Indeed, indirect markers of NO bioavailability (e.g. arterial flow-mediated dilation) have been increased following polyphenol supplementation such as apple (Peri et al., 2005), dark chocolate (Engler et al., 2004; Hooper et al., 2012), blackcurrant (Cook et al., 2017), blueberry (Rodriguez-Mateos et al., 2013), grape (Chaves et al., 2009; Li et al., 2013), pomegranate (Crum et al., 2017; Roelofs et al., 2017) and montmorency cherry supplementation (Aboo Bakkar et al., 2019; cf: Keane et al., 2018). These data suggest the potential for polyphenol compounds in beetroot juice to increase NO bioavailability and to induce subsequent NO-mediated physiological effects. However, given that NO_3^--rich beetroot juice has been reported to induce more pronounced favourable physiological effects compared to NO_3^- salts (Flueck et al., 2016; Thompson et al., 2018) and that the influence of NO_3^- supplementation on the O_2 cost of exercise is equivocal, further research is required to understand the potential influence of the polyphenol content of NO_3^- provided in vegetable juice form.

Current literature suggests that dietary NO_3^- supplementation may improve $\dot{V}O_2$ kinetics (i.e. phase II kinetics; $\dot{V}O_2$ τ_p) when O_2 delivery is impaired (Kelly et al., 2014) and during exercise protocols that require a relatively greater recruitment of type II muscle fibres such as during step transitions from moderate- to severe-intensity cycling (Breese et al., 2013) or when performing a step transition at a higher cadence (Bailey et al., 2015). This is supported by studies that have not observed changes to $\dot{V}O_2$ kinetics following NO_3^- supplementation during step transitions from unloaded cycling to moderate-intensity exercise (Bailey et al., 2009; Bescós et al., 2011; Breese et al., 2013; Ghiarone et al., 2017; Kelly et al., 2014) given that transitioning to moderate-intensity exercise would be expected to have a relatively lower reliance on type II muscle fibre recruitment compared

to higher intensities (Bottinelli et al., 2000) and that there would not be an impairment to O_2 availability (Poole & Jones, 2012). Although there is no evidence to suggest that NO_3^- supplementation influences $\dot{V}O_2$ τ_p during moderate-intensity exercise, whether NO_3^- supplementation lowers the O_2 cost of exercise remains equivocal. An important consideration that may have contributed to disparate results is that the measurement of pulmonary $\dot{V}O_2$ responses inherently contains noise (i.e. variability) such that experimental designs employing only a one-step transition may be insufficient to characterize an accurate response (Linnarsson, 1974; Özyener et al., 2001; Whipp et al., 1982). A high signal-to-noise ratio can be achieved by repeating the exercise protocol and averaging the responses of all transitions to enhance the underlying $\dot{V}O_2$ (Lamarra et al., 1987). Some earlier studies that have observed a reduced $\dot{V}O_2$ used four transitions (Bailey et al., 2009; Lansley et al., 2011; Porcelli et al., 2015) or two transitions (Porcelli et al., 2016; Rienks et al., 2015; Wylie et al., 2013; 2016; Vanhatalo et al., 2010) whilst many studies that have reported no effect only employed one transition (Bescós et al., 2011; Betteridge et al., 2016; Boorsma et al., 2014; Flueck et al., 2016; Kocoloski et al., 2018; Muggeridge et al., 2015; Nyakayiru et al., 2017; Wickham et al., 2019; Wilkerson et al., 2012). These studies highlight that since dietary NO_3^- supplementation is expected to lower $\dot{V}O_2$ by ~3% and that the typical noise in the $\dot{V}O_2$ response with one transition is high, pulmonary $\dot{V}O_2$ measurements need to be accurate to observe an effect. However, there is currently no data on whether the number of step transitions influences the $\dot{V}O_2$ response (i.e. steady-state $\dot{V}O_2$ and $\dot{V}O_2$ τ_p) following NO_3^--rich beetroot juice supplementation.

Therefore, the purpose of this study was two-fold: 1) to investigate whether PL supplementation would influence indices of NO bioavailability or the pulmonary $\dot{V}O_2$ response compared to a water control condition (CON); and 2) to investigate whether the number of transitions to a moderate work rate would influence the pulmonary $\dot{V}O_2$ response outcome following NO_3^--rich beetroot juice supplementation (BR) compared to NO_3^--depleted beetroot juice supplementation (PL). We hypothesized that firstly, PL supplementation would not significantly increase resting plasma [NO_3^-] and [NO_2^-] or influence $\dot{V}O_2$ or

$\dot{V}O_2$ τ_p during moderate-intensity exercise compared to CON. Secondly, we hypothesized that the O_2 cost of exercise would be significantly lower following BR supplementation when 4 step transitions were averaged but not when averaged with fewer than 4 step transitions and that BR supplementation would not influence the $\dot{V}O_2$ kinetics response with any number of transitions.

Methods

Subjects

Twelve recreationally active males and females (mean ± SD: age 29 ± 7 years, body mass 71 ± 11 kg, height 1.72 ± 0.09 m, $\dot{V}O_{2peak}$ 44 ± 9 mL·kg^{-1}·min^{-1}, females *n*=4) volunteered to participate for this study. A priori power analysis showed that a sample size of twelve would provide a power = 0.80, alpha = 0.05, an effect size = 0.92. The protocols, risks, and benefits of participating were explained prior to obtaining written informed consent. This study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki.

Experimental overview

Subjects reported to the laboratory on 7 separate occasions over a ~4 to 5 week period. Prior to experimental testing, subjects completed a ramp incremental cycling exercise test for the determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). Breath-by-breath pulmonary gas exchange was measured continuously during the incremental test and averaged over consecutive 10 s periods. The data collected during the incremental tests were used to calculate individualized work rates at 80%GET for subsequent moderate-intensity step tests. Over the remaining six laboratory visits, subjects completed moderate-intensity step exercise tests during three separate 4-day supplementation periods with water supplementation (CON), NO_3^--depleted placebo (PL) or NO_3^--rich beetroot juice (BR) for the determination of the O_2 cost of moderate-intensity exercise and $\dot{V}O_2$ kinetics response. The three supplementation periods were administered in a randomized, double-blinded, crossover experimental design.

Subjects completed two moderate-intensity step tests on *days 3* and *4* of each

dietary condition (CON, PL and BR). The step tests comprised 3 min of baseline cycling at 20 W, followed by a step increment to a moderate-intensity (80%GET) constant work rate bout for 6 min, followed by a period of 7 min rest. Subsequently, subjects performed another 3 min of baseline cycling at 20 W, followed by a step increment to a moderate-intensity (80%GET) constant work rate bout, totalling two moderate-intensity step exercise bouts over *day 3* and *4* during each condition. In total, subjects completed four bouts of transitions to average the pulmonary $\dot{V}O_2$ responses in the same experimental condition to enhance signal-to-noise ratio (Lamarra et al., 1987).

Supplementation

Following the initial ramp test, subjects underwent three 4-day supplementation periods in which they consumed 2 x 70 mL doses per day of CON (negligible NO_3^- content per 50 mL; Buxton Natural Still Water, Nestlé UK Ltd., UK), PL (~0.04 mmol of NO_3^- per 70 mL; Beet It Sport, James White Drinks Ltd., Ipswich, UK) or BR (~6.2 mmol of NO_3^- per 70 mL; Beet It Sport, James White Drinks Ltd., Ipswich, UK) separated by at least a 5-day wash-out period. Each 70 mL beetroot juice beverage contained 72 kcal energy and 15.4 g of carbohydrate. The CON was provided in 50-mL plastic tubes which were placed in a small transparent plastic bag. On *day 1* and *2* of each supplementation period, subjects consumed one 70 mL beverage in the morning and one in the evening, whereas on the experimental days (*days 3* and *4*), subjects consumed 2 x 70 mL of their allocated beverage in the morning ~2.5 h prior to the exercise (Webb et al., 2008; Wylie et al. 2013a). For the duration of the study, subjects were asked to maintain their habitual physical activity and dietary intake. However, subjects were instructed to avoid consuming foods high in dietary nitrate such as beetroot, kale, spinach, and rocket, and to refrain from taking any other dietary supplements or using antibacterial mouthwash (Govoni et al., 2008). Experimental visits were performed at the same time of day (± 2 h). Subjects recorded their activity and diet during the 24 h prior to the first experimental visit and were asked to repeat these for subsequent visits. Subjects were also instructed to arrive at the laboratory having avoided strenuous exercise and alcohol in the 24 h preceding, and caffeine in the 8 h preceding, each experimental visit.

Measurements

On *days 3* and *4* of each supplementation period, subjects completed two identical exercise bouts interspersed by a 7 min period of rest. The exercise protocol consisted of a one-step transition from 3 min of unloaded (20 W) pedalling followed by 6 min of moderate-intensity cycling (80%GET) for the determination of steady-state $\dot{V}O_2$ and the $\dot{V}O_2$ kinetics response. Venous blood samples were drawn into a 6-mL lithium-heparin tube (Vacutainer, Becton-Dickinson, New Jersey, USA) and centrifuged at 4000 rpm and 4°C for 8 min, within 2 min of collection. Plasma was subsequently extracted and frozen at -80°C for later deproteinization and analysis of [NO_3^-] and [NO_2^-] as described previously (Tan et al., 2018). The [NO_3^-] in CON was analyzed using methods as described previously (Tan et al., 2018).

During all tests, breath-by-breath pulmonary gas exchange was measured with subjects wearing a nose clip and breathing through a low-dead-space, low-resistance mouthpiece and impeller turbine assembly (Jaeger Triple V). The inspired and expired gas volume and gas concentration signals were continuously sampled at 100 Hz, the latter with paramagnetic O_2 and infrared CO_2 analyzers (Jaeger Oxycon Pro, Hoechberg, Germany) via a capillary line connected to the mouthpiece. The gas analyzers were calibrated before each test with gases of known concentration, and the turbine volume transducer was calibrated with a 3-L syringe (Hans Rudolph, Kansas City, MO). The volume and concentration signals were time aligned by accounting for the delay in the capillary gas transit and the analyzer rise time relative to the volume signal.

Data analysis procedures

The breath-by-breath $\dot{V}O_2$ data from each test were analyzed as previously described (Bailey et al., 2009). Briefly, a single-exponential model was used to characterize the $\dot{V}O_2$ responses to moderate-intensity exercise as described in the following equation:

$$\dot{V}O_2(t) = \dot{V}O_{2\,\mathrm{baseline}} \quad A_{\mathrm{p}}\left[1 - e^{-(t-\mathrm{TD}_{\mathrm{p}}/\tau_{\mathrm{p}}}\right]$$

where $\dot{V}O_2(t)$ represents the absolute $\dot{V}O_2$ at a given time *t*; $\dot{V}O_{2baseline}$

represents the mean $\dot{V}O_2$ in the baseline period; A_p, TD_p, and τ_p the amplitude, time delay, and time constant, respectively, describing the phase II increase in $\dot{V}O_2$ above baseline.

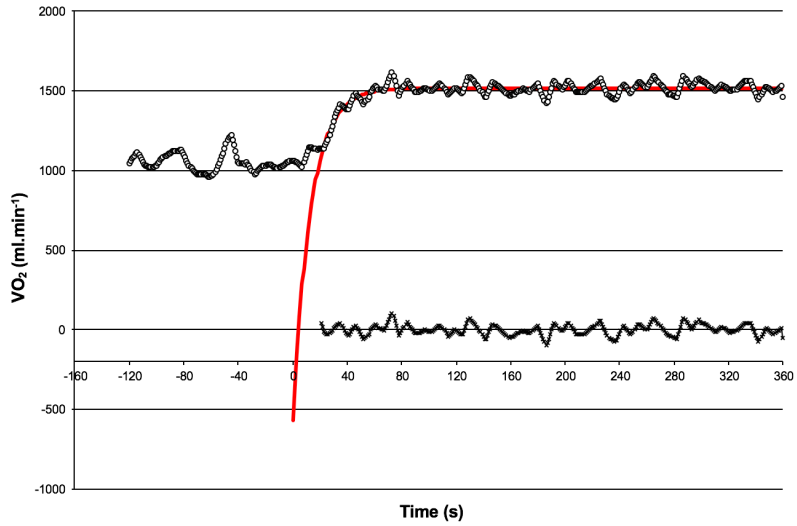

Figure 1. Example $\dot{V}O_2$ kinetics fit of one subject.

$\dot{V}O_{2baseline}$ was defined as the mean $\dot{V}O_2$ measured over the final 60 s of baseline pedaling. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 60 s of exercise.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to analyze differences in the O_2 uptake responses and plasma [NO_3^-] and [NO_2^-] between CON, PL, and BR. The coefficient of variation (CV%) for plasma [NO_3^-] and [NO_2^-] were calculated between duplicate samples and between trials. Significant main and interaction effects were further explored using Fisher's LSD post hoc comparisons. A one-way ANOVA was used to analyze differences in the $\dot{V}O_2$ responses between CON, PL, and BR. In addition, a paired t-test was used to analyze differences in the end-exercise steady-state $\dot{V}O_2$ response between BR vs. PL to provide a comparable approach to literature which commonly test only two conditions. Data for $\dot{V}O_2$ kinetics were analyzed for n=9 due to n=3 containing high noise and unreliable data. The CV% for steady-state $\dot{V}O_2$ using the average results of 1, 2, 3, and 4 transitions were calculated using data from CON and PL provided that there was no significance between conditions. Statistical significance was

accepted at $P<0.05$. Results are presented as mean ± SD unless otherwise stated.

Results

All subjects reported consuming all servings of each supplement at the correct times and confirmed that they had maintained their exercise and dietary habits prior to each testing visit. There were no reports of adverse reactions, gastrointestinal distress or discomfort following the ingestion of supplements.

Plasma [NO_3^-] and [NO_2^-]

The CV% for duplicate samples was 6.0 ± 3.2% and 3.0 ± 0.2% for plasma [NO_3^-] and [NO_2^-], respectively. The CV% for between day 3 and day 4 injections was 16.7 ± 35.5% and 18.9 ± 55.2% for plasma [NO_3^-] and [NO_2^-], respectively. There was a main effect by condition ($P<0.01$), but no effect by time ($P>0.05$) on plasma [NO_3^-] and [NO_2^-] (Fig. 2A and B). Plasma [NO_3^-] was higher in BR (831 ± 225 μM) compared to PL (45 ± 53 μM, $P<0.01$) and CON (45 ± 8 μM, $P<0.01$) and there was no significant difference between PL and CON ($P>0.05$). Plasma [NO_2^-] was higher in BR (569 ± 225 nM) compared to PL (117 ± 53 nM, $P<0.01$) and CON (107 ± 24 nM, $P<0.01$) and there was no significant difference between PL and CON ($P>0.05$).

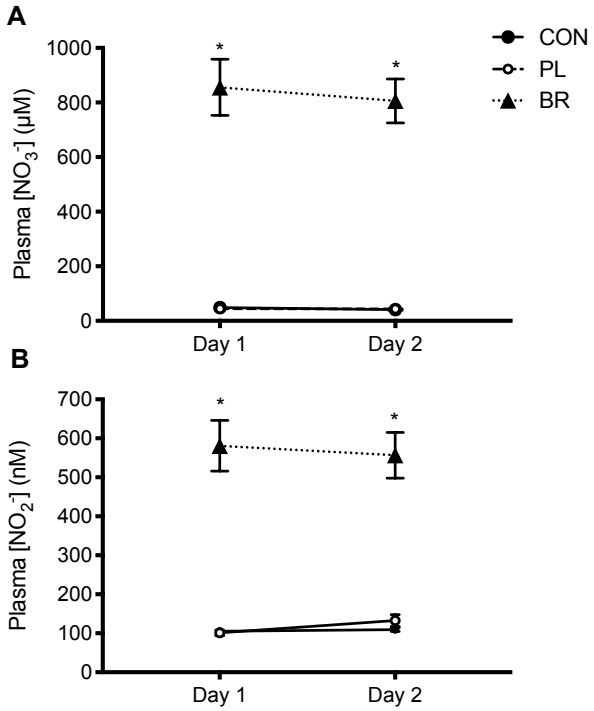

Figure 2. Mean ± SE plasma nitrate (NO_3^-; panel A) and nitrite (NO_2^-; panel B) at rest following: 1) CON, water supplementation consumed before exercise (solid circle and solid line); 2) PL, nitrate-depleted beetroot juice consumed before exercise (open circle and dashed line); and 3) BR, nitrate-rich beetroot juice consumed before exercise (solid triangle and dotted line). * = significantly different from CON and PL (P<0.05).

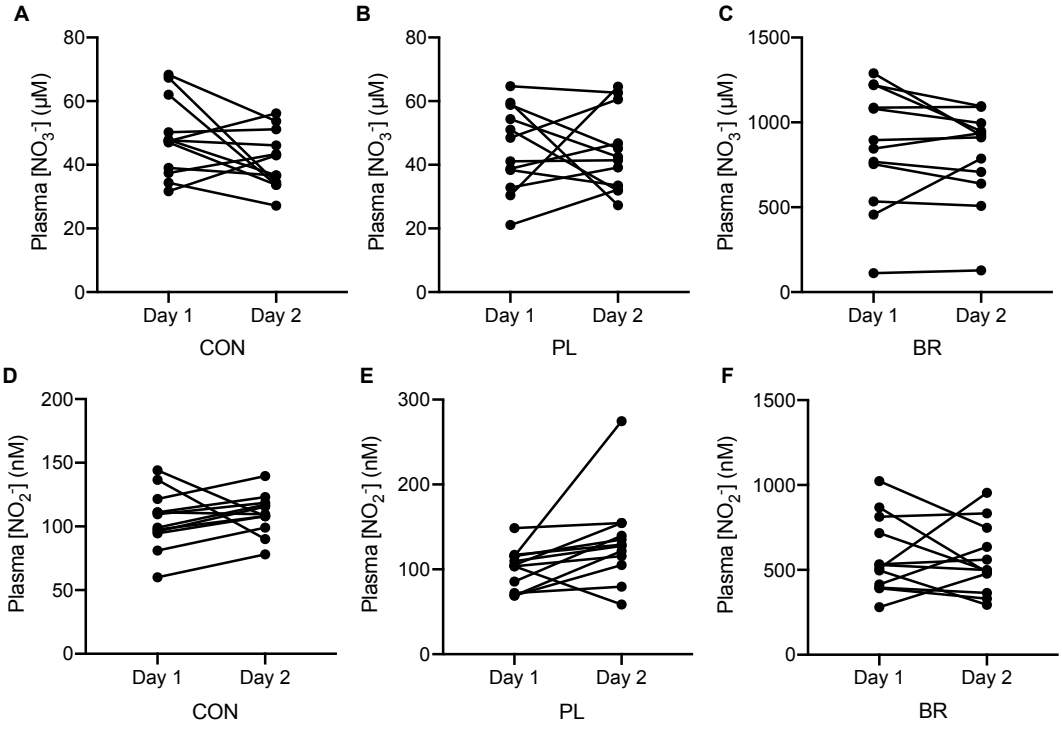

Figure 3. Individual responses of plasma nitrate (NO_3^-; panel A, B, C) and nitrite (NO_2^-; panel D, E, F) on day 1 and 2 following: 1) CON, water supplementation consumed before exercise; 2) PL, nitrate-depleted beetroot juice consumed before exercise; and 3) BR, nitrate-rich beetroot juice consumed before exercise.

Pulmonary gas exchange

There was no effect by condition (CON, PL, BR) on the pulmonary $\dot{V}O_2$ responses at baseline or during moderate-intensity cycling exercise when data were averaged over 4 step transitions (Table 1 and 3.). The CV% for steady-state $\dot{V}O_2$ was 2.5 ± 2.6%, 2.8 ± 2.4%, 2.2 ± 1.7%, and 2.1 ± 1.7% when results were averaged over 1, 2, 3, and 4 transitions, respectively. The end-exercise $\dot{V}O_2$ response was significantly different between BR and PL following the average over 4 transitions (P<0.05) but not over 1, 2, or 3 transitions (P>0.05, Table 2.). The $\dot{V}O_2$ τ_p was not significantly different between BR and PL following the average over 1, 2, 3 or 4 transitions (P>0.05, Table 3.). The baseline and end-exercise values of $\dot{V}CO_2$, minute ventilation ($\dot{V}E$), and respiratory exchange ratio were not significantly different between conditions (P>0.05, Table 1.).

Table 1. *Mean ± SD ventilatory and gas exchange dynamics during moderate-intensity exercise following supplementation with water (CON), nitrate-depleted beetroot juice (PL), and nitrate-rich beetroot juice (BR) over 4 step transitions.*

	CON	**PL**	**BR**
$\dot{V}O_2$			
Baseline ($L \cdot min^{-1}$)	0.90 ± 0.13	0.90 ± 0.14	0.89 ± 0.13
End-exercise ($L \cdot min^{-1}$)	1.33 ± 0.34	1.33 ± 0.34	1.31 ± 0.34
Phase II time constant (s)	18 ± 10	15 ± 6	19 ± 9
Mean response time (s)	38 ± 13	36 ± 8	37 ± 11
Primary amplitude ($L \cdot min^{-1}$)	0.47 ± 0.30	0.46 ± 0.28	0.46 ± 0.26
Gain ($ml \cdot min^{-1} \cdot W^{-1}$)	8.26 ± 1.28	8.06 ± 0.94	8.31 ± 0.57
$\dot{V}CO_2$			
Baseline ($L \cdot min^{-1}$)	0.83 ± 0.12	0.85 ± 0.13	0.83 ± 0.13
End-exercise ($L \cdot min^{-1}$)	1.26 ± 0.30	1.28 ± 0.33	1.27 ± 0.30
$\dot{V}E$			
Baseline ($L \cdot min^{-1}$)	23 ± 3	23 ± 4	22 ± 4
End-exercise ($L \cdot min^{-1}$)	32 ± 8	32 ± 10	32 ± 9
Respiratory exchange ratio			
Baseline	0.93 ± 0.06	0.95 ± 0.04	0.94 ± 0.05
End-exercise	0.96 ± 0.04	0.97 ± 0.03	0.97 ± 0.04

Values are means ± SD. $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, expired carbon dioxide; $\dot{V}E$, minute ventilation. Data for $\dot{V}O_2$ kinetics data were analyzed for *n*=9.

Table 2. *Mean ± SD steady-state end-exercise $\dot{V}O_2$ response averaged over 1, 2, 3, and 4 step transitions from unloaded to moderate-intensity exercise following supplementation with nitrate-rich beetroot juice and nitrate-depleted beetroot juice*

	1 transition	2 transitions	3 transitions	4 transitions
Nitrate-rich beetroot juice				
End-exercise $\dot{V}O_2$ ($L \cdot min^{-1}$)	1.31 ± 0.34	1.31 ± 0.34	1.31 ± 0.34	1.31 ± 0.34*
Nitrate-depleted beetroot juice				
End-exercise $\dot{V}O_2$ ($L \cdot min^{-1}$)	1.32 ± 0.33	1.33 ± 0.33	1.33 ± 0.33	1.33 ± 0.34
P value	0.30	0.32	0.13	0.04

Values are means ± SD. $\dot{V}O_2$, oxygen uptake. * = Significantly different to nitrate-depleted beetroot juice ($P<0.05$).

Table 3. *Mean ± SD $\dot{V}O_2$ kinetics response averaged over 1 and 4 step transitions from unloaded to moderate-intensity exercise following supplementation with nitrate-rich beetroot juice and nitrate-depleted beetroot juice*

	1 transition	4 transitions
Nitrate-rich beetroot juice		
$\dot{V}O_2$ τ_p (s)	22 ± 15	19 ± 9
Nitrate-depleted beetroot juice		
$\dot{V}O_2$ τ_p (s)	20 ± 9	15 ± 6

Values are means ± SD. $\dot{V}O_2$ τ_p, phase II time constant.

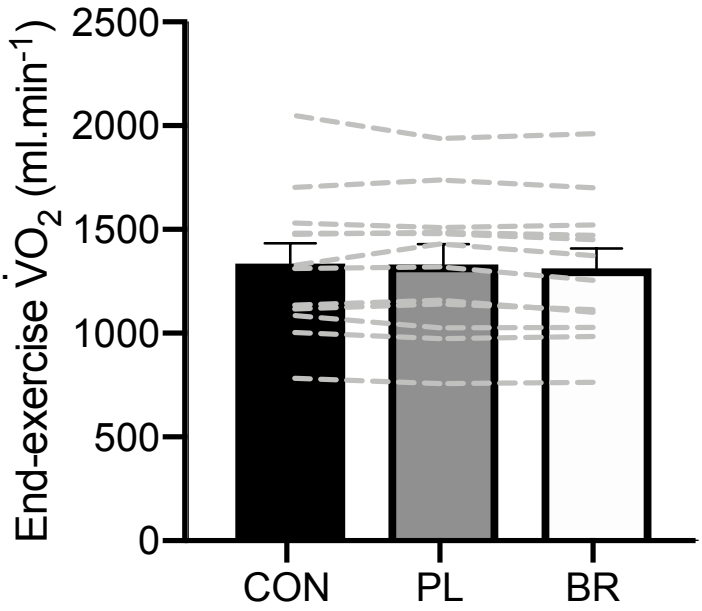

Figure 4. Mean ± SE and individual responses of pulmonary O_2 uptake averaged over 4 step transitions (panel A) following: 1) CON, water supplementation consumed before exercise; 2) PL, nitrate-depleted beetroot juice consumed before exercise; and 3) BR, nitrate-rich beetroot juice consumed before exercise.

Discussion

This study investigated the effect of NO_3^--depleted beetroot juice on NO bioavailability and pulmonary $\dot{V}O_2$ responses during moderate-intensity exercise compared to a water control condition in healthy humans, as well as the influence of the number of transitions on the pulmonary $\dot{V}O_2$ responses during moderate-intensity exercise following NO_3^--rich and NO_3^--depleted beetroot juice. The principal novel findings of the present study were that: 1) PL supplementation did not elevate NO bioavailability or influence the pulmonary $\dot{V}O_2$ responses compared to a water control condition (CON); and 2) NO_3^--rich beetroot juice (BR) lowered the O_2 cost of exercise compared to NO_3^--depleted beetroot juice (PL) when 4 step transitions were averaged but not with 1, 2, or 3 transitions. These findings indicate that despite NO_3^--depleted beetroot juice containing bioactive polyphenolic compounds (i.e. flavonoids, betalains, ascorbic acid; Shepherd et al., 2015), NO_3^--depleted beetroot juice is an appropriate placebo control for scientific investigations examining the influence of concentrated NO_3^-

-rich beetroot juice on physiological responses to exercise. In addition, these results are consistent with our hypotheses and indicate that the number of transitions may be important in detecting any influence of NO_3^- on the O_2 cost of submaximal exercise.

The effect of NO_3^--depleted beetroot juice supplementation on indices of NO bioavailability and pulmonary O_2 responses during moderate-intensity exercise

Short-term NO_3^--depleted beetroot juice supplementation (i.e. placebo) did not influence plasma [NO_3^-] and [NO_2^-] which is consistent with previous reports (Lansley et al., 2011). Importantly, there was no difference in plasma [NO_3^-] and [NO_2^-] between PL and CON, suggesting that concentrated NO_3^--depleted beetroot juice is not effective at altering indices of NO bioavailability despite beetroot juice containing phytochemical compounds (Georgiev et al., 2010; Ninfali & Angelino, 2013; Shepherd et al., 2015; Wootton-Beard & Ryan, 2012). However, consistent with previous studies, NO_3^--rich beetroot juice supplementation increased plasma [NO_3^-] and [NO_2^-] by ~1750% and ~420%, respectively, compared to CON and PL (Wylie et al., 2013a). Recent evidence suggests that more pronounced effects may occur following NO_3^- supplementation administered in vegetable juice form (i.e. beetroot juice) compared to a pharmaceutical form (i.e. NO_3^- salts) which could indicate an influence of polyphenols within the juice form (Flueck et al., 2016; Jonvik et al., 2016; McIlvenna et al., 2017; Thompson et al., 2018). For example, Thompson et al. (2018) reported that 4 weeks of sprint training combined with NO_3^--rich beetroot juice supplementation lowered systolic blood pressure by ~5% and increased exercise tolerance by ~71% whereas in contrast, 4 weeks of sprint training combined with NO_3^- salt supplementation only lowered systolic blood pressure by ~2% and increased exercise tolerance by ~42%. Although it is possible that physiological effects following NO_3^--rich beetroot juice supplementation may be partly to phytochemicals interacting synergistically with NO_3^- (Kanner et al., 2001; Simon et al., 1993), the data from the present study provide evidence that NO_3^--depleted beetroot juice does not contribute to the elevation of plasma [NO_3^-] and [NO_2^-] and thus is not likely to induce NO-mediated effects.

A possible explanation for the lack of effect of NO_3^--depleted beetroot juice supplementation on NO bioavailability could be due low phytochemical bioavailability (Clifford et al., 2015). To our knowledge, only two studies that have investigated polyphenol supplementation have measured plasma [NO_3^-] and/or [NO_2^-] and these found no influence of montmorency tart cherry powder (~256 $mg \cdot d^{-1}$ anthocyanins; Aboo Baker et al., 2019) or concentrate (74 mg cyanidin-3-glucoside and 179 mg gallic acid; Keane et al., 2016; 2018) on plasma [NO_3^-] and/or [NO_2^-]. However, the relative concentration and type of each phytochemical within beetroot juice could be important for any potential physiological effects to occur. In support of this interpretation, a meta-analysis concluded that ~500 mg of flavonoids was required to influence vascular function (Kay et al., 2012), which is an indirect measure of NO bioavailability given that improved vascular function is an NO-mediated process. However, beetroot juice contains drastically less, containing only ~4 mg of flavonoids per 100 ml (Shepherd et al., 2015). Therefore, although it is acknowledged that phytochemicals may impact physiological responses to exercise through NO-mediated mechanisms (Bowtell & Kelly, 2019), it is likely that NO_3^--depleted beetroot juice (i.e. placebo) and its type and concentration of bioactive constituents, do not independently contribute to NO bioavailability following NO_3^--rich beetroot juice supplementation.

The lack of effect of NO_3^--depleted beetroot juice supplementation on the pulmonary $\dot{V}O_2$ responses during moderate-intensity exercise is not surprising given that NO_3^--depleted beetroot juice supplementation did not elevate biomarkers of NO bioavailability. Indeed, the increase in plasma [NO_2^-] following NO_3^--rich beetroot juice supplementation is important for inducing subsequent favourable physiological and ergogenic effects (Dreissigacker et al., 2010; Porcelli et al., 2015; Wilkerson et al., 2012; Wylie et al., 2013a). Elevated NO bioavailability could induce NO-mediated effects such as reversible inhibition of O_2 binding on cytochrome C oxidase (Brown & Cooper, 1994) which could subsequently increase homogeneity of oxygenation in muscle fibres more distal to the capillaries (Hagen et al., 2003) and/or improve mitochondrial function by downregulating uncoupling protein content (Larsen et al., 2011; Li et al., 2006). In addition, NO may induce reversible S-nitrosylation to impact structure and

function of key contractile proteins (Aghdasi et al., 1997; Galler et al., 1997; Stoyanovsky et al., 1997).

The influence of the number of transitions on the pulmonary O_2 responses during moderate-intensity exercise following NO_3^--rich beetroot juice supplementation

We observed that BR supplementation significantly lowered the O_2 cost of exercise by ~2% compared to PL supplementation when averaged over 4 step transitions but not when averaged over 1, 2, or 3 step transitions. These results are consistent with earlier studies but are slightly less than the previously reported 3 to 5% reduction in $\dot{V}O_2$ (Bailey et al., 2009; Larsen et al., 2007; Lansley et al., 2011; Vanhatalo et al., 2010) which could be due to differences in cohort muscle fibre-type composition (Jones et al., 2016), fitness (Porcelli et al., 2015) and/or muscle redox status (Whitfield et al., 2016). Mechanistically, the lowering of the O_2 cost of exercise following NO_3^- ingestion may be underpinned by decreased mitochondrial uncoupling (Larsen et al., 2011), reduced ATP cost of force production (Bailey et al., 2010), and/or redox signalling (Whitfield et al., 2016). Interestingly, significance was only detected following the averaged response of 4 step transitions, which could be related to an improvement in the amount of error (i.e. improved CV%) with each additional transition. Together, our findings indicate that inherent noise from the pulmonary O_2 response may be a factor which obscures identifying any significant physiological effect following NO_3^- supplementation (Lamarra et al., 1987). Accordingly, it could be possible that previous investigations that employed few (Breese et al., 2013; Christensen et al., 2013; Kelly et al., 2014) and especially one transition (Bescós et al., 2011; Kocoloski et al., 2018; Wickham et al., 2019) who did not observe a significant effect on steady-state $\dot{V}O_2$ required at least four-step transitions to accurately characterize the response.

The $\dot{V}O_2$ kinetics during the step transition from unloaded to moderate-intensity exercise were not influenced by BR compared to PL which is consistent with previous studies (Bailey et al., 2009; Bescós et al., 2011; Breese et al., 2013; Ghiarone et al., 2017; Kelly et al., 2014). Moreover, increasing the averaged number of transitions from 1 to 4 had no effect on the influence of NO_3^- supplementation on $\dot{V}O_2$ τ_p. These results are in agreement with the “tipping

point" theory, which suggests that when O_2 delivery is sufficient (e.g. healthy individuals performing moderate-intensity exercise), increases to O_2 delivery (e.g. following NO_3^- supplementation) would not be expected to influence $\dot{V}O_2$ τ_p (Poole & Jones, 2012). In addition, given that NO_3^- supplementation improves blood flow and contractile function specifically in type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012) or exercise protocols which require a greater proportional contribution of type II muscle fibres (Bailey et al., 2015; Breese et al., 2013; Coggan et al., 2015) and that moderate-intensity exercise would be expected to recruit a relatively lower proportion of type II muscle fibres (Bottenelli et al., 2000), the lack of effect of NO_3^- supplementation on $\dot{V}O_2$ kinetics could be partly underpinned by the relatively smaller contribution of type II muscle fibres required when transitioning to low work rates (i.e. unloaded to moderate-intensity exercise).

Strengths and limitations

Although the results of this Experimental Chapter suggest that NO_3^--depleted beetroot juice does not influence NO bioavailability or physiological responses during exercise, the design of the study has an important limitation that should be addressed in future investigations. This Experimental Chapter is lacking a condition that administers NO_3^- in a pharmaceutical form, which would not contain other antioxidant constituents such as beetroot juice. The addition of this condition would provide further confidence in the interpretation of the results of this Experimental Chapter.

Conclusion

In conclusion, NO_3^--depleted beetroot juice supplementation did not significantly influence NO bioavailability, as reflected by plasma [NO_3^-] and [NO_2^-] or the pulmonary $\dot{V}O_2$ responses measured during moderate-intensity exercise when compared to a water control condition. In addition, short-term NO_3^--rich beetroot juice supplementation lowered the O_2 cost of moderate-intensity cycling exercise when averaged over four-step transitions but not 1, 2, or 3 step transitions. These data have important methodological and practical implications which suggest that NO_3^--depleted beetroot juice is an effective placebo for investigating the potential physiological effects following NO_3^- supplementation and also indicate that

numerous step transitions (i.e. 4) may be required to detect significant changes to the O_2 cost of exercise following NO_3^- supplementation.

Experimental Chapter 4 achieved the first aim of the thesis as results suggest that NO_3^--depleted beetroot juice does not have any impact on NO bioavailability or any $\dot{V}O_2$ responses, and that potential favourable effects following NO_3^--rich beetroot juice may be ascribed to the NO_3^- content in the juice. Therefore, all subsequent Experimental Chapters in the present thesis have administered NO_3^--rich beetroot juice to investigate the potential physiological responses during prolonged exercise following NO_3^- supplementation.

Beetroot juice ingestion *during* prolonged moderate-intensity exercise attenuates progressive rise in O_2 uptake

The second aim of the present thesis was to investigate the potential physiological effects of nitrate-rich beetroot juice before and *during* prolonged moderate-intensity exercise. Given that intramuscular metabolite concentrations are important for prolonged exercise and that the elevation of nitric oxide bioavailability is thought to be crucial in the efficacy of nitrate supplementation, Experimental Chapter 5 provided an additional dose of nitrate during the prolonged exercise bout and measured intramuscular muscle metabolic variables.

Introduction

Nitric oxide (NO) is recognized as a ubiquitous signaling molecule fundamental to regulating many physiological functions including vasodilation (Cosby et al., 2003), skeletal muscle contraction (Reid, 1998), mitochondrial respiration (Brown & Cooper, 1994), and glucose uptake (Balon & Nadler, 1997). In humans, NO bioavailability can be increased through exogenous consumption of inorganic nitrate (NO_3^-) which can be reduced to nitrite (NO_2^-) by bacterial NO_3^- reductases in the oral cavity and further reduced into NO and other reactive nitrogen species under appropriate physiological conditions (Lundberg et al., 2008). In addition to reducing resting blood pressure (Webb et al., 2008), dietary NO_3^- supplementation has been reported to reduce the O_2 cost of exercise (Bailey et al., 2009; Lundberg et al., 2008; Vanhatalo et al., 2010) and to enhance skeletal muscle contractile function (Haider & Folland, 2014; Hernánadez et al., 2012; Whitfield et al., 2017), effects which might be expected to result in improved exercise performance.

Several studies indicate that NO_3^- supplementation can enhance short duration (<30 min) exercise performance (Bailey et al., 2009; 2010; Cermak et al., 2012, Lansley et al., 2011; Porcelli et al., 2016). However, the efficacy of NO_3^- supplementation in improving longer duration exercise performance is less clear (Betteridge et al., 2016; Cermak et al., 2012; Coggan et al., 2015; Lane et al., 2014; Wilkerson et al., 2012). This disparity in the efficacy of NO_3^-

supplementation in shorter vs. longer endurance exercise may be related to the metabolism of NO_3^- and NO_2^- during exercise. The pre-exercise elevation in plasma [NO_2^-] following NO_3^- supplementation has been shown to be associated with the magnitude of performance enhancement during long duration cycling (Wilkerson et al., 2012). However, following NO_3^- supplementation, plasma [NO_2^-] declines over the course of short duration moderate- and severe-intensity exercise (Kelly et al., 2014; Shannon et al., 2017), as well as during repeated sprints (Thompson et al., 2015; 2016; Wylie et al., 2013b). Indeed, this decline in plasma [NO_2^-] with time during exercise, which may reflect the use of nitrite as a 'substrate' for NO production, is correlated with enhanced high-intensity exercise performance following NO_3^- supplementation (Thompson et al., 2015; Wylie et al., 2013b). It is possible, therefore, that long duration endurance exercise results in a progressive, and perhaps substantial, depletion of plasma [NO_2^-] such that the potential benefits of NO_3^- supplementation on performance later in exercise are no longer elicited (Christensen et al., 2013; Wilkerson et al., 2012). Ingesting NO_3^- *during* longer duration exercise might maintain plasma [NO_2^-] at an elevated level and provide the potential for performance to be improved.

During prolonged, constant-work-rate exercise, an upward drift in pulmonary O_2 uptake ($\dot{V}O_2$) is typically observed (Brueckner et al., 1991; Hopker et al., 2017). The O_2 cost of such exercise may increase with time due to a shift in substrate utilization towards fat oxidation, a progressive recruitment of type II muscle fibers, or a decline in skeletal muscle mitochondrial and/or contractile efficiency (Jones et al., 2011). Muscle glycogen depletion during prolonged exercise may also contribute to the loss of efficiency over time (Ørtenblad et al., 2011). Dietary NO_3^- supplementation has the potential to lower O_2 demand during prolonged exercise (Bailey et al., 2009; Jones, 2014). Specifically, NO_3^- supplementation has been reported to enhance the mitochondrial P/O ratio (Larsen et al., 2011; cf. Whitfield et al., 2017) and to reduce the ATP cost of muscle force production (Bailey et al., 2010). In animal studies, NO_3^- supplementation has been reported to improve intracellular calcium (Ca^{2+}) handling and increase force production at low frequencies of contraction in type II muscle fibers (Hernández et al., 2012) and to lead to preferential blood flow (and O_2) distribution to type II muscle (Ferguson et al., 2013; 2015). Given that: 1) fatigue development and the progressive increase

in $\dot{V}O_2$ during prolonged exercise may be related, at least in part, to the recruitment of type II muscle fibers (Krustrup et al., 2004); and that 2) NO_3^- supplementation positively impacts muscles comprised predominantly of type II fibers (Jones et al., 2016); it is possible that ingesting NO_3^- during as well as before such exercise may be better than pre-exercise NO_3^- ingestion alone in limiting fatigue development, minimizing $\dot{V}O_2$ and enhancing performance.

Another mechanism by which NO_3^- supplementation might potentially alter the O_2 cost of exercise is via effects on carbohydrate metabolism. NO has been shown to play an important role in regulating skeletal muscle glucose uptake (Balon & Nadler, 1997). Wylie et al. (2013b) reported lower blood [glucose] during high-intensity intermittent exercise following NO_3^- supplementation, which might suggest enhanced skeletal muscle glucose uptake; however, this was not confirmed during longer duration moderate-intensity exercise (Betteridge et al., 2016). It therefore remains unclear whether dietary NO_3^- supplementation before, and especially *during*, prolonged exercise can affect carbohydrate metabolism or muscle glycogen utilization. A lower metabolic cost of exercise as reflected by a lower $\dot{V}O_2$ and/or increased muscle glucose uptake from the blood might reduce muscle glycogen utilization during prolonged exercise and enhance endurance performance.

The purpose of the present study was, therefore, to investigate whether ingestion of NO_3^--rich beetroot juice (BR) before, and also *during*, 2 h of moderate-intensity cycle exercise influences physiological responses and improves performance in a subsequent target-work (100 kJ) cycling performance test relative to a placebo condition. We hypothesized that BR supplementation before and during 2-h moderate-intensity exercise would: 1) preserve an elevated plasma [NO_2^-]; 2) attenuate the expected progressive increase in $\dot{V}O_2$ with time; 3) reduce muscle glycogen depletion; and, therefore, 4) improve TT performance.

Methods

Subjects

Twelve recreationally-active males (mean ± SD: age 21 ± 1 years, body mass 78

± 11 kg, height 1.77 ± 0.07 m, $\dot{V}O_{2peak}$, 45 ± 4 $mL \cdot kg^{-1}min^{-1}$) volunteered to participate in this study, nine of whom volunteered for invasive measurements (muscle biopsies and blood sampling). The protocol, risks, and benefits of participating were explained prior to obtaining written informed consent. This study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki. A priori power analysis showed that a sample size of nine would provide a power = 0.80, alpha = 0.05, an effect size = 0.50.

Experimental overview

Subjects reported to the laboratory on 5 separate occasions over a 5-week period. On the first visit, subjects completed a ramp incremental exercise test for the determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). During the second visit, subjects were familiarized to the exercise testing procedures, including completion of a moderate-intensity exercise bout (at a work rate of 80% of the GET) for 30 min before completing a target-work (100 kJ) cycling performance test designed to simulate a 4-km TT.

For the duration of the study, subjects were asked to avoid consuming NO_3^--rich foods such as spinach, rocket (arugula), kale, and beetroot, and to refrain from taking any other dietary supplements or using antibacterial mouthwash as the latter affects the commensal bacteria in the oral cavity, resulting in the inhibition of NO_3^- reduction into NO_2^- (Govoni et al., 2008). In a double-blind, randomized, crossover design, subjects were assigned to receive dietary supplementation for 3 days. On day 3 of each supplementation period (See Supplementation), subjects reported to the laboratory to complete the experimental protocol. Experimental visits were performed at the same time of day (± 2-h). Subjects recorded their activity and diet during the 24-h prior to the first experimental visit and were asked to repeat these for subsequent visits. Subjects were also instructed to arrive at the laboratory following a 10-h overnight fast, having avoided strenuous exercise and alcohol in the 24-h preceding, and caffeine in the 8-h preceding, each experimental visit. The subjects were provided with a standardized breakfast consisting of 2 porridge oats sachets (Quaker Oats Ltd, Leicester, UK; containing 54 g of oats, 200 kcal, 4.2 g fat, 31.8 g carbohydrate,

5.6 g fibre, 6.0 g protein) mixed with 180 mL of water, 1-h prior to exercising.

Supplementation

Subjects were randomly assigned to three 3-day supplementation periods in which they consumed 2 x 70 mL doses per day of either NO_3^--rich BR: (~6.2 mmol NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK) or a NO_3^--depleted placebo (PL: ~0.04 mmol NO_3^- per 70 mL; Beet it, James White Drinks Ltd., Ipswich, UK) separated by a 5-day wash-out period. The three supplementation conditions were: 1) BR supplementation both before and at 1-h into exercise (BR+BR); 2) BR supplementation before and PL at 1-h into exercise (BR+PL); and 3) PL before and at 1-h into exercise (PL+PL). Each 70 mL beverage contained 72 kcal energy and 15.4 g of carbohydrate. On the first two days of each supplementation period, subjects consumed one 70 mL beverage in the morning and one in the evening, whereas on the experimental day, subjects consumed 2 x 70 mL of their allocated beverage in the morning 2.5-h prior to the exercise and 1 x 70 mL of their allocated beverage at 1-h into exercise. This 3-day protocol was chosen to simulate the approach to supplementation that an athlete might take prior to competition with the time frame for supplement ingestion on the final morning selected because peak plasma [NO_2^-] occurs ~2-3-h following NO_3^- intake (Webb et al., 2008; Wylie et al., 2013a).

Exercise procedures

All exercise tests were performed on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). On the first visit, subjects completed a ramp incremental test, involving 3 min of baseline cycling at 20 W, after which the work rate was increased by 30 W/min until task failure. Task failure was recorded once the pedal rate fell by >10 rpm below the target cadence. The self-selected cadence (70-90 rpm) and seat height and handle bar configuration were recorded and reproduced on subsequent visits. Breath-by-breath pulmonary gas exchange data were collected continuously during the incremental test and averaged over 10-s periods. $\dot{V}O_{2peak}$ and GET were determined as previously described (Vanhatalo et al., 2010). Heart rate (HR) was measured during all tests using short-range radio telemetry (Polar S610, Polar Electro, Kempele, Finland).

During the experimental visits, subjects performed baseline cycling at 20 W for 3 min. Following this, subjects completed 2-h of cycling at 80% GET (91 ± 24 W) at their self-selected cadence. A 1-min rest period followed the end of the 2-h bout during which a muscle biopsy was obtained (see *Muscle Biopsy*). The 100 kJ TT commenced immediately after the 1-min period. Subjects were provided with a 5-s countdown prior to the commencement of all cycling trials. The resistance on the pedals during the TT was set for each individual using the linear mode of the Lode ergometer so that the subject would attain the power output associated with GET plus 65% of the difference between GET and peak power output (65%Δ) on reaching a cadence of 90 rpm (Lansley et al., 2011). Subjects were deprived of visual performance cues and did not receive notification on elapsed time but they received consistent verbal encouragement for each TT and were informed when 75, 50, 25 and 10 kJ of work remained to be completed. Pulmonary gas exchange was measured for discrete 6-min time periods (from 0-6 min, 27-33 min, 60-66 min, 87-93 min, and 114-120 min) during the 2-h exercise bout (the first 2 min of each period was not used in analysis), and continuously during the TT.

Measurements

Muscle biopsy

Skeletal muscle samples were obtained from two incisions made in the m. *vastus lateralis* under local anesthesia (1% lidocaine) using the percutaneous Bergström needle biopsy technique with suction (Bergstrom, 1962). Muscle samples were obtained at rest (10 min prior to the start of the 2-h moderate-intensity exercise bout), within 15 s of the completion of the 2-h exercise bout and within 15 s of the completion of the TT. Muscle samples were immediately snap-frozen in liquid nitrogen before being stored at -80°C for subsequent analysis.

Muscle metabolites

Muscle samples were freeze-dried and dissected to remove visible fat, blood, and connective tissue using forceps. 200 µL of 3 M perchloric acid was added to ~2 mg dry weight (DW) of muscle tissue. Samples were incubated on ice for 30 min, then centrifuged for 3 min at 4000 rpm. 170 µL of supernatant was transferred

over to a fresh microcentrifuge tube, and 255 µL of cooled 2 M potassium hydrogen carbonate ($KHCO_3$) was added. This was centrifuged, and the supernatant was analyzed for [PCr], [ATP], and [lactate] by fluorometric assays as described by Black et al. (2017).

Muscle glycogen

~ 1 mg DW muscle tissue was hydrolysed in 500 µL of 1 M hydrochloric acid at 100 °C for 3-h to release glycosyl units, and immediately measured using an automated glucose analyzer (YSI 2900 Biochemistry Analyzer, Yellow Springs Instruments, Yellow Springs, OH). The precision of this method of analysis within this physiological range (0.05 to 0.55 mmol/L) was checked by measuring the glucose concentration across a range of solutions made up using glucose diluted in hydrochloric acid; the measured vs. expected values lay on the line of identity with an R^2 of 0.99.

Blood analysis

Venous blood was sampled at baseline, 30, 60, 90 and 120 min during the 2-h moderate-intensity exercise bout, and immediately following the completion of the TT. All blood samples were obtained from a cannula (Insyte-W™ Becton-Dickinson, Madrid, Spain) that was inserted in the subject's antecubital vein, and were drawn into 6 mL lithium-heparin vacutainers (Becton-Dickinson, New Jersey, USA). For blood [lactate] and [glucose] analysis, 200 µL of blood was immediately hemolyzed into 200 µL of cold Triton X-100 buffer solution (Triton X-100, Amresco, Salon, OH) and then measured using an automated glucose and lactate analyzer (YSI 2300, Yellow Springs Instruments, Yellow Springs, OH). The remaining whole blood samples were centrifuged within 2 min of collection at 4000 rpm and 4°C for 10 min and then the plasma was immediately extracted and frozen at -80°C. Before the analysis of plasma [NO_3^-] and [NO_2^-], samples were deproteinized using cold ethanol precipitation. Specifically, thawed samples were centrifuged at 14000 g for 10 min, before 200 µL of sample was added to 400 µL of chilled ethanol and incubated on ice for 30 min. After further centrifugation at 14000 g for 10 min, the supernatant was removed for the subsequent determination of [NO_3^-] and [NO_2^-] via gas phase chemiluminescence as described by Wylie et al. (2013).

Statistical Analysis

A two-way (condition x time) repeated measures analysis of variance (ANOVA) was used to analyze differences in physiological and performance responses during the 2-h moderate-intensity exercise bout and the TT. Significant main and interaction effects were further explored using Fisher's Least Significant Difference test. In addition, one-way repeated measures ANOVAs were used to determine physiological and performance differences in the mean and change values from pre- to post- 2h moderate exercise, and post-TT. The relationship between $\dot{V}O_2$ and muscle [glycogen] was explored using the Pearson product moment correlation coefficient. Statistical significance was accepted at $P \leq 0.05$. Results are presented as mean ± SD unless otherwise stated.

Results

All subjects reported consuming all servings of each supplement at the correct times and confirmed that they had maintained their exercise and dietary habits prior to each testing visit. There were no reports of gastrointestinal distress or discomfort following the ingestion of BR or PL either before or during exercise.

Plasma [NO_3^-] and [NO_2^-]

There was an interaction effect (condition x time) ($P<0.01$), main effect of time ($P<0.01$), and main effect of condition ($P<0.01$) for plasma [NO_3^-] (Fig. 1A). At baseline, plasma [NO_3^-] was significantly elevated in BR+BR (315 ± 57 μM; $P<0.01$) and BR+PL (302 ± 88 μM; $P<0.01$) compared to PL+PL (16 ± 7 μM). Plasma [NO_3^-] in BR+BR and BR+PL were elevated at all time points compared to PL+PL. In PL+PL, plasma [NO_3^-] was unchanged throughout exercise. In BR+PL, plasma [NO_3^-] was unchanged from baseline to 90 min ($P>0.05$). However, compared to baseline, plasma [NO_3^-] in BR+PL decreased by ~16% at 120 min (254 ± 56 μM, $P<0.05$). In BR+BR, plasma [NO_3^-] was unchanged from baseline to 60 min (317 ± 52 μM*; P*>0.05) but then increased by ~41% at 90 min (448 ± 51 μM, $P<0.0001$) and remained elevated until 120 min (463 ± 70 μM, $P>0.05$). Plasma [NO_3^-] was significantly elevated at 90 min, 120 min, and post-TT in BR+BR compared to BR+PL ($P<0.01$).

There was an interaction effect (condition x time) (P<0.05) and main effect of condition (P<0.01) for plasma [NO_2^-] (Fig. 1B). At baseline, plasma [NO_2^-] was significantly greater in BR+BR (482 ± 211 nM; P<0.01) and BR+PL (484 ± 188 nM; P<0.01) compared to PL+PL (203 ± 63 nM), with no significant difference between BR+BR and BR+PL. Plasma [NO_2^-] was unchanged throughout exercise in PL+PL. In BR+PL, plasma [NO_2^-] tended to decrease by ~17% from baseline to 120 min (P=0.07). In contrast, in BR+BR, plasma [NO_2^-] increased by ~8% from baseline to 120 min. Plasma [NO_2^-] tended to be elevated at 90 min in BR+BR (491 ± 157 nM) compared to BR+PL (405 ± 188 nM, P=0.09), and was significantly elevated at 120 min in BR+BR (519 ± 152 nM) compared to BR+PL (400 ± 158 nM, P<0.05). Plasma [NO_2^-] fell significantly (by ~35%) from 120 min to post-TT in BR+BR (P<0.001), BR+PL (P<0.01) and PL+PL (P<0.05).

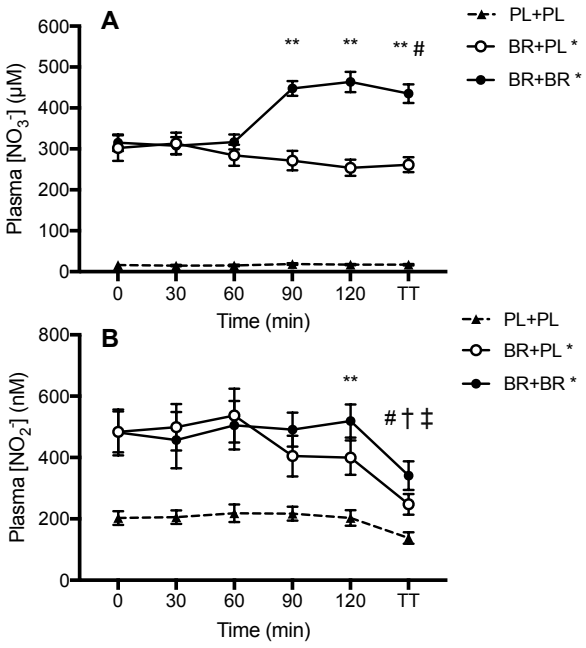

Figure 1. Mean ± SE plasma nitrate (panel A) and nitrite (panel B) concentrations over 120 min of moderate-intensity cycle exercise and a subsequent 4-km TT following: PL+PL: placebo consumed before and during exercise (solid triangle and dotted line); BR+PL: NO_3^- -rich beetroot juice consumed before and placebo consumed during exercise (open circle and solid line); and BR+BR: NO_3^- -rich beetroot juice before and during exercise (solid circle and solid line), (n = 9). * = significantly different from PL+PL, ** = BR+BR significantly different from BR+PL, # = significantly different from 120 min to end of TT in BR+BR, † = significantly different from 60 min to end of TT in BR+PL, ‡ = significantly different from 90 min to TT in PL+PL.

Pulmonary gas exchange during prolonged moderate-intensity exercise

$\dot{V}O_2$ measured at baseline was not different between conditions (P>0.05). There was a main effect of time (P<0.01) and an interaction effect (condition x time) for $\dot{V}O_2$ (P<0.05; Fig. 2A). *Post hoc* analyses revealed that the change in $\dot{V}O_2$ from 30 min to 120 min (P<0.05) was lower in BR+BR compared to PL+PL (P<0.05) and tended to be lower compared to BR+PL (P=0.07, Fig. 2B); there was no difference between BR+PL and PL+PL (P>0.05). At 120 min, $\dot{V}O_2$ was lower in BR+BR compared to PL+PL (P<0.01), and tended to be lower than BR+PL (P=0.08); (P>0.05). There was a main effect of time on RER (P<0.01), with RER declining from ~0.93 at 30 min to ~0.89 at 120 min, but no effect of condition and no interaction (P>0.05). Mean RER was not significantly different between conditions at 30 min (PL+PL: 0.93 ± 0.04 vs. BR+PL: 0.92 ± 0.04 vs. BR+BR: 0.93 ± 0.03), 60 min (PL+PL: 0.90 ± 0.03 vs. BR+PL: 0.89 ± 0.02 vs. BR+BR: 0.89 ± 0.03), 90 min (PL+PL: 0.91 ± 0.04 vs. BR+PL: 0.90 ± 0.06 vs. BR+BR: 0.91 ± 0.04) or 120 min (PL+PL: 0.90 ± 0.04 vs. BR+PL: 0.89 ± 0.03 vs. BR+BR: 0.90 ± 0.04). Similarly, there was a main effect of time (P<0.05) but no effect of condition or interaction for HR or minute ventilation. There was a main effect of time (P<0.05) but no effect of condition or interaction for blood [glucose] (P>0.05; Table 1). There was no effect of time or condition and no interaction effect for blood [lactate] (P>0.05; Table 1).

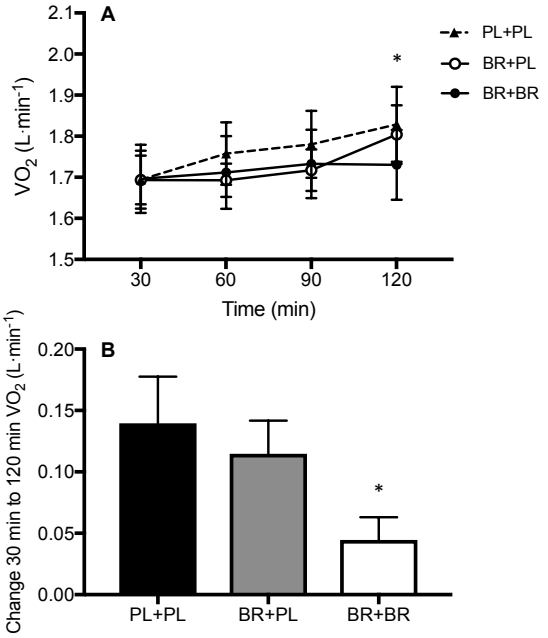

Figure 2. Mean ± SE O_2 uptake over 120 min of moderate-intensity cycle exercise (panel A) and the change in O_2 uptake from 30 min to 120 min (panel B) following PL+PL, PL+BR and BR+BR. * = significantly different in BR+BR compared to PL+PL.

Muscle metabolic variables

There was a main effect of time (P<0.01) and a trend for an interaction effect (P=0.06) on muscle [glycogen] measured at baseline, 120 min, and post-TT (Fig. 3). At baseline, there was no significant difference in muscle [glycogen] between conditions (BR+BR: 383 ± 105 vs. BR+PL: 383 ± 144 vs. PL+PL: 412 ± 121 mmol·kg^{-1} DW, P>0.05). *Post hoc* tests revealed that in all conditions, muscle [glycogen] was significantly lower at 120 min compared to resting baseline (P<0.01), and at post-TT compared to 120 min (P<0.01). At 120 min, muscle [glycogen] tended to be greater in BR+BR (283 ± 103 mmol·kg^{-1} DW) compared to BR+PL (215 ± 102 mmol·kg^{-1} DW; P=0.08) and PL+PL (226 ± 90 mmol·kg^{-1} DW; P=0.08) There was no difference between conditions at post-TT (BR+BR: 161 ± 79 vs. BR+PL: 127 ± 65 vs. PL+PL: 132 ± 69 mmol·kg^{-1} DW, P>0.05). The absolute muscle [glycogen] at 120 min was inversely correlated with the absolute $\dot{V}O_2$ at 120 min (r = -0.71; P<0.01). There was a tendency for a main effect of condition in the change in muscle [glycogen] from baseline to 120 min (P=0.09), where the ~28% decline in BR+BR was significantly less compared to the ~44% decline in PL+PL (P<0.05) and tended to be less than the ~44% decline in BR+PL

(*P*=0.07). The change in muscle [glycogen] from 120 min to post-TT were not significantly different between conditions (*P*>0.05).

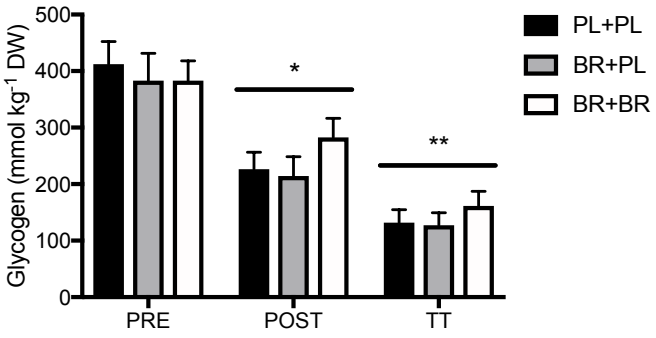

Figure 3. Mean ± SE muscle [glycogen] at rest (PRE), after 120 min moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n = 9). * = significantly different from PRE to POST, ** = significantly different from POST to TT. There were no significant differences between the three conditions at any discrete time point but the change in muscle [glycogen] was significantly less in BR+BR compared to PL+PL (*P*<0.05; see text for details).

There was a main effect of time on muscle [PCr] (*P*<0.01; Fig 4A.), [ATP] (*P*<0.01; Fig. 4B) and [lactate] (*P*<0.01; Fig. 4C). Baseline muscle [PCr] and [ATP] were not different between conditions (*P*>0.05). There was no effect of condition and no interaction for muscle [PCr] or [ATP] (*P*>0.05). *Post hoc* tests revealed that in all conditions, muscle [PCr] declined from baseline to 120 min (*P*<0.05), and from 120 min to post-TT (*P*<0.01). The mean [PCr] tended to be greater in BR+BR compared to PL+PL (*P*=0.08) but there was no difference between BR+BR and BR+PL or between BR+PL and PL+PL (*P*>0.05). Muscle [ATP] declined significantly from 120 min to post-TT in BR+BR (*P*<0.01) and BR+PL (*P*<0.05) but not PL+PL. Muscle [lactate] was not significantly different between conditions at 120 min but, compared to 120 min, muscle [lactate] increased significantly post-TT in all conditions (*P*<0.01).

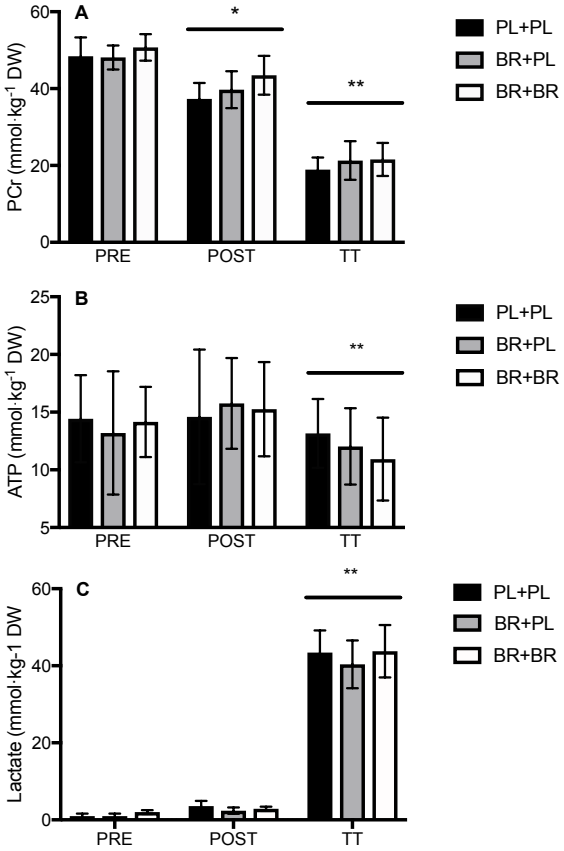

Figure 4. Mean ± SE muscle [PCr] (panel A), [ATP] (panel b), and [lactate] (panel C) at rest (PRE), after 120 min moderate-intensity exercise (POST), and after the 4-km time trial (TT), (n = 9). * = significantly different from PRE to POST, ** = significantly different from POST to TT.

TT performance

TT completion time, mean $\dot{V}O_2$ and mean power output during the TT were not significantly different between conditions (all *P*>0.05, Fig. 5). Similarly, maximal HR, blood [lactate] and blood [glucose] were not different between conditions (*P*>0.05; Table 1).

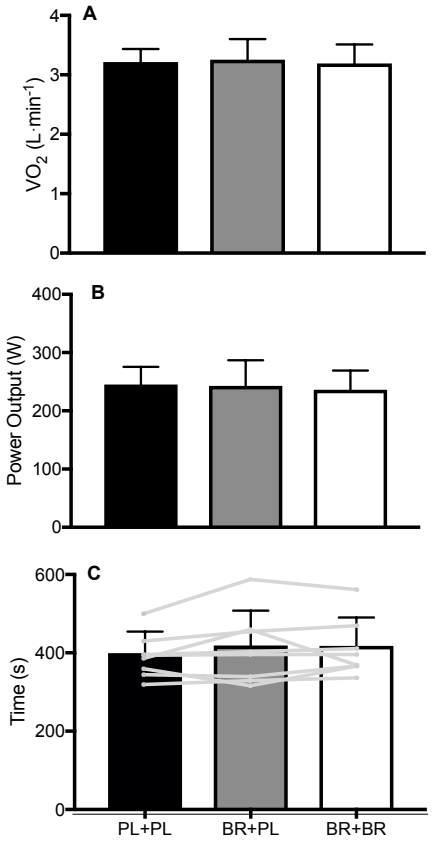

Figure 5. Mean ± SE O_2 uptake (panel A), power output (panel B), and completion time (panel C) over the 4-km time trial in PL+PL (black bars), BR+PL (grey bars) and BR+BR (white bars). Completion times for individual subjects shown in grey lines.

Discussion

This is the first study to investigate the effect of BR ingestion *during* exercise, in addition to pre-exercise, on the physiological responses to prolonged moderate-intensity exercise, and subsequent TT performance. The major novel findings of this study were that, compared to pre-exercise BR supplementation alone, a 'top-up' dose of BR consumed during exercise: 1) maintained the elevation of plasma [NO_2^-]; 2) better maintained the lowered O_2 cost of exercise; 3) tended to attenuate the fall in muscle [glycogen] over 2-h of moderate-intensity cycling; but, 4) did not alter simulated 4-km TT performance. Although TT performance was not significantly improved, our findings indicate that the ingestion of BR during prolonged exercise, in addition to short-term BR supplementation, may attenuate the rise in $\dot{V}O_2$ that typically develops during such exercise.

Plasma [NO_3^-] and [NO_2^-] during prolonged moderate-intensity exercise

It is well established that pre-exercise BR supplementation elevates resting plasma [NO_3^-] and [NO_2^-] (Bailey et al., 2009; Kelly et al., 2014; Vanhatalo et al., 2010), and the results of the present study were consistent with these previous reports. After reaching peak values at ~2-3 h following ingestion, plasma [NO_2^-] then declines with time (Webb et al., 2008; Wylie et al., 2013) as well as during exercise (Kelly et al., 2014; Thompson et al., 2015). Assuming that plasma [NO_2^-] reflects the potential for O_2-independent NO synthesis in the vasculature and skeletal muscle (Gladwin et al., 2000; Webb et al., 2008), a decline in plasma [NO_2^-] over time and during exercise may impact on the efficacy of BR supplementation in long-duration exercise bouts. Changes in plasma [NO_2^-] during exercise may reflect the utilization of NO_2^- to produce NO, conversion of NO_2^- to NO_3^- or other reactive nitrogen species, or transport to other body compartments including skeletal muscle (Piknova et al., 2015). In the present study, when BR was only consumed pre-exercise (i.e., in the BR+PL condition), both plasma [NO_3^-] (by 16%; $P<0.05$) and [NO_2^-] (by 17%; $P=0.07$) declined from baseline to 120 min. However, when BR was also consumed at 60 min into exercise (i.e. in the BR+BR condition), plasma [NO_3^-] was increased above baseline by 41% at 90 min and 120 min and plasma [NO_2^-] was increased above baseline by 8% at 120 min (Fig. 1). Plasma [NO_2^-] was therefore significantly greater at 120 min in BR+BR compared to BR+PL. These results indicate that, following pre-exercise BR supplementation, prolonged moderate-intensity exercise can lead to a substantial reduction in plasma [NO_3^-] and [NO_2^-], but that this decline can be negated by BR ingestion during exercise. The results of the present study demonstrate, for the first time, that BR ingestion during exercise can lead to relatively rapid changes in plasma [NO_3^-] and [NO_2^-]. The pharmacodynamics and pharmacokinetics of plasma [NO_3^-] and [NO_2^-] following dietary NO_3^- ingestion have been described at rest (Webb et al., 2008; Wylie et al., 2013a) but not during exercise, and further research is warranted to determine whether, and to what extent, the NO_3^- - NO_2^- - NO pathway is impacted by exercise and its sequelae (including, for example, changes in metabolic rate, core and oral temperature, distribution of cardiac output, and salivary flow rate).

Influence of BR on metabolic responses during prolonged moderate-intensity

exercise

In the present study, $\dot{V}O_2$ was not significantly different between conditions until 120 min of exercise, at which point it was lower in BR+BR compared to BR+PL and PL+PL. The increase in $\dot{V}O_2$ as exercise progressed in BR+PL and PL+PL was therefore attenuated in BR+BR (Fig. 2). An increasing O_2 cost of maintaining the same work rate during long-duration exercise may be related to an increased O_2 cost of mitochondrial ATP production and/or an increased ATP cost of force production and could reflect changes over time in substrate utilization, mitochondrial function and motor unit recruitment (Jones et al., 2011).

Dietary NO_3^- supplementation has been reported to reduce the O_2 cost of exercise in many (Bailey et al., 2009; 2010; Lansley et al., 2011; Larsen et al., 2007; 2011; Vanhatalo et al., 2010; Whitfield et al., 2016), though not all (Betteridge et al., 2016; Thompson et al., 2015) studies, but the mechanistic basis for this effect is not fully resolved. Larsen et al. (2011) reported that $NaNO_3$ supplementation enhanced mitochondrial P/O ratio *in vitro* and found that this was significantly correlated with the reduction in the O_2 cost of cycling *in vivo*. In contrast, Whitfield et al. (2016) reported that, while BR reduced the O_2 cost of exercise, it did not alter indices of mitochondrial efficiency. Another explanation for a lower O_2 cost of exercise following NO_3^- supplementation is a reduced ATP cost of muscle contraction. Consistent with this, it has been reported, using ^{31}P magnetic resonance spectroscopy, that muscle PCr depletion is reduced during exercise following BR supplementation (Bailey et al., 2010; Fulford et al., 2013). In the present study, muscle [PCr] determined from biopsy samples tended to be higher at 120 min of moderate-intensity exercise in BR+BR compared to PL+PL (P=0.08). Given that the depletion of PCr during exercise reflects the energy cost of contraction (Jubrias et al., 2008), these results suggest that BR supplementation may have reduced the metabolic cost of force production. For the same mitochondrial P/O, a lower ATP requirement at the same power output would dictate a lower $\dot{V}O_2$ (Wilson, 2017).

It has been reported in rodents (Hernández et al., 2012; Ivarsson et al., 2017) and in humans (Coggan et al., 2015; Haider & Folland, 2014; Whitfield et al., 2017), that muscle contractile force is increased following NO_3^- supplementation.

However, the mechanism responsible for this effect remains to be elucidated given that modifications to key contractile proteins related to intracellular Ca^{2+} handling have been observed in rodents (Hernández et al., 2012) but not humans (Whitfield et al., 2017). Whitfield et al. (2016) reported an increased emission of hydrogen peroxide following BR supplementation, suggesting a potential role for redox signaling in augmenting contractile efficiency (Ferreira & Reid, 2008). Moreover, at least in rodents, BR supplementation preferentially increases blood flow to (Ferguson et al., 2013), and increases microvascular O_2 pressure surrounding (Ferguson et al., 2015), type II muscle fibers, which could contribute to enhanced contractile function. It is possible that, collectively, these effects lower the O_2 cost of long-duration exercise by reducing or delaying the recruitment of motor units that are higher in the recruitment hierarchy and that may be less efficient (Barstow et al., 1996; Jones et al., 2011).

In the present study, we found that muscle glycogen declined by ~28% over 120 min of exercise in BR+BR, compared to ~44% decline in both BR+PL and PL+PL (Fig. 3). This tendency for muscle glycogen sparing could be reflective of a reduction in overall metabolic demand (from mitochondrial and/or contractile efficiency improvements), and therefore a lower absolute requirement for carbohydrate oxidation. This is supported by the existence of a significant negative correlation between the absolute $\dot{V}O_2$ and muscle [glycogen] measured at 120 min of exercise. It has been reported that muscle glycogen content is positively correlated with sarcoplasmic reticulum Ca^{2+} release rate, which may affect skeletal muscle contractile function (Ørtenblad et al., 2011). The tendency for muscle glycogen sparing in the BR+BR condition of the present study suggests a possible new mechanism by which dietary NO_3^- might enhance efficiency during long-duration exercise, with implications for exercise performance in such events, and is worthy of further investigation.

There was no difference in RER or blood [glucose] between conditions in the present study. In some previous studies, RER has been observed to be slightly (Bailey et al., 2010; Larsen et al., 2011) or significantly (Wylie et al., 2013a) higher following NO_3^- compared to PL supplementation, although most studies have not found significant differences in RER (Bailey et al., 2009; Betteridge et al., 2016;

Christensen et al., 2013; Vanhatalo et al., 2010; Whitfield et al., 2016). Wylie et al. (2013b) reported a lower blood [glucose] during high-intensity intermittent exercise following BR compared to PL supplementation and suggested that this may be due to an increased skeletal muscle glucose uptake. It is possible that this effect is intensity-dependent given that other studies have reported no effect of BR on glucose handling during moderate-intensity exercise (Betteridge et al., 2016; Christensen et al., 2013). Given that we did not observe differences between conditions in blood [glucose] or RER, the sparing of muscle glycogen in BR+BR would appear to be related to a reduced overall muscle metabolic demand as reflected in the lower O_2 cost of exercise. Alternatively, the tendency for muscle [PCr] to be somewhat better maintained during exercise in BR+BR compared to PL+PL, which is consistent with the lower $\dot{V}O_2$ in BR+BR (Bailey et al., 2010), indicates that muscle energy charge may have been higher when BR was ingested such that the stimulation of glycogenolysis was reduced (Hargreaves & Richter, 1988). In contrast to our findings, Betteridge et al. (2016) reported no effect of pre-exercise BR supplementation on muscle [glycogen] (or $\dot{V}O_2$) during 60 min of moderate-intensity cycling. The reason for this difference is unclear but, in addition to the longer exercise duration and the inclusion of BR ingestion *during* as well as pre-exercise, our subjects consumed 12.4 mmol NO_3^- per day for 3 days whereas the subjects in the study of Betteridge et al. (2016) consumed an acute 8 mmol dose of NO_3^- 2.5 hours pre-exercise. The dose and duration of NO_3^- supplementation is one factor that is likely to influence efficacy (Jones, 2014) since it may influence NO_3^- and NO_2^- storage in skeletal muscle as well as blood (Nyakayiru et al., 2017; Piknova et al., 2015; Wylie et al., 2016). Recent studies indicate that rat (Piknova et al., 2015) and human (Nyakayiru et al., 2017) skeletal muscle has high [NO_3^-] relative to the blood, that the muscle NO_3^- store decreases substantially during exercise in rats (Piknova et al., 2016) and that muscle [NO_3^-] can be modulated by dietary NO_3^- content (Gilliard et al., 2018; Nyakayiru et al., 2017).

Influence of BR on metabolic responses and performance during TT exercise

Plasma [NO_2^-] declined markedly during the TT (Fig. 1B). This greater rate of decline in plasma [NO_2^-] from 120 min to post-TT is in contrast to the more gradual decline in plasma [NO_2^-] observed from baseline to 120 min in the BR+PL

condition, which may suggest an exercise-intensity dependency of plasma [NO_2^-] dynamics. Indeed, previous research has reported significant reductions in plasma [NO_2^-] following high-intensity exercise of shorter duration (Kelly et al., 2014; Shannon et al., 2017; Thompson et al., 2015; Wylie et al., 2013b). It is possible that the greater degree of hypoxia and acidosis that would be expected to develop in skeletal muscle during high-intensity exercise, such as TT, compared to moderate-intensity exercise, facilitates or dictates a greater reduction of NO_2^- to NO (Modin et al., 2001). Moreover, a greater recruitment of type II muscle fibers, which have a lower microvascular O_2 pressure compared to type I fibers (Ferguson et al., 2015), during higher intensity exercise may also result in a greater reduction of NO_2^- to NO.

It is perhaps surprising that, despite evidence that the metabolic cost of the initial long-duration exercise bout was reduced in BR+BR (i.e. lower end-exercise $\dot{V}O_2$ and trends for a sparing of muscle [PCr] and [glycogen]), subsequent simulated 4-km TT performance was not different between the three conditions. Our results are consistent, in part, with those of Christensen et al. (2013) who reported that performance in a 400-kcal cycle TT, which began after a 2-h moderate-intensity 'pre-load', was not significantly altered by BR compared to PL in elite cyclists (18.3 vs. 18.6 min, respectively). The influence of NO_3^- supplementation on TT performance is controversial (Cermak et al., 2012a; Cermak et al., 2012b; Christensen et al., 2013; Lane et al., 2014; Lansley et al., 2011a; McQuillan et al., 2017; Peeling et al., 2015; Shannon et al., 2017; Wilkerson et al., 2012) and whether or not NO_3^- ingestion is performance-enhancing appears to depend on factors such as subject training status, the dose and duration of NO_3^- supplementation, and the intensity, duration, and modality of exercise (Jones, 2014). Positive effects of NO_3^- supplementation are more likely to be exhibited in tests of exercise capacity rather than TT efforts (McMahon et al., 2017). When observed, the ergogenic effect of NO_3^- supplementation on TT performance, while relatively small (~2%; Cermak et al., 2012a; Lansley et al., 2011a; Peeling et al., 2015; Shannon et al., 2017), may be meaningful in terms of competitive performance. However, as is the case for the majority of putative nutritional ergogenic aids, the magnitude of this effect is within the sensitivity of most laboratory tests (Jonvik et al., 2015) and may be obscured by intrinsic variability

in performance as well as subject motivation. It is possible that the apparently positive effects of BR on some physiological variables during prolonged exercise that we found were simply too small to impact on TT performance. However, it is also possible that a greater exercise pre-load, resulting in greater glycogen depletion, and/or the inclusion of a longer duration TT, or a higher-sensitivity test of exercise capacity (McMahon et al., 2017), might have enabled the detection of a beneficial effects of BR on exercise performance. Administering the top-up dose of BR earlier than 60 min and/or increasing the duration of the moderate-intensity exercise bout might have enabled plasma [NO_2^-] to reach a higher value prior to the TT and perhaps resulted in a performance benefit.

Experimental Considerations

Although there was no significant difference in muscle [glycogen] between conditions at 120 min of exercise, the decline in muscle [glycogen] between resting baseline and 120 min was significantly attenuated in BR+BR compared to PL+PL. The changes in muscle [PCr] and $\dot{V}O_2$ during exercise were also significantly smaller in BR+BR compared to PL+PL. Although statistical significance was not attained, the changes in muscle [glycogen], muscle [PCr] and $\dot{V}O_2$ over time also tended to be smaller in BR+BR compared to BR+PL. The significant inverse correlation across conditions between the absolute $\dot{V}O_2$ and the absolute muscle [glycogen] at 120 min lends confidence to the interpretation that the sparing of muscle glycogen utilization was related to changes in oxidative metabolic demand following BR ingestion. However, it should be acknowledged that the extent of the sparing of muscle glycogen utilization between baseline and 120 min in BR+BR (~100 mmol·kg^{-1} DW) compared to PL+PL (~186 mmol·kg^{-1} DW) and BR+PL (~168 mmol·kg^{-1} DW) was much greater than would be expected based on the comparatively small differences in $\dot{V}O_2$ and [PCr] we measured. There is the possibility, therefore, that the differences in muscle [glycogen] may have been overestimated in the present study. Additional studies are required to investigate the influence of pre- and in-exercise NO_3^- supplementation on changes in muscle [glycogen] in a larger sample and in trained as well as untrained participants.

If a glycogen sparing effect of BR ingestion during exercise can be confirmed,

this may have important implications not just for single long-endurance events but also for multi-day endurance events such as cycle tours and expeditions, wherein muscle [glycogen] may fall progressively over consecutive days of exercise. It is also possible that consuming BR during arduous endurance training programs might attenuate fatigue development related to glycogen availability and enable additional training to be completed.

Strengths and limitations

This Experimental Chapter provides interesting insight to the muscle metabolic response during prolonged exercise following NO_3^-. However, despite the results suggesting the potential for NO_3^- to preserve intramuscular glycogen and PCr concentractions, it is important to note that these were statistically trends. Therefore, it could be possible that limitations associated with the experimental design need to be considered for future investigations. Firstly, it could be possible that more pronounced effects of NO_3^- would be observed with greater muscle glycogen depletion. In addition, it could be possible that the exercise performance task required a longer duration to observe more pronounced effects. Lastly, the timing in the adminstration of the 'top up' dose could influence the physiological responses measured.

Conclusion

A single dose of BR ingested *during* exercise in addition to pre-exercise BR supplementation increased plasma [NO_3^-] and preserved an elevated plasma [NO_2^-] during prolonged moderate-intensity exercise. This was associated with an attenuated upward drift in the O_2 cost of exercise, and a tendency for a sparing of muscle glycogen and PCr, effects which might be expected to predispose to enhanced exercise tolerance. In conclusion, BR supplementation *during* exercise can modulate plasma [NO_3^-] and [NO_2^-] dynamics and attenuate the progressive rise in $\dot{V}O_2$ during prolonged moderate-intensity exercise. However, under the conditions of the present study, subsequent TT performance was not enhanced by BR supplementation.

The results of Experimental Chapter 5 suggest that NO_3^--rich beetroot juice ingested before and *during* exercise have the potential to preserve intramuscular

glycogen and phosphocreatine although it had no effect on subsequent exercise performance. Experimental Chapter 5 achieved the second aim of the present thesis by suggesting that NO_3^--rich beetroot juice could influence prolonged type exercise and that changing the supplementation strategy may have beneficial effects. Given that the muscle metabolic cost was improved from Experimental Chapter 5, Experimental Chapter 6 sought to investigate if the improvements to muscle metabolic demand were related to changes to skeletal muscle contractile efficiency.

The effect of dietary nitrate supplementation on skeletal muscle contractile efficiency and O_2 cost of prolonged single-leg knee extensor exercise

The third aim of the present thesis was to investigate whether skeletal muscle contractile efficiency changed over prolonged exercise and if short-term ingestion of nitrate had any influence on this physiological response. Given that Experimental Chapter 5 provided insight to the potential of nitrate in preserving muscle metabolite concentrations during prolonged exercise, Experimental Chapter 6 sought to achieve the third aim of the present thesis by measuring intramuscular phosphocreatine over prolonged knee extensor exercise.

Introduction

The efficiency of skeletal muscle contraction is an important determinant of endurance exercise performance (Joyner & Coyle, 2008). Muscle efficiency can be understood in terms of contractile efficiency, which reflects the adenosine triphosphate (ATP) cost of muscle force production, and metabolic efficiency, which reflects the energy or oxygen (O_2) cost of resynthesizing ATP (Whipp & Wasserman, 1969). In humans performing whole body exercise, gross efficiency (GE), defined as the ratio of mechanical work output to total metabolic cost, is typically reported to approximate 18-25% (Poole & Richardson, 1997; Joyner & Coyle, 2008) and this appears to be relatively insensitive to factors such as age, aerobic fitness and training status (Moseley et al., 2004). There is evidence, however, that GE declines during both high-intensity exercise (Bangsbo et al., 2001; Krustrup et al., 2003), and prolonged, fatiguing endurance exercise (Brueckner et al., 1991; Hopker, O'Grady & Pageaux, 2017) as a consequence of factors such as impaired skeletal muscle contractile function (Allen & Trajanonovska, 2012; Vøllestad et al., 1990), a progressive recruitment of type II muscle fibres (Endo et al., 2007; Krustrup et al., 2004), decreased mitochondrial efficiency (Fernström et al., 2007), reduced muscle glycogen stores and changes in metabolic substrate (Ørtenblad et al., 2011). It is unclear whether the fall in GE during prolonged endurance exercise is related more to changes in muscle contractile efficiency or metabolic efficiency.

Supplementation of the diet with inorganic nitrate (NO_3^-), which is reduced *in vivo* to nitrite (NO_2^-) and thence to nitric oxide (NO; Lundberg et al., 2008), lowers the O_2 cost of submaximal exercise (Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010). There are several possible mechanisms, that are not mutually exclusive, that may contribute to this phenomenon (Jones, 2014). Dietary NO_3^- supplementation may: 1) lower the ATP cost of muscle force production, reflecting improved skeletal muscle contractile efficiency (Bailey et al., 2010); 2) lower the O_2 cost of ATP resynthesis, indicating improved mitochondrial efficiency (i.e. P/O ratio; Larsen et al., 2011; cf. Whitfield et al., 2016); and 3) enhance skeletal muscle O_2 availability (Ferguson et al., 2013; Ferguson et al., 2015), thereby offsetting the development of muscular fatigue and better preserving GE (Grassi et al., 2015).

^{31}Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) enables non-invasive quantification of intramuscular high-energy phosphate compounds (i.e. ATP, adenosine diphosphate (ADP), phosphocreatine (PCr), inorganic phosphate (P_i) and pH in skeletal muscle with high temporal resolution at rest and during exercise and subsequent recovery (Arnold et al., 1984; Kemp, Taylor & Radda, 1993). ^{31}P-MRS can be used to estimate the rate of ATP turnover for a given external power output, which is proportional to changes in muscle [PCr], and to estimate muscle mitochondrial oxidative capacity from the time constant (τ) of post-exercise muscle [PCr] recovery kinetics (Arnold et al., 1984; Haseler et al., 1999). When combined with measurements of pulmonary gas exchange, ^{31}P-MRS has the potential to differentiate between contractile and metabolic determinants of changes in GE during exercise.

We have previously reported, using ^{31}P-MRS, that dietary NO_3^- supplementation enabled a higher steady-state muscle [PCr] compared to a placebo condition during short duration (6-10 min), low-intensity, single-leg knee-extensor exercise (Bailey et al., 2010; Vanhatalo et al., 2014). The lower PCr cost of exercise following NO_3^- supplementation was proportional to the lower O_2 cost of exercise (Bailey et al., 2010), implying that improvements in GE with NO_3^- supplementation are related to an improved muscle contraction coupling

efficiency. We have also reported that dietary NO_3^- supplementation resulted in faster muscle [PCr] recovery kinetics, indicative of a greater maximal rate of mitochondrial ATP resynthesis, in hypoxia but not normoxia (Vanhatalo et al., 2011; 2014). There are contrasting reports on whether NO_3^- supplementation influences *ex vivo* biomarkers of mitochondrial efficiency (Hezel et al., 2015; Larsen et al., 2011; 2014; Ivarsson et al., 2017; Whitfield et al., 2016). To date, the effects of NO_3^- supplementation on contractile and/or metabolic efficiency and mitochondrial function have been investigated under resting conditions or during short (<10 min) bouts of submaximal exercise. Given that prolonged (1-2 hours) endurance exercise results in a progressive loss of GE, a combined assessment of muscle energetics using ^{31}P-MRS and pulmonary gas exchange would facilitate investigation of changes in contractile efficiency, metabolic efficiency, and muscle oxidative capacity both with respect to exercise duration and as a consequence of dietary NO_3^- supplementation.

Accordingly, the purpose of the present study was to investigate the influence of dietary NO_3^- supplementation on skeletal muscle contractile efficiency, metabolic efficiency and oxidative capacity during a 2 h constant-work-rate exercise protocol. We hypothesised that: 1) over the duration of exercise, GE would decline, as reflected by a progressive fall in muscle [PCr] and a progressive rise in pulmonary $\dot{V}O_2$, and that [PCr] recovery kinetics would become slower; and 2) dietary NO_3^- supplementation would attenuate the decline of GE by enabling a better maintenance of muscle [PCr] and pulmonary $\dot{V}O_2$. We also hypothesised that [PCr] recovery kinetics would become slower over time during the prolonged exercise bout but that this would be attenuated by dietary NO_3^- supplementation.

Materials and Methods

Subjects

Eleven recreationally active subjects volunteered to take part in this study, including five males (mean ± SD: age 22 ± 4 years, body mass 83 ± 10 kg, height 1.82 ± 0.08 m) and six females (mean ± SD: age 21 ± 2 years, body mass 68 ± 7 kg, height 1.67 ± 0.04 m). The protocols, risks, and benefits of participating were explained prior to obtaining written informed consent. This study was approved by the Institutional Research Ethics Committee and

conformed to the code of ethics of the Declaration of Helsinki. A priori power analysis showed that a sample size of eleven would provide a power = 0.95, alpha = 0.05, an effect size = 1.25.

Experimental Protocol

The subjects reported to the laboratory on seven occasions over a 9-wk period. MRS-based exercise tests for the determination of *in vivo* skeletal muscle energetics were conducted using a custom-designed knee ergometer with subjects positioned in the prone position inside a whole-body MRS system (1.5T, Intera, Philips, The Netherlands) at the Exeter MR Research Centre. Subjects performed knee extensions in which the range of motion was dependent on individual limb length (Figure 1A).

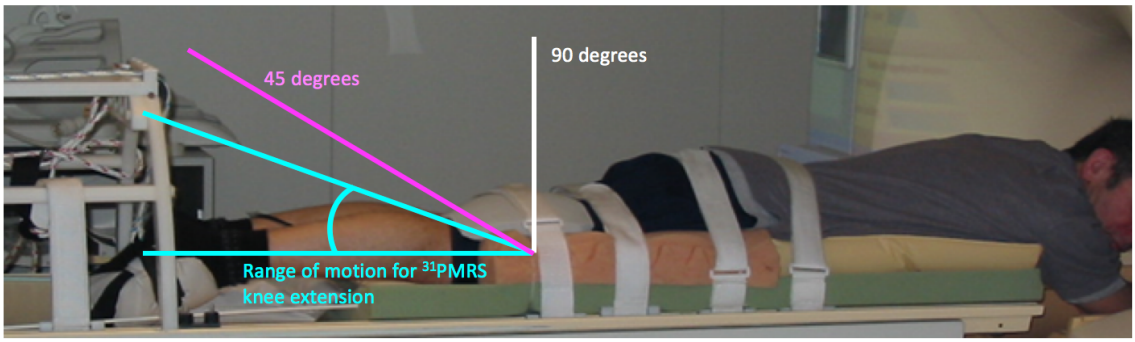

Figure 1A. Approximate range of motion of the knee extension exercise protocol.

Due to restrictions in operating the equipment within a magnetic environment, pulmonary gas exchange was measured and blood samples were collected using the same exercise equipment placed within a custom-built simulation of the MRS system situated in a separate research laboratory. Subjects were secured to the ergometer bed via Velcro straps at the thigh, buttocks and lower back to minimise extraneous movement. The right foot was fastened to a padded foot brace, which was connected to the ergometer load basket using a rope and pulley system. This allowed for the performance of rhythmic single-leg knee-extension exercise over a distance of ~ 0.22 m. Subjects lifted the weight in accordance with an auditory cue at a frequency of 40/min. For the exercise tests conducted inside the whole-body MRS system, a 6-cm ^{31}P transmit/receive surface coil was placed within the scanner bed and the subject asked to lie upon it such that the coil was centred over the quadriceps muscle of the right leg.

All subjects were familiarised to the exercise protocol prior to data collection to minimise learning effects and ensure accurate work rates could be determined for each individual. During the first visit within the whole-body MRS system, each subject completed a step incremental exercise test to volitional exhaustion for the determination of the work rate to be used during the prolonged constant work-rate exercise bouts of the experimental visits. Following a 1 min period of rest, subjects commenced single-leg knee extension exercise against an initial basket load of 2 kg. Thereafter, the basket load was increased by 0.5 kg at the end of each 5 min stage until the subjects were no longer able to maintain the required frequency (40/min) with appropriate form (i.e. smooth, continuous movement over the full range of motion). Pilot experiments indicated that an appropriate sustainable work rate during a prolonged continuous exercise bout was 1 kg below the highest work rate at which muscle [PCr] remained stable during the step incremental test.

During the second laboratory visit, the work rate to be used for the assessment of the PCr recovery time constant (τ) was determined. This was defined as the highest work rate that the subject could maintain for 24 s without compromising the timing and range of motion of the knee-extension task. This approach enables a marked reduction in [PCr] in a short period of time without pH falling below the resting baseline (Holliss et al., 2013; Vanhatalo et al. 2011).

The work rates determined for the prolonged continuous exercise bout and the 24 s high-intensity exercise bouts for each subject during visit 1 and 2 were used during all subsequent visits. During the third visit to the laboratory, subjects performed a familiarisation to the 2 h exercise protocol, consisting of prolonged single-leg knee extensions, to ensure the work rate was sustainable for the entire duration. In addition, subjects performed four 24 s high-intensity exercise bouts separated by 2 min 36 s of rest at 1, 53, and 105 min. Due to the exercise being performed in the prone position, the subject's ankle was in the plantar-flexed position and three subjects were unable to complete the full 2 h at their calculated work rate owing to ankle pain. The work rate for these subjects were reduced by 0.5 kg and the familiarisation repeated to ensure the completion of the exercise.

Work done was calculated as the product of force and displacement. The work rates were 19 ± 4 W for the prolonged continuous exercise bout, and 58 ± 13 W for the 24 s high-intensity exercise bouts.

Following completion of familiarisation, subjects were randomly allocated in a double-blind, crossover fashion to receive either NO_3^--rich beetroot juice or a NO_3^--depleted placebo juice (*see Supplementation*). Within each condition, subjects completed two 4-day supplementation periods separated by 7-day wash-out period such that subjects performed the experimental protocol in the whole-body MRS for one supplementation period, and in the simulation MRS for the remaining supplementation period (Fig. 1B).

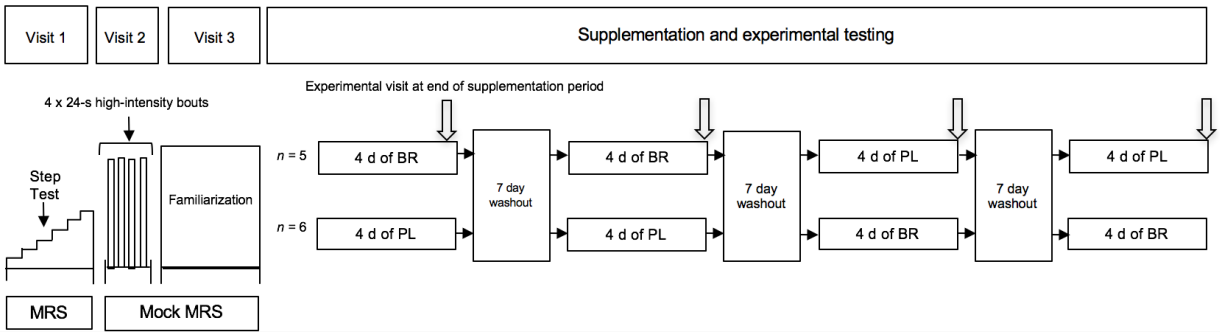

Figure 1B. Schematic diagram of the experimental protocol. BR = beetroot supplementation; PL = placebo supplementation; MRS = magnetic resonance spectroscopy.

Subjects recorded their activity and diet during the 24-h prior to the first experimental visit and were asked to repeat these for subsequent visits. Subjects were also instructed to arrive at the laboratory having avoided strenuous exercise and alcohol in the 24-h preceding, and caffeine in the 8-h preceding, each experimental visit. The experimental protocol was performed on *day 4* of each supplementation period. During the experimental visits in the whole-body MRS system, a resting blood sample was obtained, and ^{31}P-MRS was used to measure muscle high-energy phosphates and to calculate pH. During the experimental visits in the simulation MRI, gas exchange was measured and blood samples were obtained at baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min, Fig. 1C). The experimental protocol consisted of a 1 min period of rest, followed by a prolonged continuous bout of prone knee extensor exercise

for 80-min interspersed with four 24 s bouts of high-intensity exercise at 1, 53, and 105 min, separated by periods of rest lasting 2 min 36 s. Experimental visits were performed at the same time of day (± 2 h). Subjects recorded their activity and diet during the 24 h prior to the first experimental visit and were asked to repeat these for subsequent visits.

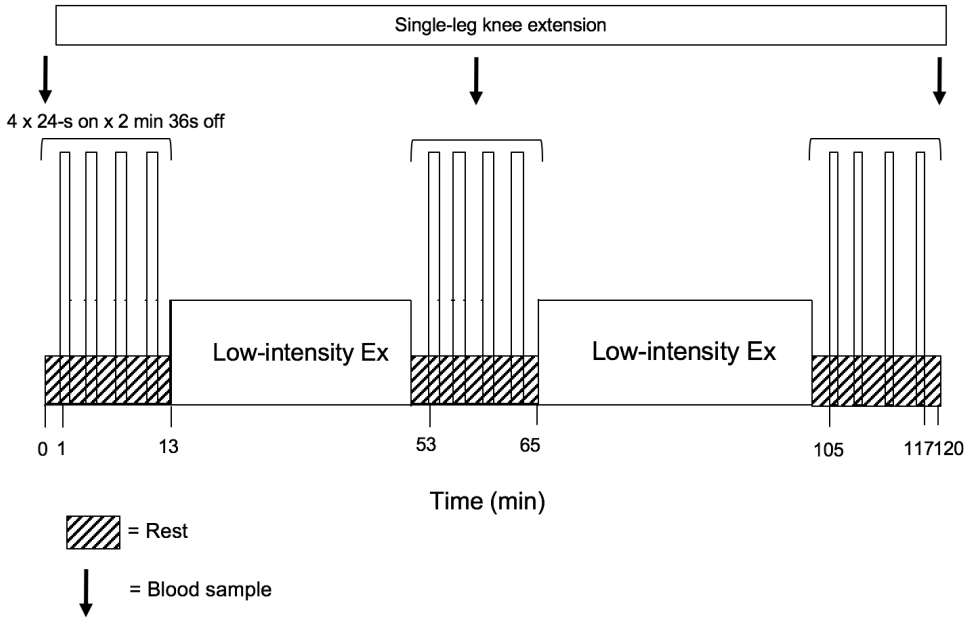

Figure 1C. Schematic diagram of the exercise protocol.

Supplementation

Subjects were randomly allocated to four 4-day supplementation periods (Fig. 1B) in which they consumed 2 x 70 mL doses per day of either concentrated NO_3^--rich (BR: ~6.2 mmol NO_3^- per 70 mL; Beet It Sport, James White Drinks, Ipswich, UK) or NO_3^--depleted placebo (PL: ~0.04 mmol NO_3^- per 70 mL; Beet It Sport, James White Drinks, Ipswich, UK) separated by a minimum of 7 days for a wash-out period. On the first three days of each supplementation period, subjects consumed one 70-mL beverage in the morning and one in the evening, whereas on the experimental day, subjects consumed 2 x 70 mL of their allocated beverage in the morning 2.5 h prior to the exercise. This time frame was chosen because peak plasma [NO_2^-] occurs ~2 to 3 h following NO_3^- supplementation (Wylie et al., 2013a).

^{31}P-MRS data

^{31}P-MRS data, with a spectral width of 1,500 Hz and 1,000 data points, were acquired every 1.5 s. Phase cycling with four phase cycles led to a spectrum

being acquired every 6 s. The subsequent spectra were quantified by peak fitting using the AMARES fitting algorithm in the jMRUI (v3) software package. Absolute values of [PCr] and [Pi] were subsequently calculated from the PCr-to-ATP and Pi-to-ATP ratios, with the assumption of 8.2 mM ATP. Intracellular pH was calculated using the chemical shift of the Pi spectra relative to the PCr peak. [ADP] was calculated as described by Kemp et al. (27). In all cases, relative amplitudes were corrected for partial saturation resulting from the short repetition time relative to T1 relaxation time via a spectrum consisting of 24 averages that was acquired with a TR of 20 s before the commencement of exercise testing.

To derive the [PCr] τ values following the 24 s exercise bouts, PCr recovery was fitted with Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA) with a single exponential of the form:

$$PCr_{(t)} = PCr_{(end)} + PCr_{(0)}\left(1 - e^{(-t/\tau}\right)$$

where PCr_{end} is the value at the end of exercise, $PCr_{(0)}$ is the difference between the PCr at end exercise and fully recovered, *t* is the time from exercise cessation and τ is the time constant for the exponential recovery of PCr. After each 24 s exercise period, recovery kinetics were fitted over the subsequent 150 s and τ determined. The four separate τ values for the four 24 s exercise bouts at each sampling window (baseline, half-way, and end-exercise) were then averaged to generate a single value.

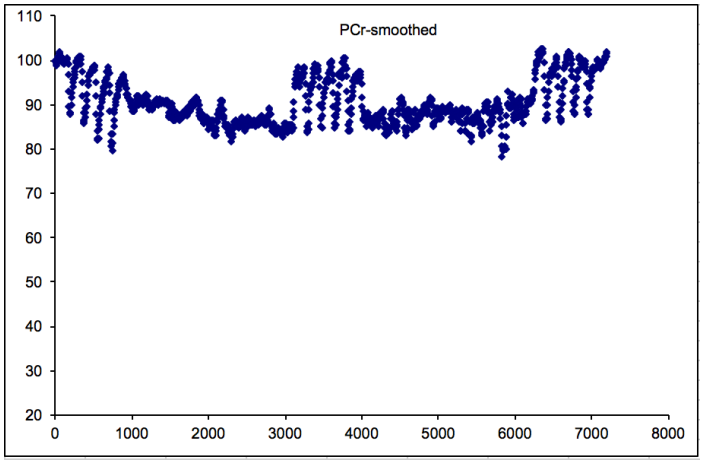

Figure 1D. Example fit of PCr recovery kinetics of an individual subject.

Blood data

During experimental visits where ^{31}P-MRS measures were made, a baseline blood sample was obtained via venepuncture at the antecubital vein prior to the exercise protocol being completed. During laboratory visits, prior to the commencement of the exercise protocol, a cannula (Insyte-W^{TM} Becton-Dickinson, Madrid, Spain) was inserted in the subject's antecubital vein for blood sampling at 0, 60 and 120 min of the exercise. Owing to difficulties with drawing from the cannula, blood data are reported for *n*=10.

All blood samples were drawn into 6 mL lithium heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). The samples were centrifuged at 4000 rpm and 4°C for 10 min and plasma was then immediately extracted and frozen at -80°C for [NO_3^-] and [NO_2^-] analysis via gas phase chemiluminescence as previously described (Wylie et al., 2013a). Prior to analysis, samples were deproteinized using procedures outlined in Tan et al. (2018).

Pulmonary gas exchange and gross efficiency

Subjects wore a nose clip while breathing through a low-dead space mouthpiece and impeller turbine assembly (Triple V, Jaeger, Hoechburg, Germany) connected to paramagnetic (O_2) and infrared (CO_2) analyzers (Oxycon Pro; Jaeger, Hoechburg, Germany) through a capillary line. Prior to each test, the gas analysers were calibrated with gases of known concentration, and the volume transducer was calibrated with a 3 L syringe (Hans-Rudolph, Kansas City, MO).

The gas exchange data were averaged in 10 s time bins for further analysis. GE was calculated as the ratio of work done to energy expenditure as described in Hopker et al. (2017).

Statistical analyses

A two-way repeated measures analysis of variance (ANOVA) was used to assess differences between BR and PL conditions and across time for [PCr] recovery τ, pulmonary $\dot{V}O_2$ and GE at baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min). A two-way repeated measures ANOVA (condition x time) was used to assess differences between BR and PL conditions an across time for plasma [NO_3^-], [NO_2^-] measured at 0, 60, and 120 min. A two-way repeated measures ANOVA was used to examine any differences between BR and PL conditions in the muscle metabolic milieu ([PCr], [P_i], pH) and were analysed in 1 min bouts from 14 to 15 min, 33 to 34 min, 48 to 49 min, 69 to 70 min, 88 to 89 min, and 103 to 104 min. A one-way repeated measures ANOVA was used to examine the effect of time on the muscle metabolic milieu prior to the 24 s high-intensity exercise bouts. Significant main and interaction effects were further explored using one-way repeated measures ANOVA and Fisher's Least Significant Difference test. Differences in the change in [NO_3^-], [NO_2^-] and pulmonary $\dot{V}O_2$ between male and females were assessed using Student's paired t-tests and an analysis of covariance (ANCOVA). Statistical significance was accepted at $P<0.05$. Results are presented as mean ± SD unless otherwise stated.

Results

Plasma [NO_3^-] and [NO_2^-]

Plasma [NO_3^-] and [NO_2^-] data are shown in Fig. 2. There was a main effect of condition only ($P<0.0001$) for plasma [NO_3^-]. During the laboratory test, resting plasma [NO_3^-] was significantly elevated in BR (439 ± 173 μM) compared to PL (75 ± 170 μM, $P<0.001$, Fig. 2A). Plasma [NO_3^-] did not change during the duration of the exercise protocol ($P>0.05$). During the MRS test, resting plasma [NO_3^-] was significantly elevated in BR (458 ± 104 μM) compared to PL (20 ± 3 μM, $P<0.0001$, Fig. 2B). There was an interaction effect (condition x time, $P<0.05$) and a main effect of condition ($P<0.05$) for plasma [NO_2^-]. During the

laboratory test, resting plasma [NO_2^-] was elevated in BR (433 ± 299 nM) compared to PL (149 ± 76 nM, *P*<0.05, Fig. 2C), and tended to be greater in BR at half-way (589 ± 496 nM, *P*=0.06) and at end-exercise (599 ± 481 nM, *P*=0.07) compared to at rest. Plasma [NO_2^-] did not change during the duration of the exercise protocol in PL (*P*>0.05). During the MRS test, resting plasma [NO_2^-] was elevated in BR (529 ± 266 nM) compared to PL (98 ± 31 nM, *P*<0.001, Fig. 2D). There was no difference between males and females in the change in plasma [NO_3^-] (M: 347.15 ± 215.33 vs. F: 382.23 ± 232.92 μM, *P*>0.05) and [NO_2^-] (M: 127.5 ± 104 vs. F: 478.0 ± 384.76 nM, *P*>0.05); BR – PL) at rest (Fig. 3). A one-way ANCOVA was conducted to compare the change in oxygen uptake following BR and PL, whilst controlling for sex. Levene's test and normality were carried out and the assumptions met. There was no significant difference in the change in oxygen uptake between conditions (P>0.05) although there was a significant effect of sex (P<0.05).

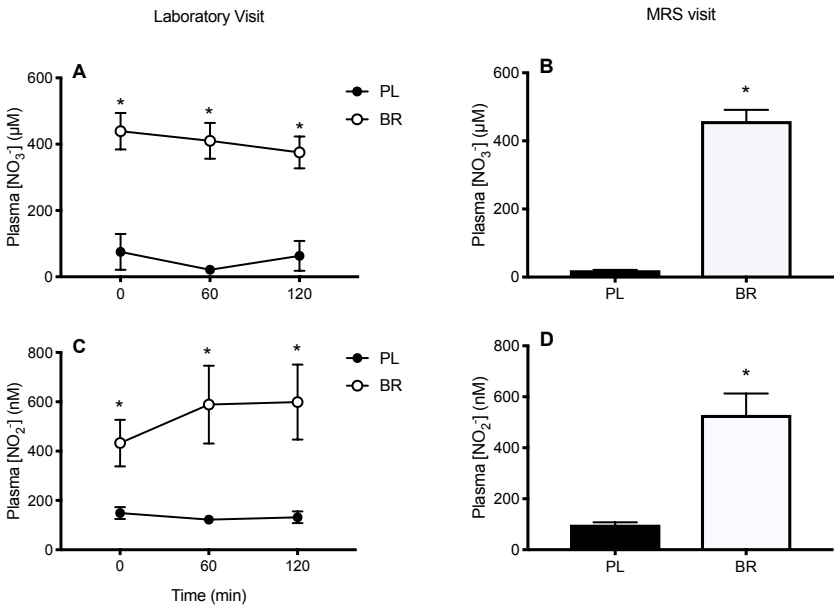

Figure 2. Mean ± SEM of plasma nitrate concentrations ([NO_3^-]) during the laboratory visit over a 2-h single-leg knee extensor exercise protocol (panel A), and at rest during the MRS visit (panel B) following nitrate-rich beetroot juice (BR; open circle and bar), and nitrate-depleted beetroot juice supplementation (PL; solid circle and bar). Mean ± SEM of plasma nitrite concentrations ([NO_2^-]) during the laboratory visit over a 2-h single-leg knee extensor exercise protocol (panel C), and at rest during the MRS visit (panel D) following BR (open circle and bar), and PL (solid circle and bar) supplementation. * = significantly different from

PL.

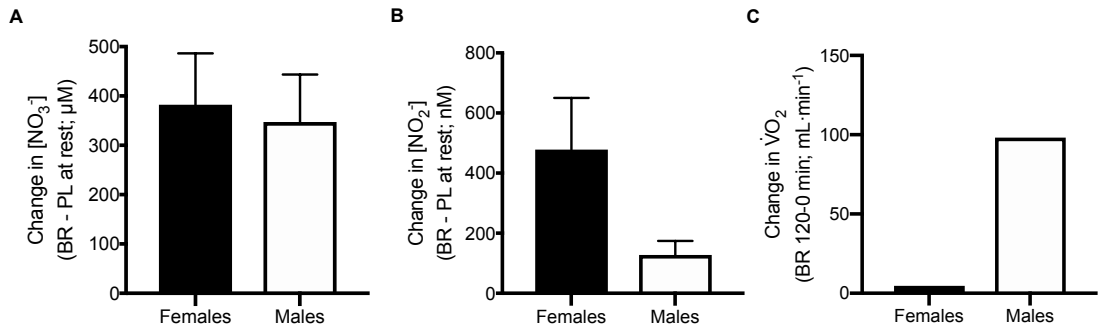

Figure 3. Change in plasma nitrate ($[NO_3^-]$; panel A) and nitrite ($[NO_2^-]$; panel B) following BR (BR – PL; µM, nM) in males (*n*=5) and females (*n*=5) and the change in pulmonary $\dot{V}O_2$ measured over time in BR (120 – 0 min; $ml \cdot kg^1 \cdot min^{-1}$; panel C) between males (*n*=5) and females (*n*=6).

Muscle metabolic responses during prolonged continuous exercise

The muscle metabolic responses to prolonged exercise are shown in Fig. 4. There was an interaction effect (condition x time, P<0.05) for [PCr] during prolonged continuous exercise such that [PCr] declined from 14-15 min to 69-70 min in placebo only (P<0.05). There was an interaction effect (condition x time, P<0.01) and a main effect of time for [P_i] (P<0.01). There was a main effect of time only for pH (P<0.0001) where pH decreased in both conditions over the prolonged exercise bout (Fig. 4B and 4C).

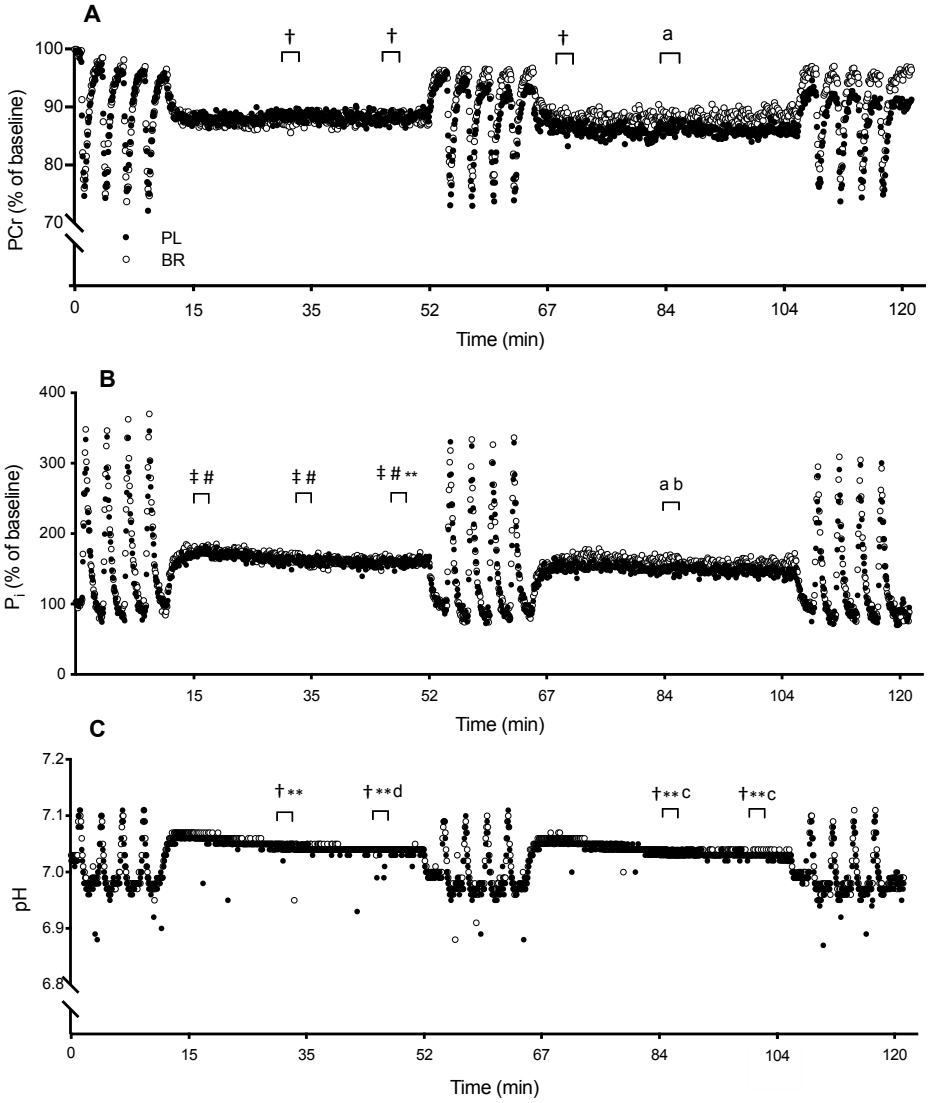

Figure 4. Group mean of representation of a 2-h continuous low-intensity prolonged single-leg knee extensor exercise protocol for the determination of intramuscular [phosphocreatine] ([PCr], panel A), [inorganic phosphate] ([P_i], panel B), and pH (panel C), interspersed with four 24-s high-intensity bouts for the determination of [PCr] τ from baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min). Intramuscular [PCr], [Pi] and pH

were analyzed in 1 min bouts from 14 to 15 min, 30 to 31 min, 48 to 49 min, 69 to 70 min, 88 to 89 min, and 103 to 104 min. † = significantly different from 14 to 15 min in PL (P<0.05), ** = significantly different from 69 to 70 min in PL (P<0.05), # = significantly different from 88 to 89 min in PL (P<0.05), ‡ = significantly different from 103 to 104 min in PL (P<0.05), a = significantly different from 103 to 104 min in BR (P<0.05), b = significantly different from 69 to 70 min in BR (P<0.05), c = significantly different from 30 to 31 min (P<0.01), d = significantly different from 30 to 31 min (P<0.05).

[PCr] recovery kinetics

There was no main effect of time on baseline [PCr] during the rest periods prior to the 24 s high-intensity bouts in either condition (P>0.05). The amplitude of [PCr] degradation during the 24 s high-intensity bouts were not different between conditions (P>0.05) or over time (P>0.05). The amplitude of pH reduction during the 24 s high-intensity bouts was not different between conditions (P>0.05) or over time (P>0.05).

The [PCr] τ measured during recovery is shown in Fig. 5. There was no effect of condition on [PCr] recovery τ (P>0.05) but [PCr] recovery τ decreased over time in both conditions (P<0.001).

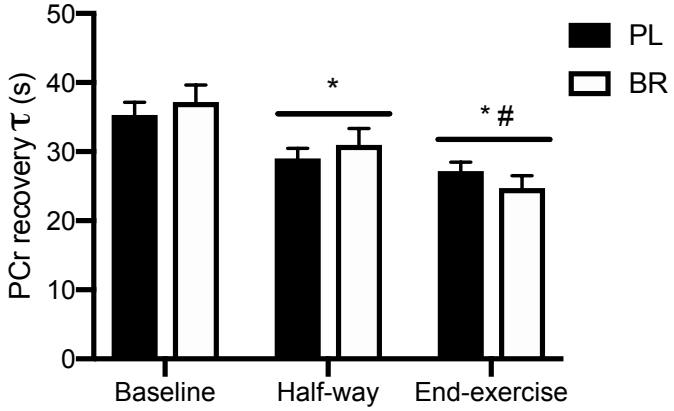

Figure 5. Mean ± SEM of muscle [PCr] recovery kinetics (τ) averaged over the four 24-s high-intensity bouts during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min). In BR, [PCr] recovery τ sped up from baseline to end-exercise (35 ± 6 vs. 27 ± 4-s, P=0.001), baseline to half-way (35 ± 6 vs. 29 ± 5-s, P<0.01), and was not different from half-way to end-exercise (29 ± 5 vs. 27 ± 4-s, P>0.05). In PL, [PCr] recovery τ sped up from baseline to end-exercise (37 ± 8 vs. 25 ± 6-s, P<0.0001), and tended to speed up from baseline to half-way (37 ± 8 vs. 31 ± 8-s, P=0.06), and from half-way to end-exercise (31 ± 8 vs. 25 ± 6-s, P=0.06). * = significantly different from baseline (P<0.01), # = significantly different from half-way (P<0.01).

Pulmonary gas exchange and gross efficiency

The pulmonary $\dot{V}O_2$ responses during the prolonged exercise bout are illustrated in Fig. 6. There was a main effect of time (P<0.01) on $\dot{V}O_2$ measured at baseline, half-way, and end-exercise. There was a main effect of time (P<0.05) only on RER. RER declined in both PL (baseline: 1.06 ± 0.16 vs. half-way: 0.98 ± 0.19 vs. end-exercise: 0.93 ± 0.17) and BR (baseline: 1.03 ± 0.14 vs. half-way: 0.95 ± 0.13 vs. end-exercise: 0.91 ± 0.13).

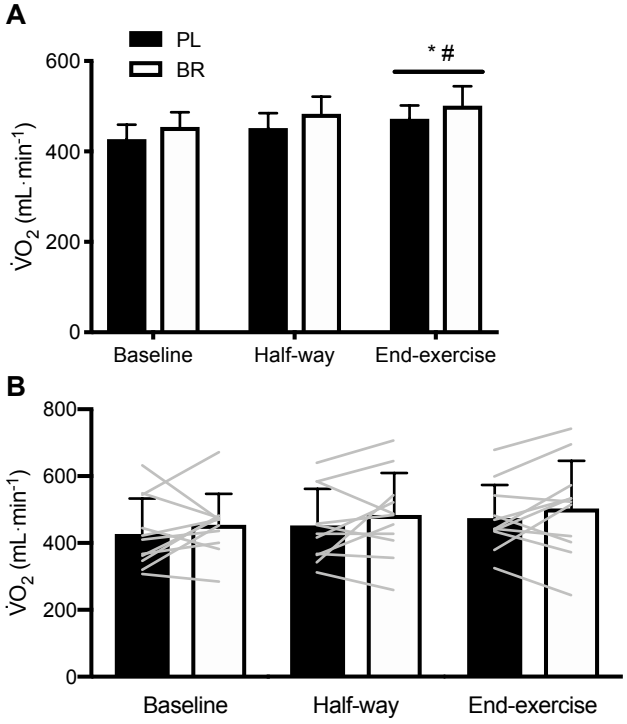

Figure 6. Mean ± SEM of pulmonary $\dot{V}O_2$ measured during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min) during the four 24-s high-intensity bouts following nitrate-rich beetroot juice (BR; open bar) and nitrate-depleted beetroot juice (PL; solid bar) supplementation (panel A), and individual pulmonary $\dot{V}o_2$ responses over time (panel B). * significantly different from baseline (P<0.001); # = significantly different from half-way (P<0.05).

GE during the prolonged exercise protocol is illustrated in Fig. 6. There was a main effect of time (P<0.05) on GE. GE declined in both PL (baseline: 6.43 ± 1.82% vs. half-way: 6.14 ± 1.56% vs. end-exercise: 5.89 ± 1.36%) and BR (baseline: 5.91 ± 0.97% vs. half-way: 5.73 ± 0.98% vs. end-exercise: 5.63 ±

0.90%).

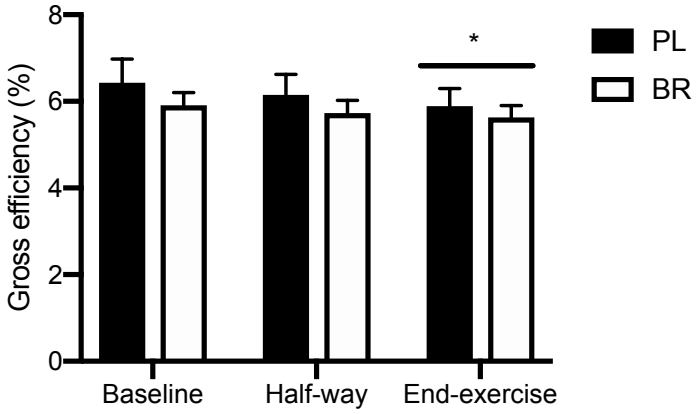

Figure 7. Group mean ± SEM GE calculated during baseline (1 to 13 min), half-way (53 to 65 min), and end-exercise (105 to 117 min) during the four 24-s high-intensity bouts interspersed with 2 min 36 s of recovery following nitrate-rich beetroot juice (BR; open bar) and nitrate-depleted beetroot juice (PL; solid bar) supplementation. # = significantly different to baseline (P<0.05); ‡ = significantly different to half-way (P<0.05).

Discussion

The major novel finding of this study was that dietary NO_3^- supplementation did not influence the PCr cost of force production or GE during a 2 h exercise protocol consisting of continuous single-leg knee extensor exercise interspersed with intermittent high-intensity exercise. This finding is not consistent with the hypothesis that dietary NO_3^- supplementation improves skeletal muscle contractile efficiency during prolonged exercise. Another novel, and surprising, finding of this study was that [PCr] recovery τ became shorter (i.e., [PCr] recovery was accelerated) over time in both conditions, suggesting that, while the maximal rate of oxidative ATP resynthesis was improved over time during the prolonged exercise bout, this was not impacted by NO_3^- supplementation. In agreement to our hypothesis, we observed that the PCr cost of force production declined over time in the placebo condition and that pulmonary $\dot{V}O_2$ increased over time during prolonged exercise. Collectively, these results suggest that dietary NO_3^- supplementation does not influence skeletal muscle efficiency or GE during 2 h of prolonged, low-intensity knee extensor exercise.

Effects of dietary nitrate supplementation on PCr cost of force production

In the present study, we observed that muscle [PCr] declined over time in the

placebo condition, but there was no significant difference following 4 days of dietary NO_3^- supplementation compared to placebo throughout 2 h of constant work-rate, single-leg knee extensor exercise. This is in contrast to previous studies showing that NO_3^- supplementation reduced the PCr cost of force production during shorter (4-10 min) bouts of moderate- and severe-intensity (Bailey et al., 2010) and heavy-intensity knee extensor exercise (Vanhatalo et al., 2014), reflecting a reduction in the ATP cost of force production (Bailey et al., 2010; Jubrias et al., 2008). It is known that GE deteriorates during prolonged, fatiguing exercise (Brueckner et al., 1991; Hopker, O'Grady & Pageaux, 2017) and thus important novel findings of the present study include that: 1) muscle contractile efficiency, as reflected by [PCr], may decline over time, and muscle metabolic efficiency, as reflected by $\dot{V}O_2$, declines with time during prolonged exercise; and 2) [PCr] and $\dot{V}O_2$ are not influenced by dietary NO_3^- supplementation compared to placebo. These results are in contrast to the commonly observed lowering of $\dot{V}O_2$ and higher muscle [PCr] following NO_3^- supplementation during shorter bouts of exercise (Larsen et al., 2007; Bailey et al., 2009; 2010; Pawlak-Chaouch et al., 2016) driven by the effects of NO_3^- on muscle contractile efficiency (Bailey et al., 2010; Coggan & Peterson, 2018; Hernández et al., 2012; Whitfield et al., 2016), mitochondrial efficiency (Larsen et al., 2011) and/or a combination of the two mechanisms. In turn, this emphasises the importance of considering the exercise duration and intensity as factors that may influence the ergogenic properties of dietary NO_3^-. The present study showed that dietary NO_3^- supplementation does not affect the [PCr] and O_2 cost during prolonged, small muscle mass exercise.

In the present study, dietary NO_3^- supplementation significantly increased plasma [NO_2^-] and therefore NO bioavailability. In contrast to our hypotheses, elevated NO bioavailability did not modulate muscle contractility (Coggan & Peterson, 2018) or the O_2 cost of exercise (Pawlak-Chaouch et al., 2016). The production of NO through the sequential NO_3^--NO_2^--NO pathway (Lundberg et al., 2008) is potentiated in acidic (Modin et al., 2001) and hypoxic (Castello et al., 2006) environments, and is prudent to the efficacy of dietary NO_3^- supplementation. Therefore, the low-intensity and long-duration of the exercise protocol in the present study might not have induced an optimal metabolic environment to

facilitate the reduction of NO_3^- to NO_2^- to NO. Indeed, following NO_3^- ingestion, muscle metabolites (i.e. [PCr], [P_i], [ADP]) were reported to return to normoxic values during hypoxic knee extensor exercise (Vanhatalo et al., 2011), which suggests that exercise intensity (and subsequent muscle acidosis (Modin et al., 2001) and hypoxia (Castello et al., 2006)) could partly influence the efficacy of NO_3^- supplementation. Moreover, Whitfield et al. (2016) speculated that elevated hydrogen peroxide (H_2O_2) emission following NO_3^- supplementation may contribute to improved human muscle contractile function. Therefore, it is possible that the low-intensity exercise did not induce sufficient H_2O_2 emission to promote enhancements to muscle contractility (Andrade et al., 1998). Lastly, inorganic NO_3^- supplementation has been evidenced to enhance muscle groups comprised of a greater relative proportion of fatigue-sensitive type II fibres in rodents (Ferguson et al., 2013; 2015; Hernández et al., 2012; Ivarsson et al., 2017; cf: Whitfield et al., 2017; for review see Jones et al., 2016) and to enhance physiological responses to exercise protocols that necessitate greater recruitment of type II fibres in humans (Bailey et al., 2015; Breese et al., 2013; Peeling et al., 2015). Taken together, these data suggest that NO-induced enhancements to muscle contractility could be possible during prolonged, fatiguing endurance exercise where fatigue of initially recruited type II fibres and/or a progressive activation of type II fibres may occur. Future studies are warranted to clarify potential effects of NO_3^- supplementation on contractile efficiency during prolonged exercise.

Effects of dietary nitrate supplementation on pulmonary gas exchange

We observed that the O_2 cost of exercise was not different during the prolonged exercise bout following NO_3^- supplementation compared to placebo, which is in contrast with earlier studies that have used shorter exercise durations (Bailey et al., 2009; Cermak et al., 2012; Larsen et al., 2007; Vanhatalo et al., 2010) but in agreement with others (Betteridge et al., 2015; Kocoloski et al., 2018; Oskarsson et al., 2018; Wickham et al., 2019). A progressive recruitment of type II muscle fibres and/or fatigue of initially recruited fibres during prolonged exercise is associated with fatigue development (Krustrup et al., 2004) and may be linked to the observed rise in the O_2 cost of exercise (Jones et al., 2011; Grassi et al., 2015; Hopker et al., 2017). To our knowledge, only three studies have

investigated the pulmonary $\dot{V}O_2$ response during prolonged continuous exercise following NO_3^- supplementation and have yielded equivocal results, reporting $\dot{V}O_2$ to be unchanged (Betteridge et al., 2015; Wilkerson et al., 2012) or preserved over time (Tan et al., 2018). In the present study, it could be speculated that the lack of effect of NO_3^- supplementation on the O_2 cost of exercise could be due to the results being masked by sex-based differences in the cohort that was comprised of males (*n*=5) and females (*n*=6; Fig. 7.). It is becoming recognized that sexual dimorphisms in physiology influence NO biosynthesis (Rosselli et al., 1994), fibre type composition (Haizlip et al., 2015), contractile velocity (Haizlip et al., 2015), fatigability (Solianik et al., 2017) and skeletal muscle efficiency (Wüst et al., 2008). To date, the few studies that have examined sex-based differences with NO_3^- supplementation found differences in O_2 utilization in rodents (Craig et al., 2018; 2019) and NO_x (i.e. NO_3^- + NO_2^-) bioavailability in humans (Kapil et al., 2010). However, the present study is insufficiently powered to draw conclusive results regarding sex differences in the O_2 response during exercise. Further investigations are required to elucidate potential sex-based differences in physiological responses to NO_3^- supplementation.

In the present study, RER decreased over time reflecting increased fatty acid utilization, with a commensurate increase in $\dot{V}O_2$, but with no influence of NO_3^- supplementation. This finding is consistent with studies which have not observed changes to RER following NO_3^- ingestion during bouts of exercise of shorter (5-20 min) duration (Bailey et al., 2009; Christensen et al., 2013; Vanhatalo et al., 2010; Whitfield et al., 2016), although some studies have reported slightly higher RER values following NO_3^- supplementation compared to placebo (Bailey et al., 2010; Larsen et al., 2011; Wylie et al., 2013a). The attenuation of muscle glycogen depletion over prolonged exercise would be expected to positively influence contractile efficiency and exercise performance (Bergstrom, 1962; Ørtenblad et al., 2011). NO plays an important role in skeletal muscle glucose uptake (Merry et al., 2010), and therefore could contribute to the sparing of muscle glycogen (Tsintzas & Williams, 1998). The literature remains equivocal concerning whether dietary NO_3^- ingestion may influence glucose uptake and muscle glycogen utilization during exercise in humans (Betteridge et al., 2016; Tan et al., 2018; Wylie et al., 2013b). However, rodent studies indicate that NO-

induced modulations to GLUT4 translocation (Jiang et al., 2014) or protein expression (Gheibi et al., 2018) may be possible. Further work is required to provide insight into the potential for dietary NO_3^- supplementation to effect glycogen sparing, which may in turn enhance skeletal muscle contractile function.

Effect of dietary nitrate supplementation on [PCr] recovery kinetics

In the present study, dietary NO_3^- supplementation had no effect on the [PCr] recovery τ, which reflects the maximal rate of mitochondrial ATP resynthesis (Arnold et al., 1984; Paganini et al., 1997) over the 2 h constant work-rate exercise protocol. This is consistent with previous reports that NO_3^- ingestion did not alter [PCr] recovery τ in normoxia (Fulford et al., 2013; Vanhatalo et al., 2014), although it should be noted that NO_3^- supplementation led to a reduced [PCr] recovery τ in hypoxia (Vanhatalo et al., 2011; 2014). These findings suggest that dietary NO_3^- supplementation does not influence the maximal rate of mitochondrial ATP re-synthesis *in vivo* except in circumstances where muscle O_2 availability may be impaired.

Using an *in vivo* model, our results indicate that dietary NO_3^- supplementation does not influence mitochondrial function as we did not observe any changes to [PCr] recovery τ following NO_3^- ingestion during the 2 h exercise protocol, or in previous studies utilizing shorter exercise protocols in normoxia in healthy young adults (Vanhatalo et al., 2011; 2014). This is in contrast to Larsen et al. (2011) who reported that dietary NO_3^- supplementation reduced the amount of O_2 consumed per ATP molecule re-synthesised possibly through a reduction in ATP/ADP translocase expression which is associated with proton leakage. Whitfield et al. (2016) recently reported that the reduction in $\dot{V}O_2$ following NO_3^- ingestion in humans they observed was not accompanied by changes to mitochondrial proteins associated with efficiency, such as uncoupling protein 3 or adenine nucleotide translocase. In addition, studies using rodents yield conflicting results on whether dietary NO_3^- supplementation influences mitochondrial function (Hezel et al., 2015; Ivarsson et al., 2017; Larsen et al., 2011; 2012).

An unexpected finding of the present study was that [PCr] recovery τ became shorter over time, i.e. PCr recovery became faster, irrespective of dietary

condition. The speeding of [PCr] recovery τ following chronic training interventions is considered to reflect an increased mitochondrial mass (Constable et al., 1987; Holloszy & Coyle, 1984; Laurent et al., 1992; McCully et al., 1989), and/or improved perfusion (i.e. oxygenation) (Haseler et al., 1999; Tschakovsky & Hughson, 1999). Considering the acute nature of the present study, alterations to mitochondrial mass can be excluded as a potential explanation for the speeding of [PCr] recovery τ we observed. In addition, given that our subjects were young, healthy and recreationally active, it is unlikely they would have impairments to muscle O_2 delivery (Haseler et al., 1999). There are several possible explanations for the speeding of [PCr] recovery τ across the 2 h exercise protocol. Firstly, it is known that posture (i.e. supine vs. prone vs. upright exercise) during exercise may influence skeletal muscle perfusion (Jones et al., 2006; Goulding et al., 2017). It is possible, therefore, that the prone position required to perform the exercise protocol may have initially limited blood flow but that this was overcome over the course of exercise bout. Secondly, mitochondrial enzyme activation may have increased throughout the exercise bout, enabling enhanced O_2 utilization (Grassi, 2001; Koga et al., 2012). Indeed, prior exercise or "priming" has been suggested to increase the activation of key rate-limiting oxidative enzymes (Behnke et al., 2002). Finally, an increased muscle temperature over exercise could speed rate-limiting reactions regulating oxidative phosphorylation (Koga, 1997). Our findings indicate that, at least under the conditions of the present study (i.e., single-leg, knee-extension exercise, performed in the prone position), muscle oxidative capacity, as inferred from [PCr] recovery τ, is enhanced over the course of prolonged endurance exercise.

Strengths and limitations

In this Experimental Chapter, the use of ^{31}P-MRS provided novel information on the intramuscular [PCr] response during prolonged knee extensor exercise. A major limitation that should be considered when interpreting the results of this study is that the biomechanical strain on the ankle joint due to the prolonged exercise duration limited the exercise intensity of the protocol. Accordingly, prolonged single leg knee extension exercise protocols may not reflect prolonged exercise protocols recruiting greater muscle mass and relatively higher exercise intensities. Another limitation of the present study is that the O_2 cost of muscular

work was measured only during the high-intensity exercise bouts and not the moderate-intensity exercise bouts, limiting the interpretation of whether mitochondrial efficiency was changing over exercise and/or with NO_3^- supplementation. Lastly, the present study was not designed to detect potential sex-based differences and is underpowered for this purpose. Although an ANCOVA revealed that there was no effect of condition on the O_2 cost of exercise, future investigations should elucidate whether sex is a confounding factor.

Conclusion

This study is the first to investigate the PCr and O_2 cost of muscular work during a prolonged (2 h) exercise protocol both with and without preceding dietary NO_3^- supplementation. Over the prolonged exercise bout, the PCr cost of exercise and GE declined, while the O_2 cost was progressively increased consistent with changes in RER reflecting increased fatty acid utilization. Dietary NO_3^- supplementation did not significantly influence the [PCr] and O_2 cost of prolonged exercise compared to placebo, suggesting that dietary NO_3^- does not alter muscle contractility or metabolic efficiency over prolonged, low-intensity exercise. The accelerated PCr recovery kinetics over the course of prolonged exercise, which may be related to enhanced O_2 delivery, oxidative enzyme activity and/or muscle temperature, was not impacted by dietary NO_3^- supplementation. In conclusion, these findings indicate that dietary NO_3^- supplementation does not improve exercise efficiency during prolonged single-leg knee extension exercise.

Experimental Chapter 6 achieves the third aim of the present thesis as the results reveal that NO_3^- supplementation does not have an influence on the progressive decline in skeletal muscle contractile efficiency over prolonged knee extensor exercise. However, it is possible that the exercise intensity and mode of exercise in Experimental Chapter 6 (i.e. single leg knee extensions) compared to Experimental Chapter 5 (i.e. cycling) may have had an influence in the efficacy of NO_3^- supplementation, especially considering that NO_3^- supplementation may preferentially augment type II muscle fibres. Accordingly, it could be possible that NO_3^- supplementation may be efficacious in circumstances which require a greater relative proportion of type II muscle fibres (e.g. whole body exercise and performed in the heavy-intensity domain). Therefore, Experimental Chapter 7

sought to investigate the potential physiological effects of NO_3^- supplementation during prolonged heavy-intensity cycling exercise.

The influence of co-ingesting nitrate and N-acetylcysteine on NO bioavailability, oxidative stress, neuromuscular function and respiratory muscle fatigue during prolonged heavy-intensity exercise

The results of Experimental Chapter 6 suggest that NO_3^--rich beetroot juice did not influence skeletal muscle contractile efficiency during prolonged knee extensor exercise, but that the exercise intensity and modality may be important considerations in the efficacy of NO_3^-. Therefore, Experimental Chapter 7 investigated the potential physiological effects of NO_3^- supplementation during prolonged heavy-intensity cycling exercise. An important physiological factor during heavy-intensity exercise is the accumulation of reactive oxygen species (ROS) and subsequent ROS-induced reduction to NO bioavailability as well as potential impairments to neuromuscular and respiratory muscle function. Thus, Experimental Chapter 7 examined co-ingestion of NO_3^- with an antioxidant-like supplement, N-acetylcysteine, on the physiological responses during prolonged heavy-intensity cycling exercise.

Introduction

Reactive oxygen species (ROS) production is required for normal skeletal muscle contractile function (Reid et al., 1993). However, with increasing exercise duration and intensity, ROS production can exceed the endogenous antioxidant buffering system and contribute to a decline in skeletal muscle function over time (Powers & Jackson, 2008). The role of ROS in impairing skeletal muscle function is complex but is related to the disruption of skeletal muscle contractile structure and function (Alessio et al.,1988; Davies et al., 1982; Debold, 2015) and the impairment of neuromuscular function via inhibiton of motorneuron activity (Delliaux et al., 2009; Rossman et al., 2012). In addition, ROS-mediated fatigue development in the respiratory muscles could influence skeletal muscle contractile function through the muscle metaboreflex phenomenon (Kelly et al., 2009; Shindoh et al., 1990). The muscle metaboreflex redirects blood flow away from skeletal muscle and towards respiratory muscles as respiratory muscle fatigue manifests (Amann & Calbet, 2008; Dominelli et al., 2017; Harms et al., 1997; Romer & Polkey, 2008) with implications for exercise performance (Witt et al., 2007).

Dietary nitrate (NO_3^-) supplementation, administered in the form of beetroot juice (BR), may improve exercise performance (e.g. Jones et al., 2014; 2018; McMahon et al., 2017). This beneficial effect is attributed to elevated nitric oxide (NO) bioavailability through the serial reduction of NO_3^- to nitrite (NO_2^-) and then NO (Lundberg et al., 2008). The candidate mechanisms responsible include the independent or combined NO-induced changes to contractile efficiency (i.e. ATP cost of contraction; Bailey et al., 2010), force production (Hernández et al., 2012; Whitfield et al., 2017), and enhanced muscle oxygenation (Ferguson et al., 2013; 2015). In addition to these mechanisms, recent evidence indicates that pulmonary vasculature recruitment may be influenced by NO_3^- (Behnia et al., 2018) which could potentially influence the development of respiratory muscle fatigue and thus skeletal muscle blood flow (Romer & Polkey, 2008). To date, the mechanisms underpinning the efficacy of NO_3^- ingestion have been investigated during short duration exercise bouts lasting ≤10 min (Bailey et al., 2010; Larsen et al., 2011); however, NO bioavailability is potentially compromised during prolonged, intense exercise due to excessive ROS that could scavenge NO to form other oxidizing agents (Clanton et al., 2007; Duthie et al., 1990; Halliwell, 1994; Reid et al., 1992). Many phytochemicals with antioxidant properties are found in BR, such as betalain (Escribano et al., 1998; Georgiev et al., 2010) and have ROS scavenging properties (Butera et al., 2002; Esatbeyoglu et al., 2015; Gliszczyńska-Świgło et al., 2006; Kanner et al., 2001). Thus, NO_3^- ingestion has the potential to influence exercise-induced oxidative stress (Clifford et al., 2015). In addition, it is possible that co-ingestion of NO_3^- with another supplement with antioxidant properties could provide synergistic effects on NO bioavailability, oxidative stress, and consequently the physiological responses to exercise.

N-acetylcysteine (NAC) is a reduced thiol-donor (Ruffmann & Wendel, 1991) that has been reported to improve exercise capacity (Corn & Barstow et al., 2011; Leelarungrayub et al., 2011; Matuszczak et al., 2005; McKenna et al., 2006; Medved et al., 2004b; Slattery et al., 2014). NAC provides cysteine (CYS), the rate-limiting precursor to the most important antioxidant, glutathione (GSH, Smith & Reid, 2006). GSH is the most abundant endogenous antioxidant that quenches ROS to promote a balanced cellular redox environment (Powers & Jackson,

2008). NAC has been reported to attenuate respiratory muscle fatigue (Kelly et al., 2009; Smith et al., 2014), reduce muscle membrane depolarization (McKenna et al., 2006), as well as potentially enhance vasodilation and blood flow (Boesgaard et al., 1994) through NO-mediated activity (Simon et al., 1993) during prolonged, repetitive contractions. Given that both dietary NO_3^- and NAC supplementation induce positive physiological effects (Bailey et al., 2009; 2010; Behnia et al., 2018; Cobley et al., 2011; McKenna et al., 2006; Medved et al., 2004b), and that NAC potentially augments NO bioavailability via scavenging ROS (Deneke et al., 2000; Sen, 1997; Simon et al., 1993), combining both supplements could evoke synergistic effects, especially during prolonged exercise, and enhance exercise performance.

The purpose of the present study was, therefore, to investigate whether independent and/or concurrent pre-exercise ingestion of BR and NAC influenced NO bioavailability, oxidative stress, neuromuscular function and respiratory muscle fatigue development during a preload of 1 h of heavy-intensity cycling exercise, and subsequent exercise tolerance during a severe-intensity time-to-exhaustion (TTE) task. We hypothesized that combining BR and NAC supplementation before 1 h of cycling in the heavy-intensity domain would: 1) elevate pre-exercise plasma [NO_3^-], [NO_2^-], [CYS], and [GSH] while reducing the oxidative stress biomarker plasma malondialdehyde ([MDA]), to a greater extent compared to ingestion of BR or NAC alone; 2) attenuate the expected decline in neuromuscular and respiratory muscle function to a greater extent than ingestion of BR or NAC alone; and therefore, 3) improve TTE performance to a greater extent than ingestion of BR or NAC alone.

Methods

Subjects

Sixteen recreationally active males (mean ± SD: age 24 ± 6 years, body mass 73 ± 7 kg, height 1.78 ± 0.05 m, $\dot{V}O_{2peak}$ 52 ± 8 $mL \cdot kg^{-1} \cdot min^{-1}$) volunteered to participate in this study. All subjects were familiarized to the exercise protocol prior to data collection to minimize any learning effects throughout the study and to ensure accurate work rates could be determined for each individual. The protocols, risks, and benefits of participating were explained prior to obtaining

written informed consent. This study was approved by the Institutional Research Ethics Committee and conformed to the code of ethics of the Declaration of Helsinki. A priori power analysis showed that a sample size of fifteen would provide a power = 0.80, alpha = 0.05, an effect size = 0.80.

Experimental overview

Subjects reported to the laboratory on 6 separate occasions over a ~7-8 week period. On the first visit, subjects completed a ramp incremental cycling exercise test for the determination of $\dot{V}O_{2peak}$ and gas exchange threshold (GET). During the second visit, subjects were familiarized to the exercise testing procedures, including completion of a heavy-intensity exercise bout (at a work rate of 20%Δ, where Δ represents the difference between peak work rate and the GET) for 1 h before completing a cycling performance time-to-exhaustion (TTE) test at a work rate of 70%Δ.

For the duration of the study, subjects were asked to maintain their habitual physical activity and dietary intake; however, subjects were instructed to avoid consuming foods high in dietary nitrate such as beetroot, kale, spinach, and rocket, and to refrain from taking any other dietary supplements or using antibacterial mouthwash as the latter influences commensal bacteria in the oral cavity, resulting in the inhibition of NO_3^- reduction to NO_2^- (Govoni et al., 2008). In a double-blind, randomized, crossover design, subjects were randomly assigned to four conditions to receive NO_3^- -rich beetroot juice (BR) or placebo (PL) supplementation for 6 days, and an acute dose of NAC or maltodextrin (MAL) 1 h prior to the commencement of the exercise protocol. Each condition was separated by a wash-out period of at least 7 days (Reid et al., 1994; Wylie et al., 2013a). On day 6 of each of the four supplementation periods (see Supplementation), subjects reported to the laboratory to complete the experimental protocol. Experimental visits were performed at the same time of day ($\pm$ 2 h). Subjects recorded their activity and diet during the 24 h prior to the first experimental visit and were asked to repeat these for subsequent visits. Subjects were also instructed to arrive at the laboratory following a 10 h overnight fast, having avoided strenuous exercise and alcohol in the 24 h preceding, and

caffeine in the 8-h preceding, each experimental visit. Upon arrival at the laboratory, subjects were provided with a standardized breakfast consisting of 2 porridge oat sachets (Quaker Oats Ltd, Leicester, UK; containing 54 g of oats, 200 kcal, 4.2 g fat, 31.8 g carbohydrate, 5.6 g fibre, 6.0 g protein) mixed with 180 mL of water, 1-h prior to exercising.

Supplementation

Subjects were randomly assigned in a double-blinded fashion to four 6-day supplementation periods in which they consumed 2 x 70 mL doses per day of either NO_3^--rich beetroot juice (BR: ~6.2 mmol of NO_3^- per 70 mL; Beet It Sport, James White Drinks Ltd., Ipswich, UK) or NO_3^--depleted placebo (PL: ~0.04 mmol of NO_3^- per 70 mL; Beet It Sport, James White Drinks Ltd., Ipswich, UK), separated by a 7-day wash-out period. Each 70-mL beverage contained 72 kcal energy and 15.4 g of carbohydrate. On the first five days of each supplementation period, subjects consumed one 70-mL beverage in the morning and one in the evening, whereas on the experimental day, subjects consumed 2 x 70 mL of their allocated beverage in the morning 2.5 h prior to the exercise (Webb et al., 2008; Wylie et al. 2013a) and ingested 70 $mg \cdot kg^{-1}$ of NAC (Piping Rock Health Products, New York, USA, 600 mg NAC per capsule) or maltodextrin (MAL: Bulk Powders, Sports Supplements Ltd., Essex, UK) 1-h prior to the start of exercise (Borgstrom et al., 1986; Corn & Barstow, 2011). MAL was purchased in powdered form and was weighed out on an electronic scale and placed into capsules for the subjects to consume. Capsules for both NAC and MAL were identical in size and appearance. Peppermint-scented oil was applied to the outside of all capsules to make the supplementation homogenous in taste and smell to mask the strong sulfur smell of NAC (Bloch et al., 2013). The ingestion of capsules was consumed with 100 mL of water.

The four supplementation conditions were: NO_3^--depleted beetroot juice (PL) with maltodextrin (PL+MAL); 2) PL with NAC (PL+NAC); 3) BR with maltodextrin (BR+MAL); and 4) BR with NAC (BR+NAC). Subjects were contacted on a daily basis to ensure the consumption of supplement via a phone call, text or email, and were confirmed during pre-testing questionnaires on each experimental visit.

Experimental protocol

All exercise tests were performed on an electronically-braked cycle ergometer and controlled using the associated software computer program (Lode Excalibur Sport, Groningen, The Netherlands). On the first visit, subjects completed a ramp incremental test, involving 3 min of baseline cycling at 20 W, after which the work rate was increased by 30 W/min until task failure. Task failure was recorded once the cadence fell by >10 rpm below the target cadence. The self-selected cadence (70-90 rpm) and seat height and handle bar configuration were recorded and reproduced on subsequent visits.

Subjects commenced baseline cycling at 20 W for 3 min and then completed 1 h of cycling at the work rate associated with GET plus 20% of the difference between GET and peak work rate (20%Δ; Lansley et al., 2011) at their self-selected cadence. The TTE commenced immediately after the 1 h was completed. Subjects were provided with a 5 s countdown prior to the commencement of all cycling trials. The resistance on the pedals during the TTE was set for each individual using a power output equivalent to 70%Δ. Consistent verbal encouragement was provided for each TTE but subjects were deprived of visual performance cues and did not receive any notification on elapsed time. Blood samples were obtained for the determination of plasma [NO_3^-], [NO_2^-], [CYS], [GSH], and [MDA]. Neuromuscular function was assessed at every 1 min interval during the 3-min baseline period, as well as at 0 min, 30 min, 57 min, and at exhaustion (post-TTE). Respiratory muscle fatigue was assessed by measuring maximal expiratory pressure and was measured at baseline, 50 min during cycling, and post-TTE.

Blood analyses

Venous blood was sampled at baseline, 30, and 60 min during the 1 h heavy-intensity exercise bout, and immediately following the point of exhaustion following the TTE (post-TTE, *n*=15). All blood samples were obtained from a cannula (Insyte-W^{TM} Becton-Dickinson, Madrid, Spain) that was inserted in the subject's antecubital vein, and drawn into 6 mL lithium heparin tubes (Vacutainer, Becton-Dickinson, New Jersey, USA). For plasma [CYS] and [GSH] analysis (Newton et al., 1981; Shen et al., 2015), within 1 min of collection, 200 µL of whole

blood was added to 800 µL of ice-cold deionized water and centrifuged at 16 000 G, 4°C for 10 min to liberate and detect biological thiols through erythrocyte lysation. Following this, 60 µL of the supernatant was extracted and added to 140 µL of reaction buffer (Tris-HCl, 100 mM, pH 9.5, 0.1 mM Diethylenetriaminepentaacetic acid, DPTA), to release and stabilize thiols, and 100 µL of 10 mM monobromobimane in acetonitrile (CH_3CN) was added (Newton et al., 1981; Shen et al., 2015). After 30 min of incubation in the dark, 100 µL of ice-cold 200 mM sulfosalicyclic acid solution was added. The mixture was then incubated on ice for 10 min to stop the reaction and precipitate the proteins. After incubation, the samples were centrifuged at 16 000 G at 4ºC for 10 min and the supernatant was extracted and frozen at -80ºC for subsequent injection into the HPLC system (Perkin Elmer® Flexar™ Chomera® software version v.4.1.2.6410, Perkin Elmer Inc., MA, USA). The supernatant was diluted (1:20 fold) in deionized water, and injected (10 µL) into the HPLC for analysis. The HPLC mobile phase was 0.1% trifluoroacetic acid aqueous and 0.1% trifluoroacetic acid in acetonitrile running through a 250 x 4.6 C18 5µm Brownlee validated column (Perkin Elmer Inc., MA, USA) at 0.9 mL/min flow rate, with fluorescence detection at 390 nm excitation and 475 nm emission, determined against standard curves from commercially available CYS and GSH. Individual representative curves for plasma [CYS] and [GSH] are illustrated in Fig 1A.

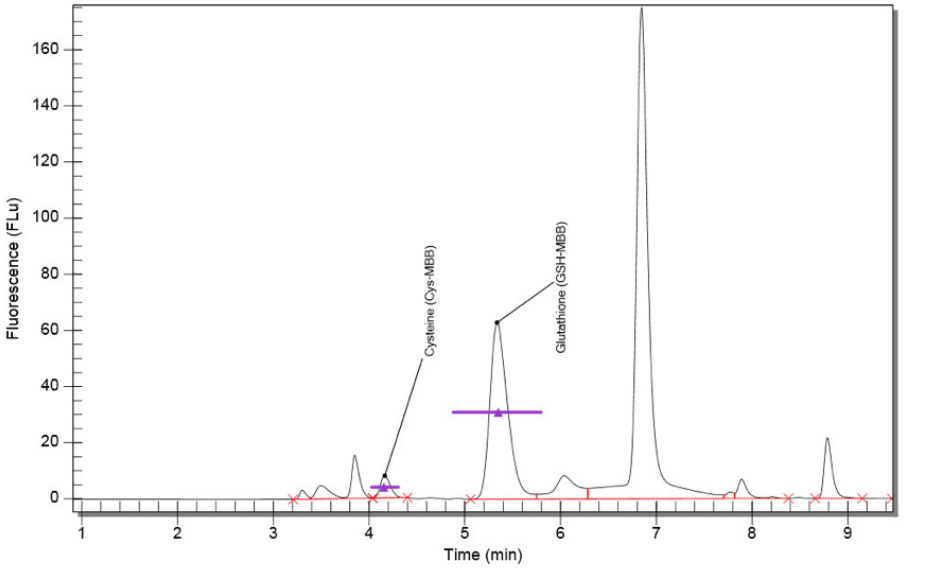

Figure 1A. Individual representative curve for plasma cysteine and glutathione assessed via high performance liquid chromatography.

The remaining whole blood samples were centrifuged within 2 min of collection at 4000 rpm and 4°C for 10 min and then the plasma was immediately extracted and frozen at -80°C. For the analysis of plasma [NO_3^-] and [NO_2^-], samples were deproteinized and analyzed via gas phase chemiluminescence as previously described (Tan et al., 2018). For plasma MDA analysis (Moselhy et al., 2013), 100 µL of plasma was added to 25 µL of 3M NaOH and heated at 60ºC in a water bath for 30 min for alkaline hydrolysis (Grotto et al., 2007). Following this, 1 mL of 0.05 sulphuric acid and 500 µL of 20% w/v trichloroacetic acid were added. The samples were centrifuged at 3000 rpm for 10 min and 1 mL of the supernatant was extracted into a new heat resistant tube. Following this, 500 µL of 0.355% w/v thiobarbituric acid was added and the samples were vortexed. Samples were heated at 90ºC for 40 min. Once cooled, the samples were centrifuged at 3000 rpm for 10 min and were injected (25 µL) into the HPLC for analysis. The HPLC mobile phase was 0.1% formic acid aqueous solution and methanol organic running through a 150 x 4.6 C18 5µm Brownlee validated column (Perkin Elmer Inc., MA, USA) at a 1.0 mL/min flow rate, with photodiode array detection analytical absorbance measured at 532 nm, reference 395 nm, determined against standard curves from commercially available MDA. An individual representative curve for plasma [MDA] is illustrated in Fig. 1B.

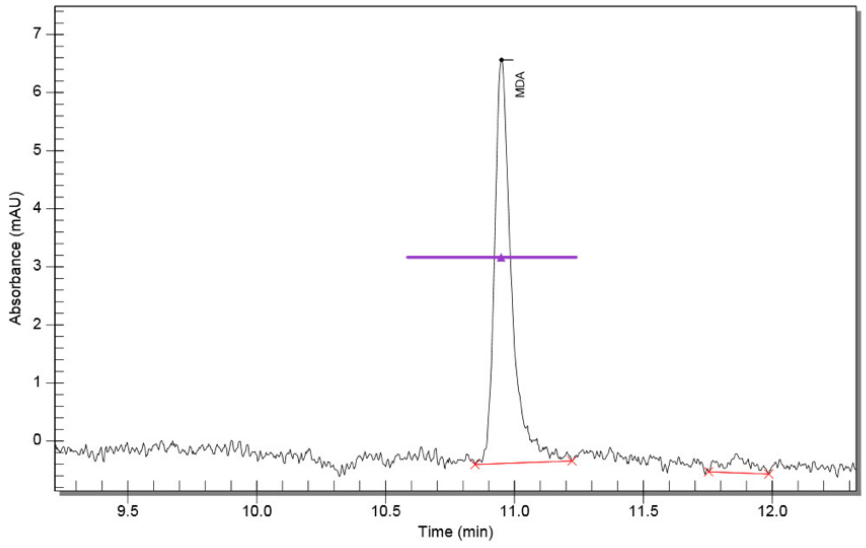

Figure 1B. Individual representative curve and plasma malondialdehyde assessed via high performance liquid chromatography.

Neuromuscular function

Neuromuscular function was assessed using methods reported previously (Black et al., 2017). Briefly, EMG was recorded continuously on the *vastus lateralis* (VL) for muscle activity during exercise using active bipolar bar electrodes with single differential configuration (DE2.1; Delsys, Boston, MA), positioned over the muscle belly [surface EMG for noninvasive measurement of muscles (SENIAM) guidelines]. The ground electrode was positioned on the patella. The skin area underneath each electrode was shaved, abraded, and cleaned with alcohol swabs before electrode placement to minimize skin impedance. The EMG signal was considered of good quality when the average rectified EMG baseline level for each muscle was below 2 μV (De Luca, 1993). The EMG signals were preamplified (1,000x), band-pass filtered (20-450 Hz, Bagnioli-8; Delsys), and digitized at a sampling rate of 2,000 Hz and resolution of 16 bits using a Power 1401 mk-II analog-to-digital converter and Spike 2 data collection software run by custom-written sampling configuration (CED; Cambridge Electronic Design, UK). The location of the optimal site for transcutaneous femoral nerve stimulation was determined while subjects were positioned on the cycle ergometer. Using an adhesive cathode (Boots UK, Nottingham, England) placed ~2 cm medial of the femoral pulse, and an adhesive anode (Boots UK) placed at the anterior aspect of the iliac crest, single electrical pulses generated by a constant current stimulator (DS7 A; Digitimer, UK) were delivered. The cathode was systematically moved vertically and horizontally, and the amplitude of the compound muscle action potential (i.e., M-wave) was monitored to identify the optimal position of the cathode for attaining maximal peak-to-peak M-wave amplitude during the cycling trials.

Following attachment of EMG and stimulation electrodes, the crank angle at which stimulation was to be delivered during the trials was determined for each subject. Subjects were positioned on the cycle ergometer and cycled at a moderate work rate (20 W below GET) for 1 min. EMG activity obtained during this period was rectified and averaged for 20 complete crank revolutions. The duration of each revolution was determined by a custom-made magnetic switch that generated an event marker signal on each occasion that the crank passed

top dead center (i.e., 0°). For each subject, the crank angle at which the rectified VL EMG activity was maximal was determined, and as performed by Sidhu et al. (2012), stimulations were delivered at the identified crank angle for all subsequent trials for that participant (65 ± 5° relative to the top dead center). A custom-written sequencer script triggered three stimulations, with at least 1 and up to 10 pedal revolutions between stimuli. The intervals were randomly determined using a random number generator incorporated within the sequencer script. This was designed to prevent subjects from anticipating the stimulus delivery, which may have affected the evoked response.

A standard M-wave recruitment curve protocol was completed during each laboratory visit. Subjects cycled at 20 W below GET throughout the recruitment curve protocol. A single-pulse electrical stimulation (200 μs) was delivered at the individually identified crank angle as described above. The current was increased in 20-mA increments until the M-wave amplitude reached a plateau at the maximal M-wave amplitude (M_{max}). A pulse of 120% M_{max} current was applied during the exercise tests (mean stimulation intensity, 290 ± 48 mA).

EMG signals from the VL were processed using a custom-written script to measure peak-to-peak M-wave amplitude and M-wave area. The root mean square (RMS) of the EMG signal (an index of the power of the signal) was calculated as the mean over a 25-ms pre-stimulation period at each stimulation time point. The EMG RMS amplitudes were normalized to the corresponding values attained after 1 min of exercise during each trial to evaluate temporal changes in the voluntary muscle activation level (i.e., EMG RMS amplitude) and M-wave parameters were normalized to the corresponding values attained during baseline exercise during each trial to evaluate temporal changes in the peripheral neuromuscular excitability (i.e., M-wave amplitude and area). In addition, the voluntary EMG RMS amplitude was normalized to the M-wave amplitude recorded at that time point to assess changes in neural drive (RMS/M; Millet & Lepers, 2004). The rates of change in M-wave and EMG parameters from baseline cycling to post-TTE were calculated for each exercise to quantify the rate of neuromuscular fatigue development.

The coefficients of variation (CV%) was 10% and 8% for between trials during unloaded cycling for M-wave amplitude and M-wave total area, respectively, and 11% and 9% between stimulations during unloaded cycling for peak-to-peak M-wave amplitude and M-wave total area, respectively.

Respiratory muscle fatigue

Maximum expiratory pressure was assessed according to published guidelines (American Thoracic Society/European Respiratory Society, 2002) using a portable handheld mouth pressure meter (Micro RPM handheld mouth pressure meter, Micro Medical Limited, Rochester, UK) to estimate exercise-induced respiratory muscle fatigue. All measurements were taken with the subjects seated and wearing a nose clip. Subjects held the rubber flanged mouthpiece tightly around their lips to prevent leaks. Subjects were verbally instructed to exhale to residual volume, then to maximally inhale to total lung capacity, immediately followed by performing the Valsalva manoeuvre (i.e. an exhalation as quickly and as hard as possible) for at least 1 s. Subjects performed this manoeuvre four times, at rest, during exercise at 50 min, and post-TTE, with the greatest value of three measurements with less than 20% variability recorded.

Statistical analyses

A two-way (condition x time) repeated measures analysis of variance (ANOVA) was used to analyse differences in plasma [NO_3^-], [NO_2^-], [CYS], [GSH], [MDA], neuromuscular function and maximal expiratory pressure during the 1 h heavy-intensity exercise bout. A one-way repeated measures ANOVA was used to analyse differences in TTE. Significant main and interaction effects were further explored using Fisher's LSD test. In addition, one-way repeated measures ANOVAs were used to determine physiological performance differences in the mean and change values from pre- to post- 1 h heavy-intensity exercise, and post-TTE. A full set of blood data were only available for n=14 for time points 0 to 60 min and n=13 for time point post-TTE due to problems with cannulation and/or blood draw. Neuromuscular data were analysed for a subset of n=12. Data for TTE are reported for n=15 owing to n=1 having foot slip out of pedal. Statistical significance was accepted at P<0.05, and P<0.10 was considered as a trend. Results are presented as mean ± SD unless otherwise stated.

Results

All subjects reported consuming all servings of each supplement at the correct times and confirmed that they had maintained their exercise and dietary habits prior to each laboratory visit. There were no reports of adverse reactions, gastrointestinal distress or discomfort following the ingestion of BR or NAC.

Plasma [NO_3^-] and [NO_2^-]

There was an interaction effect (condition x time) (P<0.05*),* main effect of condition (P<0.01), but no effect of time (P>0.05) for plasma [NO_3^-] (Fig. 2A). Plasma [NO_3^-] was higher in BR+NAC (P<0.01) and BR+MAL (P<0.01) compared to PL+NAC and PL+MAL at all time points. Plasma [NO_3^-] in BR+NAC compared to BR+MAL at tended to be higher at 0 min (P=0.07), was significantly higher at 60 min (P<0.05), and tended to be higher at post-TTE (P=0.08). There was no difference between PL+NAC and PL+MAL at any time point (P>0.05). Plasma [NO_3^-] declined from 0 min to 60 min in BR+MAL (P<0.05), PL+MAL (P<0.05), and PL+NAC (P<0.01) but not in BR+NAC (P>0.05).

There was an interaction effect (condition x time) (P=0.05), main effect of time (P<0.01), and main effect of condition (P<0.01) for plasma [NO_2^-] (Fig. 2B). At baseline, plasma [NO_2^-] was significantly elevated in BR+NAC (P<0.01) and BR+MAL (P<0.01) compared to PL+NAC and PL+MAL. There was no difference in plasma [NO_2^-] at baseline between BR+NAC and BR+MAL (P>0.05) or between PL+NAC and PL+MAL (P>0.05). There was no significant change in plasma [NO_2^-] over 1 h of exercise in all conditions (P>0.05). Plasma [NO_2^-] significantly declined from 60 min to post-TTE in BR+NAC (P<0.05), BR+MAL (P<0.05) and PL+NAC (P<0.05) but not PL+MAL (P>0.05).

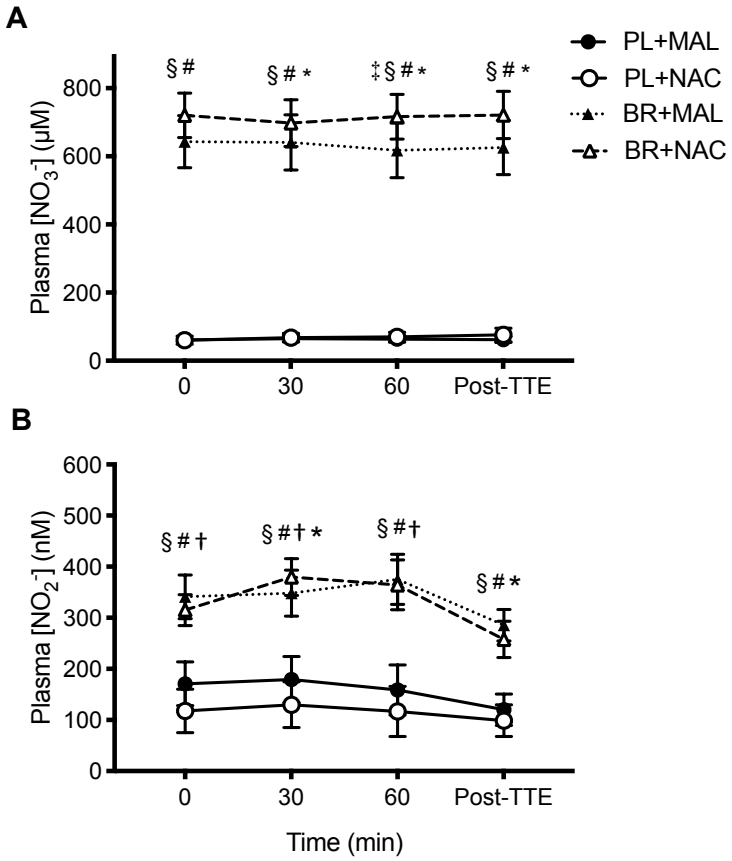

Figure 2. Mean ± SE plasma nitrate (NO_3^-; panel A) and nitrite (NO_2^-; panel B) over 1-h of heavy-intensity exercise and a subsequent TTE following: PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise (solid circle and solid line); PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise (open circle and solid line); BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise (solid triangle and dotted line); and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise (open triangle and dotted line). * = significantly different from 0 min (P<0.05), † = significantly different from TTE (P<0.05), § = significantly different in BR+NAC compared to PL+MAL and PL+NAC (P<0.01), # = significantly different

in BR+MAL compared to PL+MAL and PL+NAC (P<0.01), ‡ = significantly different in BR+NAC compared to BR+MAL (P<0.05). Data shown for a subset of n=14 from 0 to 60 min and for n=13 for post-TTE.

Plasma [CYS], [GSH] and [MDA]

There was a tendency for an interaction effect (condition x time) (P=0.06), main effect of time (P<0.01), and a tendency for a main effect of condition (P=0.06) for plasma [CYS] (Fig. 3A). Plasma [CYS] was significantly greater in PL+NAC (11 ± 5 µM, P<0.01) and BR+NAC (15 ± 6 µM, P<0.01) compared to PL+MAL (7 ± 3 µM) at rest. Plasma [CYS] increased over time compared to 0 min (P<0.01). There was a significant difference between the change in plasma [CYS] from 0 min to 60 min in BR+NAC and BR+MAL compared to PL+NAC and PL+MAL (P<0.05; Fig. 3B).

There was a main effect of time only (P<0.01) where plasma [GSH] increased from 0 min to 60 min (P<0.01) and post-TTE (P<0.01; Fig. 3C). There was no main effect of condition in the change of plasma [GSH] from 0 min to 60 min (P>0.05; Fig. 3D).

There was no interaction effect, main effect of time, or main effect of condition on plasma [MDA] (all P>0.05; Fig. 3E) or the change in plasma [MDA] from 0 to 60 min (all P>0.05; Fig. 3F).

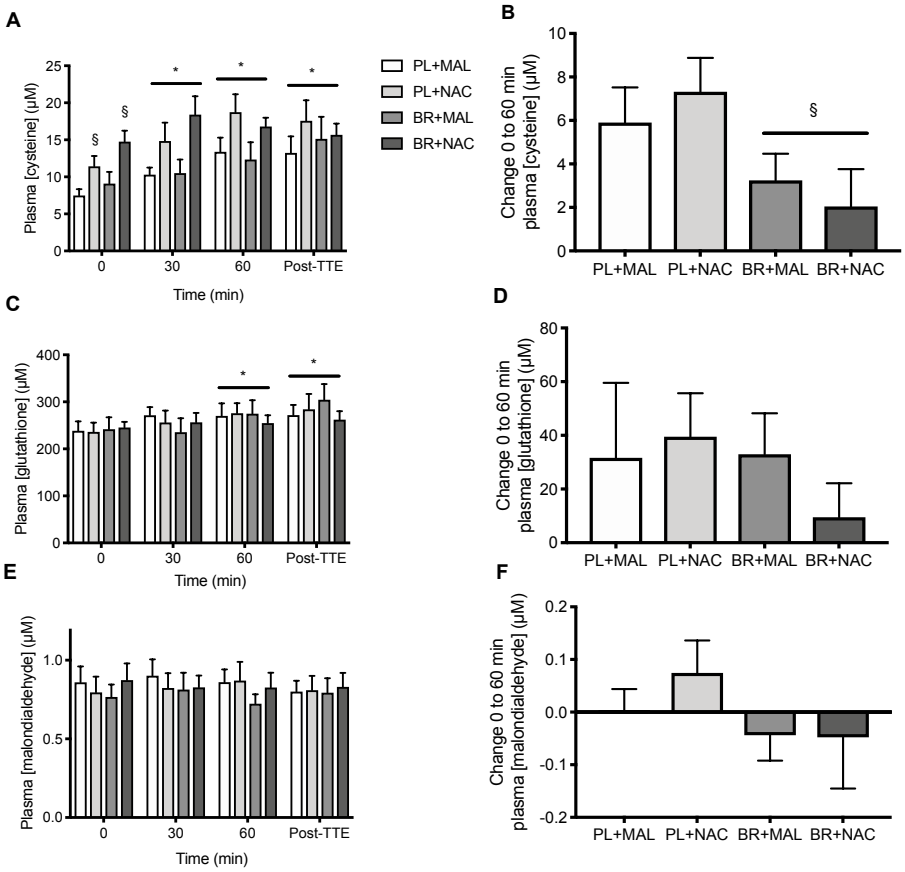

Figure 3. Mean ± SE plasma cysteine (panel A), glutathione (panel C), and malondialdehyde (panel E) over 1-h of heavy-intensity exercise and a subsequent TTE. Change from 0 to 60 min in plasma [cysteine] (panel B), change in plasma [glutathione] (panel D), and change in plasma [MDA] (panel F). PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise. * = significantly different from 0 min (P<0.01). § = significantly different to PL+NAC and PL+MAL (P<0.05). Data shown for a subset of n=14 for time points 0 to 60 min and n=13 for time point post-TTE for plasma [cysteine] and [glutathione], and a subset of n=13 for plasma [malondialdehyde].

Neuromuscular excitability: M-wave amplitude and M-wave area

Peripheral neuromuscular excitability, indicated by M-wave amplitude and M-

wave area at time points 0, 30, 57 min, and post-TTE are shown in Figure 4A and B. There was a main effect of time (P<0.001) but no effect of condition on the M-wave amplitude (P>0.05) and M-wave area (P>0.05). There was a main effect of condition (P<0.05) in the change of M-wave amplitude from 0 to 57 min (Fig. 5). The change in M-wave amplitude from 0 to 57 min was significantly greater in BR+NAC (-51 ± 41%; P<0.01) and PL+MAL (-45 ± 24%; P<0.05) compared to PL+NAC (-16 ± 28%). There was a tendency for the change in M-wave amplitude from 0 to 57 min in BR+MAL (-29 ± 27%) to be different from BR+NAC (-51 ± 41%; P=0.08). There was no effect of condition (P>0.05) or time (P>0.05) on the change from 0 to 57 min in the M-wave area.

Voluntary activation and neural drive

Voluntary muscle activation level, measured as EMG RMS amplitude, and neural drive, as indicated by RMS/M-wave amplitude, at time points 0, 30, 57 min, and post-TTE are shown in Fig. 4C and D. There was a main effect of time (P<0.001) but no effect of condition on EMG RMS (P>0.05) and RMS/M-wave amplitude (P>0.05). There was no effect of condition (P>0.05) or time (P>0.05) on the change from 0 to 57 min in the voluntary activation or neural drive.

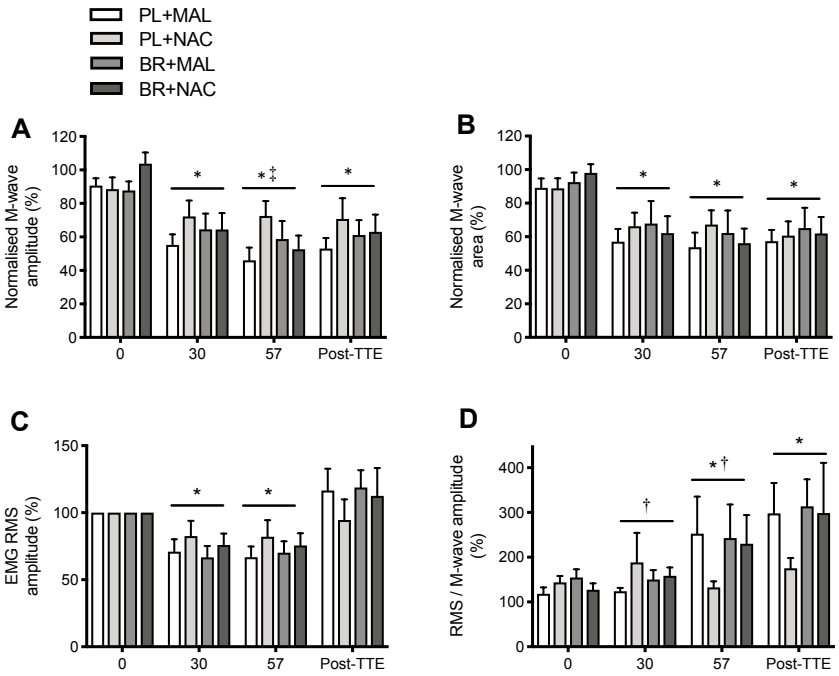

Figure 4. Compound muscle action potential (M-wave) amplitude and M-wave area (normalized to maximum M-wave during baseline pedalling) indicating peripheral neuromuscular excitability (panel A and B); voluntary electromyographic root mean square (EMG RMS) amplitude (normalized to M-wave amplitude at 1 min of exercise) indicating muscle activation level (panel C); and RMS/M-wave (normalized to corresponding M-wave amplitude at each measurement time point) indicating central fatigue (panel D) during 1-h of heavy-intensity cycling at 0, 30, and 57 min, as well as at the end of the time-to-exhaustion (Post-TTE). * significantly different from 0 min (P<0.01); ‡ = significantly different from 30 min; † = significantly different from post-TTE (P<0.01). Data shown for subset of n=12.

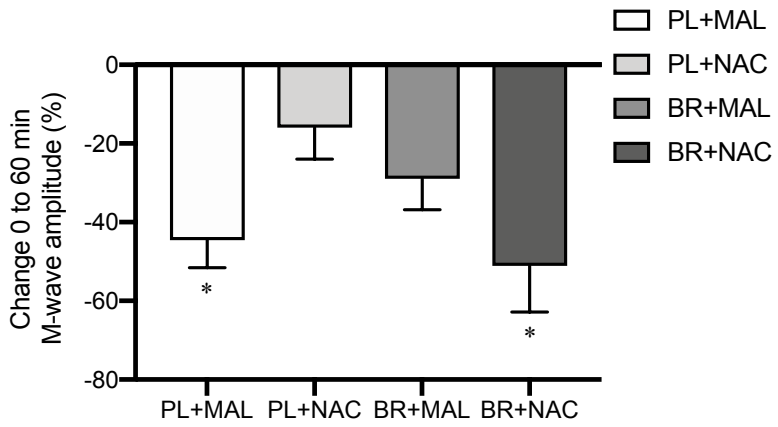

Figure 5. Change from 0 to 60 min in compound action potential (M-wave) amplitude indicating peripheral neuromuscular excitability. * = significantly different to PL+NAC. Data shown for subset of *n*=12.

Maximum expiratory pressure

There was a main effect of time (P<0.01) but no effect of condition (P>0.05; Fig. 6). Maximal expiratory pressure did not change from 0 min to 50 min (P>0.05) but declined at post-TTE (P<0.01).

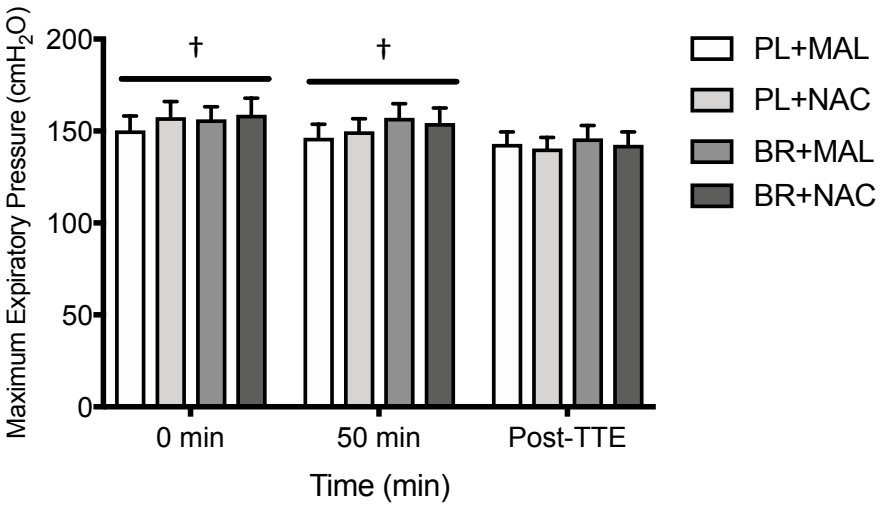

Figure 6. Mean ± SE MEP measured at 0 and 50 min during 1-h of heavy-intensity cycle exercise and post-TTE. PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise.

Time-to-exhaustion performance

No significant differences were found between conditions for TTE at 70%Δ (Fig.

7).

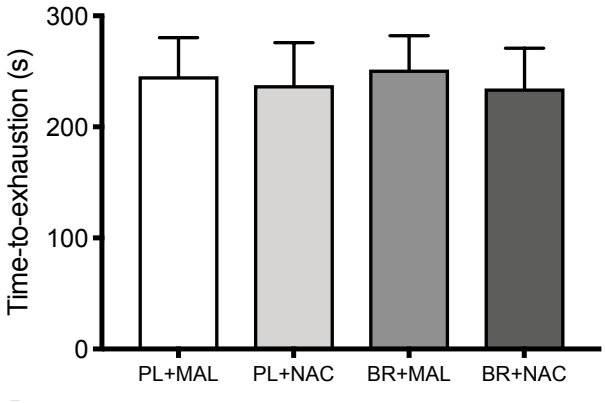

Figure 7. Mean ± SE completion time during the time-to-exhaustion at 70%Δ following 1-h of heavy-intensity cycle exercise. PL+MAL: nitrate-depleted beetroot juice and maltodextrin consumed before exercise; PL+NAC: nitrate-depleted beetroot juice and N-acetylcysteine consumed before exercise; BR+MAL: nitrate-rich beetroot juice and maltodextrin consumed before exercise; and BR+NAC: nitrate-rich beetroot juice and N-acetylcysteine consumed before exercise. Data shown for a subset of *n*=15.

Discussion

This was the first study to investigate the independent and concurrent influence of BR and NAC administration on indices of NO bioavailability, oxidative stress, neuromuscular function and respiratory muscle fatigue during a preload of 1-h of heavy-intensity cycling exercise and subsequent severe-intensity exercise tolerance in humans. The major novel findings of the present study were that: 1) co-ingestion of BR and NAC (BR+NAC) did not influence physiological responses during prolonged exercise; 2) the decline in muscle excitability over 1-h of heavy-intensity exercise was significantly attenuated in NAC alone (PL+NAC) compared to BR+NAC and PL+MAL, and tended to be attenuated in BR alone (BR+MAL) compared to BR+NAC; and 3) there was no influence of any condition on subsequent exercise tolerance. These findings are in contrast to our hypotheses, and indicate that combining BR+NAC could abolish possible physiological benefits of either supplement ingested alone.

Influence of dietary NO_3^- and NAC supplementation on indices of NO bioavailability

Both plasma [NO_3^-] and [NO_2^-] were similarly elevated following BR+NAC and BR+MAL compared to PL+MAL and PL+NAC. Results showed that plasma [NO_3^-

] declined over time in all conditions while plasma [NO_2^-] only significantly declined following the prolonged exercise bout at exhaustion. Interestingly, plasma [NO_3^-] was preserved in BR+NAC compared to BR+MAL at 60 min, suggesting that co-ingestion of BR and NAC preserves NO_3^- over prolonged exercise. Given that NO is rapidly oxidized to NO_3^- (Lundberg et al., 2008), the reduction in the relative magnitude of decline in plasma [NO_3^-] could indicate improved NO bioavailability following BR+NAC compared to BR ingested alone. Co-ingestion of BR and NAC and their associated antioxidant properties could have preserved NO from being scavenged by ROS produced over time (Clanton et al., 2007; Reid et al., 1992). Despite this, we observed no difference in plasma [NO_2^-] between BR+NAC and BR+MAL, which is consistent with previous findings (Bailey et al., 2011; Crum et al., 2018). It is unclear why plasma [NO_3^-] was further elevated by BR+NAC but not plasma [NO_2^-] but may be due to NO_2^- potentially acting as a signalling molecule (Bryan et al., 2005), participating in S-nitrosylation (Smith & Marletta, 2012), or entering muscle tissue (Piknova et al., 2015). Collectively, these data suggest that combining BR and NAC may preserve NO bioavailability, as reflected in plasma [NO_3^-], over prolonged exercise.

Influence of dietary NO_3^- and NAC supplementation on redox balance

Consistent with previous reports, we observed that plasma [CYS] was elevated following NAC supplementation in BR+NAC and PL+NAC (Ferreira et al., 2011; Medved et al., 2003; 2004b; Smith et al., 2016). However, despite the elevation in plasma [CYS], plasma [GSH] was not elevated at baseline in BR+NAC or PL+NAC which is consistent with some (Kelly et al., 2009; Trewin et al., 2013) but not all studies (Corn & Barstow, 2011; Medved et al., 2003; 2004b; Slattery et al., 2014). Interestingly, previous investigations have observed favourable effects following NAC supplementation independent of changes to plasma [GSH] (Kelly et al., 2009), which may reflect differences between the site of measurement compared to changes within the skeletal muscle following NAC administration (Medved et al., 2004b; Merry et al., 2010). Plasma [MDA] is commonly used to assess oxidative damage to lipids (i.e. lipid peroxidation) which could indicate damage to cellular membrane structure and function (Dillard et al., 1978; Halliwell & Grootveld, 1987; Hwang & Kim, 2007; Janero et al., 1990;

Valenzuela, 1991). In the present study, plasma [MDA] did not change over time and was not influenced by condition. These results are in contrast to our hypothesis that BR+NAC would produce additive favourable effects due to elevated NO and thiol bioavailability and associated antioxidant properties.

Influence of dietary NO_3^- and NAC supplementation on neuromuscular respiratory muscle function during prolonged heavy-intensity exercise

We observed a reduction in muscle excitability (M-wave amplitude and area) and voluntary activation (EMG RMS) over time, irrespective of supplementation, indicating a decline in force production, consistent with previous studies (Black et al., 2017; Lepers et al., 2000; Paris et al., 2019). At the onset of heavy-intensity exercise, both principal fibre types are activated to meet the force demands (Vøllestad et al., 1984) and accordingly, a decline in neuromuscular function over time could be interpreted as the decline or 'drop out' of higher order muscle fibres due to fatigue (Gandevia, 2001). Therefore, maintenance of muscle excitability is reflective of preserved skeletal muscle contractility (Clausen, 2003). Interestingly, the decline in muscle excitability was attenuated following PL+NAC compared to PL+MAL and BR+NAC, while BR+MAL tended to be attenuated compared to BR+NAC. These results suggest that ingesting either NAC or BR supplement alone may positively influence neuromuscular function but that combining NAC and BR negates potential benefits.

We observed that muscle excitability was preserved following PL+NAC. These results are consistent with and complements previous findings that reported attenuated plasma [K^+] disturbances and enhanced Na^+/K^+ ATPase pump activity following NAC infusion during prolonged submaximal exercise (McKenna et al., 2006). In addition, NAC supplementation has been reported to improve exercise capacity (Cobley et al., 2011; McKenna et al., 2006; Medved et al., 2004b; Slattery et al., 2014) and delay fatigue (Jannig et al., 2017; Khawli et al., 1994; Reid et al., 1994; Shindoh et al., 2013). Taken together, these studies indicate that NAC-induced modulations to the regulation of Na^+ and K^+ lead to enhanced muscle membrane excitability (Allen et al., 2008; Bangsbo et al., 2001), and that ROS, may in part, underpin the progressive dysregulation of K^+ handling over time during continuous exercise. Therefore, it could be possible that NAC

supplementation improved excitation-contraction coupling processes by preserving membrane polarization (i.e. a reduction in extracellular [K^+]) and thus preserving sarcoplasmic Ca^{2+} release rates and force production (MacIntosh et al., 2012; McKenna, 1992).

Muscle excitability tended to be better preserved during exercise in BR+MAL compared to BR+NAC. To date, this is the first investigation to examine the influence of inorganic NO_3^- supplementation on neuromuscular function during upright continuous cycling exercise. Our results suggest that independent administration of BR potentially maintains neuromuscular function during prolonged cycling exercise, but that any positive physiological effect could be abolished when co-ingested with NAC. Despite the potential for NAC to augment NO bioavailability (Simon et al., 1993), interactions between NO, thiols, and ROS are complex (Mason et al., 2016; Wynia-Smith & Smith, 2017; Xu et al., 1998). It is possible that divergent molecular signalling could occur with BR+NAC that interfere with each other, negating any effects from when either supplement is ingested alone. The potential BR+MAL to preserve muscle excitability in the present study could be due to enhanced K^+ handling (Wylie et al., 2013b). NO is a regulator of the Na^+/K^+ pump and is purported to induce effects via the cyclic guanosine monophosphate cascade and/or through post-translational protein modifications (Pirkmajer & Chibalin, 2016a). Interestingly, there is evidence to suggest that NO-mediated stimulations to Na^+/K^+ pump activity are limited to fatigue-sensitive type II fibres (Juel, 2016; McMorrow et al., 2011; Pirkmajer & Chibalin, 2016b). Further research is required to elucidate potential effects of dietary NO_3^- on muscle excitability and its underlying mechanisms.

We used maximal expiratory pressure as an index of respiratory muscle fatigue and found no effect of BR or NAC supplementation despite elevations in plasma [NO_2^-] and [CYS]. Although this is the first study to investigate the influence of NO_3^- on respiratory muscle fatigue, Behnia et al. (2018) reported that lung diffusion capacity tended to improve following BR supplementation suggesting potential for NO-induced modulations to pulmonary vascular function. Moreover, NAC supplementation has been reported to positively affect indices of respiratory muscle fatigue (Kelly et al., 2009; Shindoh et al., 1990; Smith et al., 2014;

Travaline et al., 1997). Therefore, our results contrast with current literature. This could be related to the absence of a significant decline in maximal expiratory pressure over the course of the prolonged exercise bout, reflecting limited development of respiratory muscle fatigue.

Influence of dietary NO_3^- and NAC supplementation on exercise tolerance

TTE performance was not different between conditions. This is in contrast to previous investigations reporting that BR and NAC supplementation may independently improve exercise tolerance by ~12-25% (Bailey et al., 2009; 2010; 2015; Breese et al., 2013; Lansley et al., 2011) and ~24-26% (McKenna et al., 2006; Medved et al., 2004b), respectively. Few studies are available that have employed a prolonged bout of exercise prior to the exercise performance task (Christensen et al., 2013; Tan et al., 2018). The reason for a lack of ergogenic effect in the present study is unclear but could be related to subject training status. Dietary NO_3^- supplementation is more likely to be beneficial among untrained individuals (Jones et al., 2018; Porcelli et al., 2015; Van de Walle & Vukovich, 2018), whilst NAC supplementation is more likely to be beneficial among trained individuals (Medved et al., 2003; Medved et al., 2004a; 2004b). Given that BR and NAC may be more efficacious for a different range of training statuses between the supplements, it is possible that combining both supplements has limited ergogenic potential.

Strengths and limitations

This Experimental Chapter provides insight to the potential abolishment of any benificial effect of co-ingesting NO_3^- and NAC on muscle excitability compared to when the supplements are ingested alone. However, it should be noted that a limitation to the present study is that although NO_3^- has the potential to preserve muscle excitability, the experimental design of the study does not reveal whether the effects were due to NO_3^- or the other antioxidant consituents present in beetroot juice (e.g. betanin). In addition, the present study does not provide mechanistic insight into the reason co-ingestion of NO_3^- and NAC abolishes any beneficial effect of independent ingestion. Future studies may consider including additional measures to elucidate these mechanisms.

Conclusion

In conclusion, combining inorganic NO_3^- and NAC supplementation potentially preserves NO bioavailability, but did not augment changes in redox status, or physiological responses during 1 h of heavy-intensity cycling exercise. However, independent ingestion of NO_3^- and NAC supplementation preserved muscle excitability compared to when the two compounds were ingested together. Despite the attenuated decline in muscle excitability over time, subsequent exercise tolerance was not different across conditions. Taken together, our findings indicate that co-ingestion of dietary NO_3^- and NAC supplementation may abolish any potential physiological benefit of ingesting either supplement alone. These findings have implications for supplementation strategies that are employed for athletes competing in endurance endurance athletes.

Experimental Chapter 7 achieved the fourth aim of the present thesis by providing insight suggesting that co-ingestion of NO_3^- and NAC do not have synergistic effects on neuromuscular or respiratory muscle function, and that co-ingestion potentially inhibits any potential beneficial effect of either supplement ingested independently on muscle excitability. Importantly, there was also no effect of independent or combined ingestion of NO_3^- and NAC on subsequent exercise performance. Together, Experimental Chapter 4, 5, 6, and 7 address the four aims of the thesis and contributes to current knowledge by providing insight to the potential effects of NO_3^- on preserving intramuscular glycogen, intramuscular phosphocreatine, and muscle excitability during prolonged cycling exercise, as well as the dynamic response of NO bioavailability following a change in the supplementation strategy.

Chapter 8: General Discussion

NO is an essential free radical that is continuously produced by the skeletal muscle at rest and during exercise (Balon & Nadler, 1994). NO functions to activate signalling cascades or to modulate proteins to regulate contractile function, metabolism, and perfusion (Stamler & Meissner, 2001). Recent advances in the understanding of the physiological importance of NO has fuelled research that has focused on modulating NO bioavailability, in which particular attention has been paid to dietary NO_3^- supplements, to influence physiological responses to exercise (Affourtit et al., 2015; Jones et al., 2018). NO_3^- supplements are orally administered as NO_3^--rich BR juice or NO_3^- salts as a strategy to elevate NO production, in which the elevation of NO is commonly reflected by the surrogate marker, elevated plasma [NO_2^-] (Larsen et al., 2007; Webb et al., 2008; Wylie et al., 2013a). Dietary NO_3^- supplementation has been shown to improve muscle efficiency (i.e. lower the O_2 cost of exercise; Pawlak-Chaouch et al., 2016) which could be partly explained by the independent or combined effects of a reduction in the energetic cost of contraction (Bailey et al., 2010), and/or a reduction in the O_2 cost of producing ATP (Larsen et al., 2011; Whitfield et al., 2016). Dietary NO_3^- supplementation has also been shown to induce an ergogenic effect (Jones et al., 2018), which could be associated with a preferential augmentation in the physiological responses of fatigue-sensitive type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012; Jones et al., 2016). The mechanistic bases underpinning the positive physiological effects of NO_3^- on exercise performance have yet to be fully understood. Therefore, this thesis contributes novel findings that address: 1) the physiological effects of dietary NO_3^- supplementation in prolonged continuous exercise, an exercise modality that would be expected to exhibit a progressive loss of muscle efficiency and/or recruitment of a greater proportion of type II muscle fibres; and 2) supplementation strategies to optimize NO bioavailability and associated ergogenic effects.

8.1. Research questions addressed

The aim of this thesis was to investigate the potential for dietary NO_3^- supplementation to enhance physiological responses during prolonged exercise and subsequent exercise performance, as well as potential supplementation

strategies to optimize NO bioavailability. We sought to answer the following research questions:

1) Do bioactive compounds other than NO_3^-, such as the polyphenol compounds within NO_3^--depleted beetroot juice, influence NO bioavailability and the physiological response to exercise and does the number of transitions influence the ability to detect a change in the O_2 cost of exercise following NO_3^- supplementation?
2) What are the effects of pre-exercise and *during* exercise ingestion of NO_3^- supplementation on the physiological responses during prolonged moderate-intensity exercise and on subsequent exercise performance?
3) What is the influence of pre-exercise NO_3^- ingestion on skeletal muscle contractile efficiency and muscle oxidative capacity over a prolonged exercise protocol?
4) What are the physiological and performance effects of co-ingesting pre-exercise NO_3^- and thiol supplementation on prolonged heavy-intensity exercise?

8.2. The influence of NO_3^- on physiological responses during prolonged exercise

Over a decade of groundwork has been laid by exercise physiologists and biochemists in furthering our understanding of how dietary NO_3^- supplementation influences physiological function during exercise. The earliest studies reported the surprising observation that the O_2 cost of exercise was lowered by NO_3^- ingestion using experimental designs that employed short duration constant work rate exercise bouts (Larsen et al., 2007; Bailey et al., 2009; 2010). Since then, research has continued to increase our understanding of the mechanistic bases of NO_3^- and its application. Recent evidence indicates that NO_3^- supplementation preferentially augments Ca^{2+} handling (Hernández et al., 2012) and muscle oxygenation in type II muscle fibres (Ferguson et al., 2013) suggesting the potential for NO_3^- to be efficacious in improving physiological responses in exercise modalities which require a greater relative contribution from type II muscle fibres (Bailey et al., 2015; Breese et al., 2013; Ferguson et al., 2013; Hernández et al., 2012; Jones et al., 2016). Accordingly, investigations into the effects of dietary NO_3^- supplementation in high-intensity intermittent type exercise

have drastically increased (Nyakayiru et al., 2017a; Thompson et al., 2015; Wylie et al., 2013b). However, there is also potential for NO_3^- supplementation to influence physiology during prolonged exercise since long durations could require significant type II muscle fibre activation (Krustrup et al., 2004) and that a progressive decline in muscle function occurs over long durations of exercise (Brueckner et al., 1991; Hermansen et al., 1967; Hopker et al., 2017; Ørtenblad et al., 2011). In addition, at the start of this thesis, although many studies predominantly employed submaximal continuous endurance exercise, the majority of the experimental designs lack application given that only a few investigations employed exercise durations that are applicable to popular endurance sports lasting ≥ 1 h (e.g. half marathons, marathons, triathlons). Therefore, one of the overarching themes of this thesis was to further understand the influence of dietary NO_3^- supplementation on the physiological responses in prolonged continuous type exercise, with a focus on the potential for NO_3^- supplementation to attenuate decline in physiological function over time.

Chapters 4, 5, and 6 contributes to knowledge by demonstrating that skeletal muscle function declines over prolonged exercise consistent with previous reports (Christensen et al., 2013; Hermansen et al., 1967; Hopker et al., 2017; Paris et al., 2019; Passfield & Doust, 2000). As discussed previously (see: *Literature Review*), the decline in muscle function during exercise is multi-factorial and can be reflected in numerous physiological variables. Chapter 4 revealed that a progressive rise in $\dot{V}O_2$ occurred in parallel with the depletion of muscle metabolite concentrations over 2 h of moderate-intensity cycling exercise. In Chapter 5, muscle PCr utilization progressively increased over 2 h of single-legged knee extensor exercise while in Chapter 6, neuromuscular function (i.e. muscle excitability and voluntary activation) declined over 1 h of heavy-intensity cycling exercise. Together, Chapters 4, 5 and 6 provide evidence that a decline in skeletal muscle function manifests over long duration exercise. In addition, given that the main mechanistic bases currently thought to underpin the effects of dietary NO_3^- supplementation include enhancing contractile and/or mitochondrial efficiency (Bailey et al., 2010; Hernández et al., 2012; Larsen et al., 2011; cf. Whitfield et al., 2016), as well as improving muscle oxygenation in a fibre-type specific manner (Ferguson et al., 2013) these findings provide rationale

for exploring the potential influence of NO_3^- supplementation during prolonged exercise.

The first investigation to examine the influence of NO_3^- supplementation in prolonged type exercise had trained male cyclists acutely ingest NO_3^- (0.5 L of BR juice, ~6.2 mmol of NO_3^-) prior to completing a 50-mile TT (Wilkerson et al., 2012). Results showed that following NO_3^- ingestion, $\dot{V}O_2$ tended to be lower (P=0.06) which improved the power output to $\dot{V}O_2$ ratio during the 50-mile TT (Wilkerson et al., 2012). However, despite these physiological improvements, TT performance was not significantly influenced (Wilkerson et al., 2012). Importantly, the authors observed a correlation between the relative magnitude of increase in plasma [NO_2^-] and the reduction in TT completion time (r = -0.83, P=0.01) which suggests the importance in the elevation of plasma [NO_2^-] in the efficacy of NO_3^- supplementation (Wilkerson et al., 2012). In another study, Christensen et al. (2013) had trained male cyclists perform a 2 h cycling exercise bout prior to a 400 kcal TT. The 2 h preload consisted of four identical 30 min stages whereby trained male cyclists performed 50% W_{peak} for 20 min, followed by 10 min of set variable intensities ranging from 35% to 80% W_{peak} to reflect variation in road racing (Christensen et al., 2013; Palmer et al., 1999). These authors observed that the O_2 cost of exercise increased over time but that there was no influence of 6 days of NO_3^- supplementation on exercise economy (Christensen et al., 2013). The only other available study found no change in $\dot{V}O_2$ over time and that there was no influence of acute NO_3^- ingestion (i.e. 2 h prior to exercise) on $\dot{V}O_2$ or glucose metabolism over 60 min at 65%$\dot{V}O_{2peak}$ in recreationally active males (Betteridge et al. 2016). However, the experimental designs of these studies are not in line with our recent advances in understanding of NO_3^- dosing regimens (Wylie et al., 2013a; 2016) and training status (Pawlak-Chaouch et al., 2016; Porcelli et al., 2015) which suggest that NO_3^- supplementation is most likely to be efficacious in recreationally active subjects with short-term (3 to 6 days) NO_3^- supplementation. Therefore, it cannot be excluded that dietary NO_3^- supplementation can enhance physiology during prolonged exercise when greater type II muscle fibre recruitment and a progressive decline in exercise efficiency could be expected, and/or when more appropriate supplementation strategies to manipulate NO bioavailability are employed. Accordingly, Chapters 4, 5, and 6 aimed to examine

if short-term dietary (3 to 6 d) NO_3^- supplementation could improve the physiological responses to prolonged exercise.

The novel findings from Chapter 4 were that dietary NO_3^- supplementation before and during exercise attenuated the progressive rise in the O_2 cost of exercise and tended to attenuate the decline in muscle [glycogen] (P=0.09) and [PCr] (P=0.08) during 2 h of moderate-intensity cycling exercise. Importantly, these physiological effects were only observed when a single dose of NO_3^--rich BR juice was ingested 1 h during exercise, in addition to 3 days of pre-exercise NO_3^- supplementation. Together, these data suggest that the metabolic cost of contraction was preserved over time and may be a consequence of enhanced NO bioavailability. Therefore, Chapter 4 demonstrates, for the first time, that dietary NO_3^- supplementation *during* exercise may be an effective strategy for inducing positive physiological responses, particularly in exercise protocols where NO bioavailability may decline over time (see: *Section 8.4.*). An interesting finding is that this is the first (and currently only) study to observe an influence of dietary NO_3^- supplementation on muscle glycogen depletion (cf: Betteridge et al., 2016). As previously discussed (see: *Literature Review*), muscle glycogen stores have a key role in skeletal muscle contractile function (Hermansen et al., 1967; Nielsen et al., 2009; Ørtenblad et al., 2011). Thus, the sparing of muscle glycogen, which can be interpreted to be a consequence of improved contractile and/or mitochondrial efficiency, indicates the potential for enhanced physiology following NO_3^- supplementation during prolonged exercise. In support of this, previous investigations have reported enhanced mitochondrial efficiency via reducing proton leakage (Larsen et al., 2011; cf. Whitfield et al., 2016) and a reduction in the ATP cost of contraction (Bailey et al., 2011) which provide the mechanistic bases that could underpin the glycogen sparing observed. Consistent with the findings of Bailey et al. (2010), Chapter 4 found that the decline in muscle [PCr] tended to be attenuated with NO_3^- supplementation, reflecting a lower energetic cost of force production. Although a reduction in the overall muscle metabolic cost of contraction is likely to contribute to the attenuated rise in O_2 cost of exercise, it is also possible that modulations to glucose uptake partly contributed as well. Interestingly, NO and NO_2^- molecules have been reported to influence glucose transporter protein activity (Merry et al., 2010; Jiang et al., 2014). In support of this, Wylie et al. (2013b) observed a

lowered blood glucose concentration during high-intensity intermittent type exercise, complementing the results of Chapter 4 to suggest that dietary NO_3^- supplementation has the potential to influence carbohydrate utilization. Further research is required to establish the effects of dietary NO_3^- supplementation on glycogen utilization and blood glucose concentration during exercise, and the mechanisms controlling these processes. The potential for dietary NO_3^- supplementation to influence muscle glycogen stores represents a novel mechanism that is worthy of future investigation, and has important implications for prolonged type exercise.

Chapter 5 sought to provide insight to potential mechanisms that underpinned the attenuation in the decline of exercise efficiency following dietary NO_3^- supplementation in Chapter 4. In Chapter 5, participants underwent 4 days of dietary NO_3^- supplementation prior to completing 2 h of single-legged knee extensor exercise. Non-invasive measurements of contractile efficiency and maximal oxidative capacity were performed using magnetic resonance spectroscopy, and therefore limited the exercise modality to single-legged knee extensor exercise due to the small available space within the superconducting magnet for exercise. The novel findings of Chapter 5 were two-fold: 1) that there was a decline in skeletal muscle contractile efficiency and a progressive acceleration in PCr recovery kinetics reflecting increased maximal oxidative capacity over prolonged single-legged knee extension exercise; and that 2) there was no influence of dietary NO_3^- supplementation on these physiological responses. These data are in contrast to Chapter 4, and previous investigations which observed improved contractile (Bailey et al., 2010; Fulford et al., 2013) or mitochondrial efficiency (Larsen et al., 2011) following NO_3^- supplementation. Reasons for the divergent results are unclear, but may be due to differences in participant population and/or exercise modalities between Chapters 4 and 5. Chapter 5 included males (n=5) and females (n=6) whereas Chapter 4 only included males. There is evidence to suggest that contractile function (Haizlip et al., 2015; Solianik et al., 2017; Wüst et al., 2008), NO metabolism and NO bioavailability (Kapil et al., 2010; Rosselli et al., 1994) may be different between sexes which could potentially obscure results. Indeed, a recent study by Coggan et al. (2018) reported that there tended to be a greater relative increase in maximal knee extensor power in females (P=0.06) but not males, and that the

increase in knee extension power output was correlated to the magnitude of increase in plasma [NO_2^-] (R = 0.60, P<0.01). In line with this, when the data in Chapter 5 were analysed by sex, the change in plasma [NO_2^-] and $\dot{V}O_2$ was smaller in males compared to females, suggesting that females potentially responded better to NO_3^- supplementation, although it is acknowledged that these data were non-significant. Another potential explanation to account for differences in results between Chapters 4 and 5 could be that the favourable physiological effects of dietary NO_3^- supplementation occur in muscle groups predominantly comprised of type II muscle fibres (Bailey et al., 2015; Breese et al., 2013; Coggan et al., 2015; Ferguson et al., 2013; 2015; Hernández et al., 2012; Peeling et al., 2015) and that the serial reduction of NO_3^- to NO_2^- to NO is facilitated by low PO_2 (Castello et al., 2006) and pH (Modin et al., 2001). It could be possible that the single-legged knee extensor exercise employed in Chapter 5 did not elicit an intramuscular environment conducive to facilitating NO synthesis compared to the whole-body cycling exercise employed in Chapter 4. Together, the results of Chapter 5 further the current understanding of the potential influence of dietary NO_3^- supplementation on physiological responses during prolonged exercise. Specifically, Chapter 5 highlights that dietary NO_3^- supplementation may not attenuate the decline in contractile function during long duration single-legged knee extensor exercise indicating that factors such as exercise intensity, modality, and participant population may have a role.

The primary novel finding from Chapter 6 was that 6 days of dietary NO_3^- supplementation tended to attenuate the progressive decline in neuromuscular function over 1 h of heavy-intensity cycling exercise compared to BR+NAC (i.e. co-ingestion of NO_3^--rich BR juice and N-acetylcysteine). Specifically, the M-wave amplitude (i.e. muscle excitability) declined by 29% following BR+MAL (i.e. NO_3^--rich BR juice alone) compared to a 47% decline in PL+MAL (i.e. co-ingestion of NO_3^--depleted BR juice and maltodextrin, P=0.22) and a 51% decline in BR+NAC (P=0.08). In addition, the decline in muscle excitability in PL+NAC (i.e. NAC alone) was 16% which was significantly attenuated compared to PL+MAL and BR+NAC but not BR+MAL (P=0.30). Importantly, these data suggest that both NO_3^- supplementation and NAC ingestion as independent supplementation strategies have the potential to improve neuromuscular function but that combining NO_3^- and NAC could abolish these effects. In addition, given

that the ingestion of NAC alone preserved muscle excitability to a greater extent than BR juice alone, these data suggest that ROS may play a role in the decline of muscle excitability over time, perhaps via ROS-induced dysregulation of the Na^+-K^+ pump (McKenna et al., 2006). Although there are previous investigations that examined the effect of NO_3^- supplementation on neuromuscular function (Flanagan et al., 2016; Husmann et al., 2019), Chapter 6 provides the first evidence to show that muscle excitability during cycling exercise can be modulated by NO_3^- supplementation. In support of this, Wylie et al. (2013b) observed a tendency for plasma [K^+] to be attenuated during high-intensity intermittent running exercise following NO_3^- supplementation, indicating the that muscle membrane depolarization (which contributes to muscle excitability) was preserved with BR juice. In addition, neuromuscular function was improved by BR juice in knee extension exercise (Husmann et al., 2019) and resistance exercise (Flanagan et al., 2016). Therefore, Chapter 6 provides novel information which adds to the current body of literature supporting the potential for NO_3^- supplementation to influence neuromuscular function. The mechanistic bases for potential changes following NO_3^- supplementation is relatively unexplored. NO has been reported to have a role in the regulation of the Na^+-K^+ pump (Pirkmajer & Chibalin, 2016a) by stimulating its enzymatic ATPase activity specifically in type II fibres (Juel, 2016). This fibre-type specificity could be related to differences in NOS enzyme activity between fibre-types (Kobzik et al., 1994) although this remains to be verified in human muscle (Juel, 2016). Therefore, Chapter 6 may point to a novel mechanism underpinning potential physiological enhancements following dietary NO_3^- supplementation, specifically in prolonged exercise. Collectively, Chapters 4 and 6 provide further understanding of how dietary NO_3^- supplementation potentially improves physiologic function during prolonged exercise, specifically by attenuating the progressive decline in skeletal muscle function.

In summary, the results of Chapters 4, 5, and 6 have provided novel contributions to the current body of knowledge regarding the influence of dietary NO_3^- supplementation during prolonged exercise. These results have provided evidence that, short-term (3 to 6 d) dietary NO_3^- supplementation can attenuate the decline in muscle metabolite concentration (i.e. muscle glycogen and PCr sparing), preserve neuromuscular function (i.e. muscle excitability), and

attenuate the progressive rise in overall metabolic cost of prolonged exercise. These data, from Chapters 4 and 6, provide the first evidence to support the use of dietary NO_3^- supplementation as a strategy to enhance physiologic function during prolonged exercise in recreationally active humans. Improved physiological responses following dietary NO_3^- supplementation may be due to NO-mediated modulations to carbohydrate utilization, the energetic cost of force production, and/or muscle excitability. However, the results from Chapter 5 complicate the interpretation given that that dietary NO_3^- supplementation did not influence the contractile efficiency of prolonged single-legged knee extensor exercise. The results from Chapter 5 highlight that dietary NO_3^- supplementation may not be effective in other exercise modalities (i.e. prolonged single-legged knee extensor exercise) and suggest that further investigation is required to understand which factors influence the efficacy of dietary NO_3^- supplementation during prolonged exercise. In summary, the results of Chapters 4, 5, and 6 provide important advances to our current understanding on the potential physiological effects of dietary NO_3^- supplementation in prolonged exercise in recreationally active humans. The implications of these results extend from improving endurance events, to training or multi-day events in which preserving muscle metabolites (i.e. glycogen and PCr) may allow for improved performance and total work completed.

8.3. Characterizing plasma [NO_3^-] and [NO_2^-] as markers of NO bioavailability during prolonged exercise and potential supplementation strategies to optimize NO bioavailability

It is well established that following dietary NO_3^- supplementation with concentrated and non-concentrated NO_3^--rich BR juice, an elevation in plasma [NO_3^-] and [NO_2^-] occurs, peaking within ~2 to 3 h post-ingestion (Webb et al., 2008; Wylie et al., 2013a). The elevation in resting plasma [NO_3^-] and [NO_2^-] has been repeatedly reported (Muggeridge et al., 2013; Whitfield et al., 2016; Wylie et al., 2013a) and is essential for guiding the timing of NO_3^- supplementation to ensure that peak plasma [NO_3^-] and [NO_2^-] coincides with the commencement of exercise for optimal physiological effects. The magnitude of increase of plasma [NO_3^-] and [NO_2^-] are considered important for eliciting physiological effects (Coggan et al., 2018; Dreissigacker et al., 2010; Wilkerson et al., 2012; Wylie et al., 2013a). Chapters 4, 5, 6, and 7 provide further evidence that NO_3^-

supplementation elevates plasma [NO_3^-] and [NO_2^-] at rest. However, few investigations to date have characterized the response of plasma [NO_3^-] and [NO_2^-] *during* exercise. Previous studies have observed a decline in plasma [NO_2^-] over short duration moderate-intensity (Kelly et al., 2014) and severe-intensity cycling exercise (Dreissigacker et al., 2010; Kelly et al., 2014), and during high-intensity intermittent exercise (Wylie et al., 2013b; Thompson et al., 2015). In contrast, several studies have also observed an elevation of plasma [NO_2^-] over time (Allen et al., 2010; Rassaf et al., 2007b). The reduction of NO_2^- to NO is facilitated by low PO_2 (Castello et al., 2006) and pH (Modin et al., 2001), such as occurs in contracting muscle (Richardson et al., 1999). Thus, it could be possible that a decline in plasma [NO_2^-] over exercise may reflect NO_2^- being utilized as a substrate for NO production. In addition, the NO_2^- molecule has been reported to induce signalling (Bryan et al., 2005) and cannot be ruled out in contributing to positive physiological effects observed with dietary NO_3^- supplementation. Whether the physiological modulations are underpinned by NO-mediated mechanisms (Lundberg et al., 2008), NO_2^- signalling (Bryan et al., 2005), or NO metabolites (Hogg, 2002), it is now widely accepted that the elevation in NO_2^- is crucial for inducing physiological effects (Coggan et al., 2018; Dreissigacker et al., 2010; Thompson et al., 2015; Totzek et al., 2012; Wilkerson et al., 2012; Wylie et al., 2013b). Taken together, it could be reasoned that with higher exercise intensities and/or durations, significant declines to the elevation in plasma [NO_3^-] and [NO_2^-] over exercise would consequently reduce any associated positive physiological effects of dietary NO_3^- supplementation. In particular, there is potential for plasma [NO_2^-] to decline over long duration exercise (≥ 1 h) due to a progressive loss of skeletal muscle efficiency (Hopker et al., 2017) and/or progressive type II muscle fibre activation (Krustrup et al., 2004) which could induce a low PO_2 and pH environment, facilitating the reduction of NO_2^- to NO. Therefore, Chapters 4, 5 and 6 aimed to further our understanding in the potential changes to plasma [NO_3^-] and [NO_2^-] over prolonged exercise.

Chapters 4, 5 and 6 provided novel contributions by characterizing changes in plasma [NO_3^-] and [NO_2^-] in exercise protocols lasting ≥ 1 h. Chapter 4, for the first time, demonstrated that plasma [NO_3^-] and [NO_2^-] declined over prolonged cycling exercise. Specifically, following 3 days of BR juice supplementation,

plasma [NO_3^-] and [NO_2^-] declined over 2 h of moderate-intensity exercise by ~16% and ~17%, respectively. In contrast, Chapter 5 found that plasma [NO_3^-] and [NO_2^-] non-significantly declined by ~17% and tended to increase by ~28%, respectively, over 2 h of low-intensity prolonged single-legged knee extensor exercise. In Chapter 6, following 6 days of BR juice supplementation, plasma [NO_3^-] significantly declined by ~4% while plasma [NO_2^-] non-significantly increased by ~10% over 1 h of heavy-intensity cycling exercise. Moreover, plasma [NO_2^-] declined to a greater extent during the 4 km TT and severe-intensity TTE compared to the prolonged exercise bouts, in Chapters 4 and 6, respectively. Taken together, these data suggest that change in plasma [NO_3^-] and [NO_2^-] may be dependent on factors such as exercise intensity, exercise duration, and exercise modality. As previously discussed, the muscle metabolic environment impacts the reduction of NO_2^- to NO (Castello et al., 2006; Modin et al., 2001). In addition, recent advances in our understanding of the mechanistic bases of dietary NO_3^- supplementation indicate that NO-mediated physiological effects occur in a fibre-type specific manner, favouring muscle groups comprised predominantly of type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012; Jones et al., 2016) or exercise modalities recruiting relatively more type II muscle fibres (Bailey et al., 2015; Breese et al., 2013; Coggan et al., 2015; Peeling et al., 2015). Therefore, a greater proportional recruitment of type II muscle fibres during relatively high-intensity exercise and/or a progressive increase in type II muscle fibre recruitment during long duration exercise could account for the decline in plasma [NO_3^-] and [NO_2^-] in Chapters 4 and 6. In addition, the low-intensity and the relatively smaller amount of muscle activation during single-legged knee extensor exercise in Chapter 5 compared to cycling ergometry in Chapters 4 and 6 may contribute to the disparate results observed between studies. This novel contribution to the current body of literature is important because understanding the potential decline in plasma [NO_3^-] and [NO_2^-], as well as factors that may influence this change, could lead to supplementation strategies to maintain elevated plasma [NO_3^-] and [NO_2^-], and thus NO bioavailability, during prolonged exercise.

An important finding from Chapter 4 was that altering the conventional pre-exercise NO_3^- supplementation regimen (i.e. ingesting NO_3^--rich BR juice 3 to 6 days prior, and ~2.5 h prior to exercise) could influence indices of NO

bioavailability during prolonged exercise. Specifically, Chapter 4 is the first investigation to administer a dose of NO_3^--rich BR juice *during* exercise, in addition to pre-exercise NO_3^- supplementation (3 d), with the aim of reducing potential decreases in plasma [NO_3^-] and [NO_2^-] over 2 h of moderate-intensity exercise, and therefore maintaining its associated NO-mediated physiological effects. Interestingly, plasma [NO_3^-] was elevated 30 min following ingestion of the additional top-up dose of NO_3^--rich BR juice by ~41% and remained until 120 min while plasma [NO_2^-] increased by 8% at 120 min. This is in contrast to the aforementioned decline plasma [NO_3^-] and [NO_2^-] when only pre-exercise BR juice supplementation occurred. The elevation in plasma [NO_3^-] and [NO_2^-] following BR juice ingestion during exercise is likely to be underpinned by the well characterized enterosalivary NO_3^--NO_2^--NO pathway which allows for the continual uptake, absorption, and circulation of the NO_3^- and NO_2^- anions (Lundberg et al., 2008). While these data suggest that NO bioavailability can be modulated by BR juice ingestion during exercise, it is acknowledged that the measurements in Chapter 4 are limited to NO_3^- and NO_2^- circulating in the blood. Given that other tissues (i.e. skeletal muscle, liver) have been evidenced to be endogenous reservoirs of NO_3^- and NO_2^- and likely participate in NO metabolism (Gilliard et al., 2018; Piknova et al., 2015; Nyakayiru et al., 2017b), future investigations should measure muscle [NO_3^-] and [NO_2^-] to provide further insight to NO metabolism during exercise and the response to different supplementation strategies. These findings provide important insight into the dynamic response of plasma [NO_3^-] and [NO_2^-] during exercise, and have implications for the application of dietary NO_3^- supplementation, specifically for endurance events.

Chapter 6 was the first investigation to combine pre-exercise NO_3^- supplementation (6 d) with acute NAC ingestion with the aim to further elevate plasma [NO_3^-] and [NO_2^-] at rest. Given that NAC is a reduced thiol donor which provides cysteine and glutathione, the most important antioxidant in the biological system (Powers & Jackson, 2008), it was hypothesized that elevating the antioxidant capacity of the biological system could offset potential ROS-induced decreases to NO (Aleryani et al., 1998). Chapter 6 demonstrated that following 6 days of BR juice supplementation combined with acute NAC ingestion, plasma [NO_3^-] non-significantly declined by ~0.6% and plasma [NO_2^-] non-significantly increased by ~14% over 1 h of heavy-intensity cycling exercise compared to

when only pre-exercise BR juice supplementation occurred whereby plasma [NO_3^-] significantly declined by ~4% while plasma [NO_2^-] non-significantly increased by ~9%. Importantly, plasma [NO_3^-] was significantly greater at 1 h following co-ingestion of BR juice and NAC compared to ingestion of BR juice alone (P<0.05), although there was no difference in plasma [NO_2^-] between these conditions. These data provide evidence that, for the first time, the elevation in plasma [NO_3^-] can be further enhanced by combining inorganic NO_3^- with a reduced thiol donor supplement. NO_3^- and NO_2^- are relatively stable molecules compared to NO (Gladwin et al., 2005; Hakim et al., 1996; Lundberg et al., 2008). Therefore, it could be reasoned that any additional elevations to NO bioavailability from co-ingesting BR juice and NAC could be represented as elevations in plasma [NO_3^-], given that NO is oxidized to NO_3^-. Together, Chapters 4 and 6 provide evidence that NO bioavailability can be manipulated by ingesting a source of NO_3^- during exercise, or by co-ingesting a reduced thiol donor that quenches ROS with pre-exercise NO_3^- supplementation.

As discussed in the previous section, Chapter 6 demonstrated that ROS may have an impact on NO bioavailability. In support of this, the reduction of NO_2^- to NO is facilitated by the presence of ascorbic acid and polyphenols (Gago et al., 2007; Peri et al., 2005; Weitzberg & Lundberg, 1998), which also have potential to exert antioxidant properties (Powers & Jackson, 2008). This is important to note because the BR juice (active and placebo; Lansley et al., 2011b) commonly administered in experiments investigating the effects of NO_3^- supplementation contains various potentiating bioactive constituents other than NO_3^-, such as phytochemicals including polyphenols, flavonoids, betalains, and ascorbic acid (Shepherd et al., 2015; Wootton-Beard & Ryan, 2012). Accordingly, it could be possible that the placebo juice (i.e. concentrated NO_3^--depleted BR juice) influences NO bioavailability through its phytochemical compounds. Recent evidence suggests that NO_3^-, administered as vegetable juice compared to its pharmaceutical form of NO_3^- salts, may induce divergent physiological effects such that NO_3^- in vegetable juice form may be superior to NO_3^- salts (Flueck et al., 2016; Thompson et al., 2018). Together, these data suggest that other bioactive compounds within the vegetable juice may act synergistically with NO_3^-. Therefore, Chapter 7 sought to investigate whether NO_3^--depleted BR juice

supplementation influenced plasma [NO_3^-] and [NO_2^-] at rest, as well as physiological responses during moderate-intensity exercise.

The primary novel finding of Chapter 7 was that 3 to 4 days of NO_3^--depleted BR juice supplementation did not elevate baseline plasma [NO_3^-] and [NO_2^-], or influence $\dot{V}O_2$ kinetics (i.e. phase II, τ_p) and the O_2 cost of short duration (6 min) moderate-intensity exercise. These data support that the placebo juice commonly utilized for scientific experiments is an effective placebo to study the effects of dietary NO_3^- supplementation. In addition, these results further support that positive physiological modulations following NO_3^- supplementation may be attributed to the elevation of NO_3^- itself (Lansley et al., 2011b; Lundberg et al., 2008). However, Flueck et al. (2016) observed a relatively greater lowering of the O_2 cost of exercise following NO_3^- administered in juice form compared to NO_3^- salts. In addition, Thompson et al. (2018) observed superior adaptations when sprint training was combined with NO_3^- administered as concentrated NO_3^--rich BR juice compared to NO_3^- salts. Together, the results of these previous investigations suggest that NO_3^- and the bioactive polyphenolic compounds in BR juice could act synergistically and consequently enhance its physiological effects compared to the pharmaceutical form. The type and concentration of polyphenolic compounds may be important in influencing the extent to which plasma [NO_3^-] and [NO_2^-] is further elevated by polyphenols and account for disparate findings. BR juice contains ~4 mg of flavonoids (Shepherd et al., 2015), but previous evidence suggests that ~500 mg of flavonoids is required to induce NO-mediated effects (Kay et al., 2012). It is possible that the concentration of phytochemicals within BR juice is not sufficient to induce changes to NO bioavailability; however, synergistic action between the phytochemicals and NO_3^- cannot be ruled out and could account for the disparate findings between Chapters 7 and previous investigations (Flueck et al., 2016; Thompson et al., 2018). Therefore, the phytochemical composition of BR juice may be an important factor contributing to whether NO bioavailability is elevated following NO_3^--depleted BR juice supplementation. The results from Chapter 7 further our current understanding and indicate that although BR juice may enhance physiologic function to a greater extent than NO_3^- salts, it is not necessarily due to increased phytochemical-induced elevations to NO bioavailability.

In summary, Chapters 4, 5, and 6 have furthered our understanding of how NO bioavailability changes over prolonged exercise following dietary NO_3^- supplementation. In addition, Chapters 4, and 6 are the first investigations to provide evidence that NO_3^- supplementation *during* exercise, and in combination with acute ingestion of a reduced thiol donor, have the potential to influence NO bioavailability. The mechanisms underpinning the changes in plasma [NO_3^-] and [NO_2^-] are likely due to the enterosalivary pathway and modulations to endogenous ROS bioactivity. Moreover, Chapter 7 provides important findings which indicate that bioactive phytochemical compounds that exist in concentrated NO_3^--rich and NO_3^--depleted BR juice do not influence NO bioavailability or the pulmonary $\dot{V}O_2$ responses to exercise.

8.4. No ergogenic effect of dietary NO_3^- supplementation on exercise performance following a prior bout of prolonged exercise

A main overarching theme of this thesis was to investigate whether dietary NO_3^- supplementation influenced the physiological responses during prolonged exercise. Accordingly, it was hypothesized that improved physiologic function during prolonged exercise would enable enhanced subsequent exercise performance due to a greater relative preservation of skeletal muscle function. Measuring exercise performance following a prolonged exercise bout could be reflective of real-world endurance competitive events, whereby competitors increase their pace when reaching the last stage of a race for a sprint finish. Therefore, Chapters 4 and 6 sought to investigate the influence of dietary NO_3^- supplementation on subsequent exercise performance following prolonged exercise. These data are important for furthering our understanding in the application of dietary NO_3^- supplementation and translating basic science to practice.

In Chapter 4, a 4 km TT was performed immediately following a 2 h preload at 80%GET (i.e. moderate-intensity exercise). In Chapter 6, a TTE at severe-intensity (70%Δ, where Δ represents the difference between peak work rate and the GET) was completed following a 1 h preload at 20%Δ (i.e. heavy-intensity exercise). There was no influence of dietary NO_3^- supplementation on subsequent 4 km TT despite improved indices of NO bioavailability as well as improved muscle glycogen and PCr availability during the 2 h preload in Chapter

4. Similarly, in Chapter 6 there was no significant influence on subsequent TTE despite physiological modulations during the 1 h preload, such as enhanced elevation of plasma [NO_3^-] following co-ingestion of NO_3^- and NAC and preserved muscle excitability following pre-exercise NO_3^- supplementation alone or NAC ingestion alone. Together, Chapters 4 and 6 suggest that neither exercise performance nor capacity following a prolonged exercise bout are influenced by NO_3^- supplementation. These data are consistent with Christensen et al. (2013), the only other investigation to assess exercise performance following a prolonged exercise bout. The reason for the lack of effect on subsequent exercise performance measures in Chapters 4 and 6 are unclear, especially given that there was improved physiologic function during prolonged exercise following dietary NO_3^- supplementation. In addition, dietary NO_3^- supplementation has been evidenced to enhance skeletal muscle contractile function (Bailey et al., 2010; Coggan et al., 2015; Coggan & Peterson, 2018; Fulford et al., 2013; Haider & Folland, 2014; Hernández et al., 2012; Ivarsson et al., 2017; Whitfield et al., 2017) and to induce favourable alterations to type II muscle fibres (Ferguson et al., 2013; 2015; Hernández et al., 2012; cf: Whitfield et al., 2017), which could be expected to underpin an ergogenic effect during exercise protocols such as a TT and/or a TTE (Jones et al., 2018; McMahon et al., 2017; Van de Walle et al., 2018).

A potential explanation to account for the lack of effect on subsequent exercise performance in Chapters 4 and 6 could be that the improved physiological effects during prolonged exercise were simply too small to translate into an ergogenic effect during the subsequent TT and TTE. Indeed, the attenuated decline in muscle [glycogen], [PCr], and muscle excitability in Chapters 4 and 6 were statistically trends. It could be possible that a preload that was longer in duration and/or at a relatively higher exercise intensity could have induced a greater impact and thus translated into a meaningful effect on subsequent exercise performance. In addition, the lack of effect may be related to the experimental design such that a longer TT may have revealed significant effects in Chapter 4. Therefore, it cannot be excluded that subsequent exercise performance and capacity following a prolonged exercise bout would be enhanced by dietary NO_3^- supplementation under other circumstances. An important consideration is that Chapters 4 and 6 administered BR juice as the active and placebo juice. As

previously mentioned, the main novel finding of Chapter 7 was that NO_3^--depleted BR juice supplementation, and thus the bioactive phytochemical constituents present in BR juice, did not significantly impact NO bioavailability. However, it is important to note that the phytochemical with the highest concentration in BR juice is betalain (Shepherd et al., 2015). Betalains have been reported to elicit biological activities combatting exercise-induced, fatigue-associated, physiological changes such as inflammation (Tesoriere et al., 2003; Vidal et al., 2014) and oxidative stress (Butera et al., 2002; Esatbeyoglu et al., 2014; Kanner et al., 2001). Betalain supplementation has also been reported to enhance exercise efficiency (Mumford et al., 2018) and TT performance (Montenegro et al., 2017; Mumford et al., 2018; Van Hoorebeke et al., 2016). Therefore, although the results from Chapter 7 suggest that NO_3^--depleted BR juice does not influence NO bioavailability, it cannot be excluded that the phytochemical betalain, present in NO_3^--depleted BR juice, could be influencing exercise performance independent of NO_3^- and potentially obscuring results (Georgiev et al., 2010). Together, these data provide novel contributions to current knowledge by advancing our understanding of whether dietary NO_3^- supplementation influences exercise performance following a prolonged exercise bout. Specifically, these results suggest that dietary NO_3^- supplementation may not improve 4 km TTs or severe-intensity TTE tasks following a long duration exercise preload (1 to 2 h), at least in recreationally active humans.

8.5. Future directions

8.5.1. Understanding the dynamics of plasma [NO_3^-] and [NO_2^-] during prolonged exercise and its physiological relevance

Although the responses of plasma [NO_3^-] and [NO_2^-] following dietary NO_3^- ingestion at rest are well characterized (Wylie et al., 2013a), the dynamics of plasma [NO_3^-] and [NO_2^-] during exercise are less well understood. Chapters 4, 5, and 6 complement the available literature and suggest that plasma [NO_3^-] and [NO_2^-] may decline over prolonged exercise, although factors such as exercise duration, intensity, modality, and participant population may influence the dynamics. Future investigations should seek to understand which factors have a role in the change of plasma [NO_3^-] and [NO_2^-] and if these changes influence physiological enhancements associated with elevations in plasma [NO_3^-] and [NO_2^-]. Current research suggests that the decline in plasma [NO_2^-] is reflective

of the reduction of NO_2^- into NO and important for its associated physiological effects. However, NO_3^- metabolism is complex and occurs in tissues other than blood, such as skeletal muscle and the liver (Gilliard et al., 2018; Piknova et al., 2015). Thus, it could be possible that changes to plasma [NO_3^-] and [NO_2^-] partly reflect uptake into other tissues in addition to the serial reduction to NO and increased NO_2^- from oxidation of NO produced by NOS.

Future investigations should identify what the decline in plasma [NO_2^-] over exercise represents by incorporating comprehensive measures of [NO_3^-] and [NO_2^-] dynamics in blood and other tissues. In addition, it is possible that the widely-accepted notion that the effects of NO_3^- supplementation are underpinned by increased provision of NO_2^- for NO biosynthesis only partly explains the subsequent physiological effects. This is because although NO signalling is likely to be a contributor to the observed positive physiological modulations following dietary NO_3^- supplementation, NO_2^- itself is also capable of inducing signalling to alter physiology (Bryan et al., 2005) and NO metabolites, such as S-nitrosothiols, can induce post translational protein modifications to alter protein structure and function (Hogg, 2002). Therefore, identifying the influence of NO, NO_2^-, and S-nitrosothiols could provide valuable information regarding the biochemical mechanisms underpinning the physiological effects of dietary NO_3^- supplementation.

8.5.2. Optimizing supplementation strategies for NO_3^- ingestion during exercise

Chapter 4 revealed that administering a single bolus of NO_3^-, in addition to pre-exercise NO_3^-supplementation, can enhance physiological responses during prolonged exercise compared to pre-exercise NO_3^- supplementation alone. However, it is important to note that the preservation of muscle [glycogen] and [PCr] over 2 h of moderate-intensity cycling exercise were trends. It is possible that ingesting NO_3^- earlier than when provided in the experiment (i.e. before 1 h into exercise) could have allowed for a further increase in plasma [NO_3^-] and [NO_2^-] and therefore further enhanced NO-induced physiological modulations during prolonged exercise. Further studies are required to identify the optimal timing to provide an additional NO_3^- dose during prolonged exercise and the consequent physiological effects. Moreover, the findings from Chapter 4 provide

rationale for investigating whether an additional dose of NO_3^- during other types of exercise in which muscle glycogen availability is important, such as during the half-time interval during high-intensity intermittent type sports, or for multi-day sport events, provides additional benefits to the conventional pre-exercise dietary NO_3^- supplementation regimen.

8.5.3. Exploring the influence of dietary NO_3^- supplementation on muscle glycogen availability and neuromuscular function and their mechanistic bases in prolonged exercise

Chapters 4 and 6 are the first experiments to observe that dietary NO_3^- supplementation influences muscle glycogen utilization and muscle excitability during prolonged cycling exercise. However, the mechanisms underpinning these effects were not further examined due to the experimental design. NO has many physiological functions and is purported to regulate skeletal muscle glucose uptake (Jiang et al., 2014; Merry et al., 2010) and Na^+-K^+ pump activity (Juel, 2016; Pirkmajer et al., 2016). However, few data are available which examine the potential influence of NO_3^- supplementation on carbohydrate utilization and Na^+-K^+ handling during exercise in humans (Betteridge et al., 2016; Thompson et al., 2017; Wylie et al., 2013b). To provide further understanding on these potential mechanisms, research should measure glucose transporter protein and Na^+-K^+ pump protein content and activity, in addition to muscle [glycogen], blood [glucose] and plasma [K^+] kinetics.

8.5.4. Does dietary NO_3^- supplementation influence contractile or mitochondrial efficiency in prolonged exercise?

The lack of influence of dietary NO_3^- supplementation on contractile efficiency of single-legged knee extensor exercise in Chapter 5 are in contrast to earlier studies that observed a lower energetic cost of contraction (Bailey et al., 2010; Fulford et al., 2013) and greater mitochondrial efficiency (Larsen et al., 2011). Notably, these earlier studies employed short duration exercise bouts (Bailey et al., 2010; Larsen et al., 2011). Together, these data suggest that the mechanistic bases underpinning potential positive physiological effects following dietary NO_3^- supplementation may be different and/or dependent on exercise duration and modality. Further work is required to understand whether the ATP cost of

contraction and/or mitochondrial efficiency is influenced by dietary NO_3^- supplementation during prolonged exercise.

8.5.5. Can co-ingestion of NO_3^- and reduced thiol donors further elevate NO bioavailability and influence physiological responses in prolonged exercise?

The elevation of plasma [NO_3^-] or [NO_2^-] is hypothesized to be crucial for inducing consequent physiological enhancements following dietary NO_3^- supplementation (Dreissigacker et al., 2010; Thompson et al., 2015; Wilkerson et al., 2012; Wylie et al., 2013a). Chapter 6 identified that co-ingestion of NO_3^- and NAC further enhanced plasma [NO_3^-] which indicates that NAC may have enhanced NO bioavailability by reducing ROS. However, results showed that combining NO_3^- and NAC negated potential positive physiological effects during prolonged exercise compared to NO_3^- and NAC alone. These data highlight an interesting physiological response that requires resolving given that the interaction between NO_3^- and NAC appear to interfere with each other. NAC supplementation (Cobley et al., 2011; McKenna et al., 2006) and NO_3^- supplementation (Bailey et al., 2009; Cermak et al., 2012) are both redox interventions that exert ergogenic effects and modulate physiology when independently administered, but the biochemical interaction between both compounds when co-ingested is virtually unexplored (Reid, 2016b). Further work is required to confirm the physiological responses of combining NO_3^- and NAC during exercise and the mechanisms responsible.

8.5.6. To what extent do polyphenols influence the efficacy of dietary NO_3^- supplementation?

In Chapter 7, results showed that NO_3^--depleted BR juice supplementation did not elevate plasma [NO_3^-] or [NO_2^-], suggesting that the presence of bioactive phytochemicals in BR juice other than NO_3^- alter NO_3^- or NO_2^-. In other words, NO_3^--depleted BR juice, commonly used as placebo, is not likely to induce positive NO-mediated physiological effects. However, it is acknowledged that investigations employing polyphenol supplementation (e.g. cocoa, cherry, blueberry, pomegranate, see Chapter 7) have the potential to enhance NO-dependent processes such as blood flow (Aboo Bakkar et al., 2019; Kay et al., 2012; Rodriguez-Mateos et al., 2013; Trexler et al., 2014). Moreover, consuming inorganic NO_3^- in the form of a vegetable juice has been reported to be superior

to ingesting NO_3^- salts (Flueck et al., 2016; Thompson et al., 2018). Together, these data suggest that the bioactive polyphenolic constituents of BR juice may have a synergistic interaction with NO_3^- in inducing physiological modulations following BR juice. Given that a myriad of phytochemical compounds exist in BR juice (Shepherd et al., 2015), understanding the role of the individual and aggregate effects of phytochemicals in BR juice is complex. However, it could be possible that future investigations compare NO_3^- salts to NO_3^--depleted BR juice, as well as to other polyphenol supplements, to elucidate potential effects.

8.5.7. Can dietary NO_3^- supplementation induce physiological effects in other exercise modalities in prolonged type exercise?

Chapters 4 and 6 provide evidence that the physiological responses during prolonged cycling exercise can be modulated by short term dietary NO_3^- supplementation. Specifically, muscle glycogen sparing and preserved muscle excitability following NO_3^- ingestion are factors that could contribute to delaying onset of fatigue and/or the loss of efficiency during prolonged exercise (Allen et at., 2008; Gandevia et al., 2001; Ørtenblad et al., 2011). This thesis contains the first evidence suggesting that dietary NO_3^- supplementation enhances physiological function during prolonged cycling exercise; however, further research is required to confirm whether these improvements occur in different exercise modalities. Although cycling represents one of the most popular endurance competitive events, other exercise modalities such as running (i.e. marathon, half-marathon), cross-country skiing, or combined modalities (i.e. triathlon), also have long durations (≥ 1 h). Given that the mechanisms of dietary NO_3^- supplementation potentially targets type II muscle fibres and that different exercise modalities require different metabolic demands and different muscle group activation (and thus, activation of muscles comprised of different fibre-type composition), it is important to identify sport-specific contexts in which dietary NO_3^- supplementation might enhance physiological responses.

8.5.8. Are there sexual differences in the efficacy of dietary NO_3^- supplementation?

The current literature is comprised of evidence which suggests that there are responders and non-responders to dietary NO_3^- supplementation (Campos et al., 2018; Coggan et al., 2018; Christensen et al., 2013; Thompson et al., 2015;

Wilkerson et al., 2012; Wylie et al., 2013a; 2013b). Factors that may contribute to responsiveness to dietary NO_3^- supplementation include participant training status (Porcelli et al., 2015), oral microbiome and enterosalivary pathway differences between individuals (Lundberg et al., 2008; Vanhatalo et al., 2018), and muscle fibre-type composition (Jones et al., 2016). However, results from Chapter 5 suggest that plasma [NO_2^-] dynamics and the change in the O_2 cost of exercise following NO_3^- ingestion could be enhanced to a greater extent in females. Although these data were non-significant, they complement a recent study by Coggan et al. (2018) which reported an association between increased knee extensor power output with NO_3^- in females. Future investigations should seek to elucidate whether there are sex-differences in response to dietary NO_3^- supplementation and the mechanistic bases underpinning potential divergent physiological responses.

8.6. Conclusion

The field of exercise physiology is at an exciting time. It has been 12 years since research began investigating the influence of dietary NO_3^- supplementation on physiological responses during exercise (Larsen et al., 2007). To date, our understanding of dietary NO_3^- supplementation has advanced such that potential candidate mechanisms have been identified (Affourtit et al., 2015). Specifically, dietary NO_3^- supplementation has been shown to enhance the ATP cost of force production (Bailey et al., 2010), improve mitochondrial efficiency (Larsen et al., 2011; cf: Whitfield et al., 2016), and to increase tissue perfusion (Ferguson et al., 2013), with a potential targeting of type II muscle fibres (Ferguson et al., 2013; Hernández et al., 2012). These mechanisms work independently or in combination to contribute to enhanced exercise efficiency and performance following dietary NO_3^- supplementation. In light of our recent advances in knowledge of the mechanistic bases of dietary NO_3^- supplementation, this has provided a rationale to explore exercise modalities by which the efficacy of dietary NO_3^- supplementation is likely to be enhanced. Accordingly, there has been a recent surge in publications investigating high-intensity intermittent type exercise (Jones et al., 2018). However, at the onset of this thesis, publications regarding whether dietary NO_3^- supplementation influences prolonged exercise was scarce. Therefore, the current thesis has made novel contributions which extend our understanding of dietary NO_3^- supplementation by investigating the potential

influence of dietary NO_3^- supplementation on physiological responses during prolonged exercise. In addition, in light of recent advances in our knowledge suggesting that elevated NO bioavailability is crucial for the efficacy of dietary NO_3^- supplementation, this thesis also explored potential supplementation strategies to optimize NO bioavailability.

The findings from Chapters 4, 5, and 6 demonstrated that skeletal muscle function declines over prolonged exercise, while Chapters 4 and 6 provides evidence that dietary NO_3^- supplementation has the potential to attenuate the relative magnitude of reduction in muscle metabolites and muscle excitability in prolonged exercise. Moreover, Chapters 4 and 6 explore the potential to modulate NO bioavailability by administering NO_3^- during exercise, as well as combining dietary NO_3^- supplementation with a reduced thiol donor. Specifically, the results of Chapter 4 suggest that ingesting a single dose of NO_3^- during prolonged exercise, in addition to short-term pre-exercise NO_3^- supplementation, may be effective at preserving NO bioavailability and consequently inducing further enhanced physiological effects compared to pre-exercise NO_3^- supplementation alone. The results of Chapter 6 suggest that combining pre-exercise dietary NO_3^- supplementation with acute NAC ingestion may further increase plasma [NO_3^-] but potentially abolishes positive physiological effects of dietary NO_3^- supplementation during prolonged exercise. Chapter 7 found that the number of transitions could influence detecting changes to the O_2 cost of exercise following NO_3^- supplementation. In addition, Chapter 7 indicates that NO_3^--depleted BR juice, commonly administered as the placebo juice in scientific experiments, does not influence NO bioavailability and therefore is an effective placebo. Lastly, the present thesis also contributes important information suggesting that factors such as exercise intensity, duration, modality, and sex-differences may contribute to the efficacy of dietary NO_3^- supplementation during prolonged exercise.

In summary, the information from this thesis indicates that dietary NO_3^- supplementation may be beneficial for recreationally active individuals participating in prolonged cycling exercise and that altering the supplementation strategy by ingesting a dose of NO_3^- during exercise could provide further physiological enhancements.

References

Aboo Bakkar Z, Fulford J, Gates PE, Jackman SR, Jones AM, Bond B, Bowtell JL. Montmorency cherry supplementation attenuates vascular dysfunction induced by prolonged forearm occlusion in overweight, middle-aged men. *J Appl Physiol (1985).* 126(1): 246-254, 2019.

Abraham RZ, Kobzik L, Moody MR, Reid MB, Stamler JS. Cyclic GMP is a second messenger by which nitric oxide inhibits diaphragm contraction. *Comp Biochem Physiol.* 119A: 177-183, 1998.

Abramson JJ, Salama G. Critical sulfhydryls regulate calcium release from sarcoplasmic reticulum. *J Bioenerg Biomembr.* 21: 283-294, 1989.

Adreani CM, Hill JM, Kaufman MP. Responses of group III and IV muscle afferents to dynamic exercise. *J Appl Physiol.* 82: 1811-1817, 1997.

Affourtit C, Bailey SJ, Jones AM, Smallwood MJ, Winyard PG. On the mechanism by which dietary nitrate improves human skeletal muscle function. *Front Physiol.* 6: 211, 2015.

Aghdasi B, Reid MB, Hamilton SL. Nitric oxide protects the skeletal muscle Ca^{2+} release channel from oxidation induced activation. *J Biol Chem.* 272: 25462-25467, 1997.

Ahlborg B, Bergstrom J, Ekelund LG, Hultman E. Muscle glycogen and muscle electrolytes during prolonged physical exercise. *Acta Physiol Scand*. 70: 129–142, 1967.

Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J.* 357: 593-615, 2001.

Aleryani S, Milo E, Rose Y, Kostka P. Superoxide-mediated decomposition of biological S-nitrosothiols. *J Biol Chem.* 273:6041–45, 1998.

Alessio HM, Goldfarb AH, Cutler RG. MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat. *Am J Physiol Cell Physiol.* 255: C874–C877, 1988.

Allen DG, Lamb GD, Westerblad H. Skeletal muscle fatigue: cellular mechanisms. *Physiol Rev.* 88(1): 287-332, 2008.

Allen JD, Stabler T, Kenjale A, Ham KL, Robbins JL, Duscha BD, Dobrosielski DA, Annex BH. Plasma nitrite flux predicts exercise performance in peripheral arterial disease after 3 months of exercise training. *Free Radic Biol Med.* 49(6): 1138-1144, 2010.

Allen DG, Trajanovska S. The multiple roles of phosphate in muscle fatigue. *Front Physiol.* 3: 463, 2012.

Alessio HM, Goldfarb AH, Cutler RG. MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat. *Am J Physiol Cell Physiol.* 255: C874-C877, 1988.

Amann M. Central and peripheral fatigue: interaction during cycling exercise in humans. *Med Sci Sports Exerc.* 43(11): 2039-2045, 2011.

Amann M. Significance of group III and IV muscle afferents for the endurance exercising human. *Clin Exp Pharmacol Physiol.* 39(9): 831-835, 2012.

Amann M, Calbet JA. Convective oxygen transport and fatigue. *J Appl Physiol.* 104(3): 861-870, 2008.

Amann M, Hopkins WG, Marcora SM. Similar sensitivity of time to exhaustion and time-trial time to changes in endurance. *Med Sci Sports Exerc.* 40(3): 574-578, 2008.

Amann M, Dempsey JA. Locomotor muscle fatigue modifies central motor drive in healthy humans and imposes a limitation to exercise performance. *J Physiol.* 586: 161-173, 2008.

Amann M, Blain GM, Proctor LT, Sebranek JJ, Pegelow DF, Dempsey JA. Group III and IV muscle afferents contribute to ventilatory and cardiovascular response to rhythmic exercise in humans. *J Appl Physiol.* 109:966–76, 2010.

American Thoracic Society/European Respiratory Society. ATS/ERS statement on respiratory muscle testing. *Am J Resp Crit Care Med.* 166(4): 518-624, 2002.

Andersen P, Henriksson J. Capillary supply of the quadriceps femoris muscle of man: adaptive response to exercise. *J Physiol.* 270(3): 677-690, 1977.

Andersen EJ, Neufer PD. Type II skeletal myofibers possess unique properties that potentiate mitochondrial H2O2 generation. *Am J Physiol Cell Physiol.* 290, C844–C851, 2006.

Andrade FH, Reid MB, Allen DG, Westerblad H. Effect of hydrogen peroxide and dithiothreitol on contractile function of single skeletal muscle fibres from the mouse. *J Physiol.* 509(Pt 2):565–75, 1998.

Andrade FH, Reid MB, Westerblad H. Contractile response of skeletal muscle to low peroxide concentrations: myofibrillar calcium sensitivity as a likely target for redox-modulation. *FASEB J.* 15:309–311, 2001.

Arbogast S, Vassilakopoulos T, Darques JL, Jammes Y. Influence of oxygen supply on activation of group IV muscle afferents after low-frequency muscle stimulation. *Muscle Nerve*. 23:1187–1193, 2000.

Arbogast, S, Reid, MB. Oxidant activity in skeletal muscle fibers is influenced by temperature, CO_2 level, and muscle-derived nitric oxide. *Am J Physiol Regul Integr Comp Physiol.* 287, R698–R700, 2004.

Arnold DL, Matthew PM, Radda GK. Metabolic recovery after exercise and the assessment of mitochondrial function in vivo in human skeletal muscle by means of 31PNMR. *Magn Reson Med.* 1: 307-315, 1984.

Arnold JT, Oliver SJ, Lewis-Jones TM, Wylie LJ, Macdonald JH. Beetroot juice does not enhance altitude running performance in well-trained athletes. *Appl Physiol Nutr Metab.* 40(6): 590-595, 2015.

Ashley CC, Mulligan IP, Lea TJ. Ca2+ and activation mechanisms in skeletal muscle. *Q Rev Biophys.* 24(1): 1-73, 1991.

Aucouturier J, Boissière J, Pawlak-Chaouch M, Cuvelier G, Gamelin FX. Effect of dietary nitrate supplementation on tolerance to supramaximal intensity intermittent exercise. *Nitric Oxide.* 49: 16-25, 2015.

Balog EM, Fitts RH. Effects of fatiguing stimulation on intracellular Na^+ and K^+ in frog skeletal muscle. *J Appl Physiol.* 81: 679–685, 1996.

Bailey SJ, Winyard P, Vanhatalo A, Blackwell JR, Dimenna FJ, Wilkerson DP, Tarr J, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J Appl Physiol.* 107(4): 1144-1155, 2009.

Bailey SJ, Fulford J, Vanhatalo A, Winyard PG, Blackwell JR, DiMenna FJ, Wilkerson DP, Benjamin N, Jones AM. Dietary nitrate supplementation enhances muscle contractile efficiency during knee-extensor exercise in humans. *J Appl Physiol.* 109(1): 135-148, 2010.

Bailey SJ, Varnham RL, DiMenna FJ, Breese BC, Wylie LJ, Jones AM. Inorganic nitrate supplementation improves muscle oxygenation, O2 uptake kinetics, and exercise tolerance at high but not low pedal rates. *J Appl Physiol.* 118(11): 1396-1405, 2015.

Bailey SJ, Blackwell JR, Wylie LJ, Holland T, Winyard PG, Jones AM. Improvement in blood pressure after short-term inorganic nitrate

supplementation is attenuated in cigarette smokers compared to non-smoking controls. *Nitric Oxide.* 61: 29-37, 2016.

Bailey SJ, Blackwell JR, Wylie LJ, Emery A, Taylor E, Winyard PG, Jones AM. Influence of iodide ingestion on nitrate metabolism and blood pressure following short-term dietary nitrate supplementation in healthy normotensive adults. *Nitric Oxide.* 63: 13-20, 2017.

Bailey SJ, Winyard P, Blackwell JR, Vanhatalo A, Lansley K, DiMenna FJ, Wilkerson DP, Campbell IT, Jones AM. Influence of N-acetylcysteine administration on pulmonary O_2 uptake kinetics and exercise tolerance in humans. *Resp Phys Neurol.* 175(1): 121-129, 2011.

Balon TW, Nadler JL. Nitric oxide release is present from incubated skeletal muscle preparations. *J Appl Physiol.* 77, 2519–2521, 1994.

Balsalobre-Fernández C, Romero-Moraleda B, Cupeiro R, Peinado AB, Butragueño J, Benito PJ. The effects of beetroot supplementation on exercise economy, rating of perceived exertion and running mechanics in elite distance runners: A double-blinded, randomized study. *PLoS One.* 13(7): e0200517.

Bangsbo J, Krustrup P, Gonzalez-Alonso J, Saltin B. ATP production and efficiency of human skeletal muscle during intense exercise: effect of previous exercise. *Am J Physiol Endocrinol Metab.* 280(6): E956-E964, 2001.

Barclay CJ. Mechanical efficiency and fatigue of fast and slow muscles of the mouse. *J Physiol.* 497:587–96, 1996.

Barja G. Mitochondrial oxygen radical generation and leak: sites of production in states 4 and 3, organ specificity, and relation to aging and longevity. *J Bioenerg Biomembr.* 31: 347–366, 1999.

Barnes PJ, Kharitonov SA. Exhaled nitric oxide: a new lung function test. *Thorax.* 51: 233-237, 1996.

Barreiro E, Galdiz JB, Marinan M, Alvarez FJ, Hussain SN, Gea J. Respiratory loading intensity and diaphragmoxidative stress: N-acetylcysteine effects. *J Appl Physiol.* 100(2): 555–563, 2006.

Barstow TJ, Jones AM, Nguyen PH, Casaburi R. Influence of muscle fiber type and pedal frequency on oxygen uptake kinetics of heavy exercise. *J Appl Physiol* 81(4): 1642-1650, 1996.

Barstow TJ, Lamarra N, Whipp BJ. Modulation of muscle and pulmonary O_2

uptakes by circulatory dynamics during exercise. *J Appl Physiol.* 68: 979-989, 1990.

Bassett DR Jr, Howley ET. Limiting factors for maximum oxygen uptake and determinants of endurance performance. *Med Sci Sports Exerc.* 32(1): 70-84, 2000.

Basu S, Azarova NA, Font MD, King SB, Hogg N, Gladwin MT, Shiva S, Kim-Shapiro DB. Nitrite reductase activity of cytochrome c. *J Biol Chem* 283: 32590 –32597, 2008.

Behnke BJ, Kindig CA, Musch TI, Sexton WL, Poole DC. Effects of prior contractions on muscle microvascular oxygen pressure at onset of subsequent contractions. *J Physiol.* 539(Pt 3): 927-934, 2002.

Behnke BJ, McDonough P, Padilla DJ, Musch TI, Poole DC. Oxygen exchange profile in rat muscles of contrasting fibre types. *J Physiol.* 549(Pt 2): 597-605, 2003.

Behnia C, Wheatley CM, Avolio A, Johnson BD. Influence of dietary nitrate supplementation on lung function and exercise gas exchange in COPD patients. *Nitric Oxide.* 76: 53-61, 2018.

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

Bergstrom J. Muscle electrolytes in man. *Scand J Clin Lab Med* 14: 511-513, 1962.

Bergström J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance*. Acta Physiol Scand.* 71: 140– 150, 1967.

Bergström J, Hultman E. Muscle glycogen synthesis after exercise: an enhancing factor localized to the muscle cells in man. *Nature*. 210: 309–310, 1966.

Betteridge S, Bescós R, Martorell M, Pons A, Garnham AP, Stathis CC, McConell GK. No effect of acute beetroot juice ingestion on oxygen consumption, glucose kinetics, or skeletal muscle metabolism during submaximal exercise in males. *J Appl Physiol.* 120(4): 391-398, 2016.

Bescòs R, Ferrer-Roca V, Galilea PA, Roig A, Drobnic F, Sureda A, Martorell M, Cordova A, Tur JA, Pons A. Sodium nitrate supplementation does not enhance performance of endurance athletes. *Med Sci Sports Exerc.* 44(12): 2400-2409, 2012.

Bescòs R, Rodriguez FA, Iglesias X, Ferrer MD, Iborra E, Pons A. Acute

administration of inorganic nitrate reduces VO_2 peak in endurance athletes. *Med Sci Sports Exerc.* 43: 1979–1986, 2011.

Bevilacqua L, Seifert EL, Estey C, Gerrits MF, and Harper ME. Absence of uncoupling protein-3 leads to greater activation of an adenine nucleotide translocase-mediated proton conductance in skeletal muscle mitochondria from calorie restricted mice. *Biochim Biophys Acta.* 1797: 1389– 1397, 2010.

Bigland-Ritchie B, Cafarelli E, Vøllestad NK. Fatigue of submaximal static contractions. *Acta Physiol Scand Suppl.* 556: 137– 148, 1986.

Bigland-Ritchie B, Johansson R, Lippold OC, Smith S, Woods JJ. Changes in motoneurone firing rates during sustained maximal voluntary contractions. *J Physiol* 340: 335–346, 1983.

Bigland-Ritchie B, Woods JJ. Changes in muscle contractile properties and neural control during human muscular fatigue. *Muscle Nerve.* 7: 691-699, 1984.

Biswas S, Chida AS, Rahman I. Redox modifications of protein–thiols: Emerging roles in cell signaling. *Biochem Pharmacol*. 71: 551–564, 2006.

Black MI, Jones AM, Blackwell JR, Bailey SJ, Wylie LJ, McDonagh ST, Thompson C, Kelly J, Sumners P, Mileva KN, Bowtell JL, Vanhatalo A. Muscle metabolic and neuromuscular determinants of fatigue during cycling in different exercise intensity domains. *J Appl Physiol* (1985). 122(3): 446-459, 2017. doi: 10.1152/japplphysiol.00942.2016.

Bloch MH, Panza KE, Grant JE, Pittenger C, Leckman JF. N-Acetylcysteine in the treatment of pediatric trichotillomania: a randomized, double-blind, placebo-controlled add-on trial. *J Am Acad Child Adolesc Psychiatry.* 52(3): 231-240, 2013.

Boesgaard S, Iversen HK, Wroblewski H, Poulsen HE, Frandsen H, Kastrup J, Aldershvile J. Altered peripheral vasodilator profile of nitroglycerine during long-term infusion of n-acetylcysteine. *J Am Coll Cardiol.* 23: 163-169, 1994.

Bogdanis GC, Nevill ME, Boobis LH, Lakomy HK. Contribution of phosphocreatine and aerobic metabolism to energy supply during repeated sprint exercise. *J Appl Physiol.* 80: 876-884, 1996.

Bond V Jr, Curry BH, Adams RG, Millis RM, Haddad GE. Cardiorespiratory function associated with dietary nitrate supplementation. *Appl Physiol Nutr Metab*. 39(2): 168–172, 2014.

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

Boobis LH, Williams C, Wootton SA. Influence of sprint training on muscle metabolism during brief maximal exercise in man. *J Physiol.* 342: 36P-37P, 1983.

Boorsma RK, Whitfield J, Spriet LL. Beetroot juice supplementation does not improve performance of elite 1500-m runners. *Med Sci Sports Exerc.* 46(12): 2326-2334, 2014.

Borgstrom L, Kagedal B, Paulsen O. Pharmokinetics of N-acetyl-cysteine in man. *Eur J Clin Pharmacol.* 31: 217-222, 1986.

Bottinelli R, Reggiani C. Human skeletal muscle fibres: molecular and functional diversity. *Prog Biophys Mol Biol*. 73:195-262, 2000.

Bourdillon N, Fan JL, Uva B, Müller H, Meyer P, Kayser B. Effect of oral nitrate supplementation on pulmonary hemodynamics during exercise and time trial performance in normoxia and hypoxia: a randomized controlled trial. *Front Physiol.* 6: 288, 2015.

Bowtell J, Kelly V. Fruit-derived polyphenol supplementation for athlete recovery and performance. *Sports Med.* 49(Suppl 1): 3-23, 2019.

Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, Cornwall EJ. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *J Biochem.* 392, 353–362, 2005.

Brand MD, Pakay JL, Ocloo A, Kokoszka J, Wallace DC, Brookes PS, Cornwall EJ. The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *J Biochem.* 392: 353–362, 2005.

Bredt DS. Endogenous nitric oxide synthesis: biological functions and pathophysiology. *Free Radic Res.* 31: 577–96, 1999.

Breese BC, McNarry MA, Marwood S, Blackwell JR, Bailey SJ, Jones AM. Beetroot juice supplementation speeds O2 uptake kinetics and improves exercise tolerance during severe-intensity exercise initiated from an elevated metabolic rate. *Am J Physiol Regul Integr Comp Physiol.* 305(12): R1441-1450, 2013.

Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. *Cell.* 84(5): 757-767, 1996.

Brown GC, Cooper CE. Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett*. 356: 295–98, 1994.

Brooks GA, Hittelman KJ, Faulkner JA, Beyer RE. Temperature, skeletal muscle mitochondrial functions, and oxygen debt. *Am J Physiol*. 220: 1053Y9, 1971.

Brooke MH, Kaiser KK. Three “myosin ATPase” systems: the nature of their pH lability and sulfydryl dependence. *J Histochem Cytochem*. 18, 670–672, 1970.

Brotto MAP, Nosek TM. Hydrogen peroxide disrupts calcium release from the sarcoplasmic reticulum of rat skeletal muscle fibers. *J Appl Physiol*. 81: 731–737, 1996.

Bruton JD, Place N, Yamada T, Silva JP, Andrade FH, Dahlstedt AJ, Zhang SJ, Katz A, Larsson NG, Westerblad H. Reactive oxygen species and fatigue-induced prolonged low-frequency force depression in skeletal muscle fibres of rats, mice and SOD2 overexpressing mice. *J Physiol.* 586(1): 175-184, 2008.

Brueckner JC, Atchou G, Capelli C, Duvallet A, Barrault D, Jousselin E, Rieu M, di Prampero PE. The energy cost of running increases with the distance covered. *Eur J Appl Physiol Occup Physiol* 62: 385–389, 1991.

Bryan NS, Fernandez BO, Bauer SM, Garcia-Saura MF, Milsom AB, Rassaf T, Maloney RE, Bharti A, Rodriguez J, Feelisch M. Nitrite is signalling molecule and regulator of gene expression in mammalian tissue*. Nature Chem Biol.* 1(5): 290–297, 2005.

Buck CL, Henry T, Guelfi K, Dawson B, McNaughton LR, Wallman K. Effects of sodium phosphate and beetroot juice supplementation on repeated-sprint ability in females. *Eur J Appl Physiol.* 115(10): 2205-2213, 2015.

Burnley M, Jones AM. Oxygen uptake kinetics as a determinant of sports performance. *Eur J Sport Sci.* 7: 63-79, 2007.

Burton FL, Smith GL. The effect of cromakalim on intracellular [Ca2+] in isolated rat skeletal muscle during fatigue and metabolic blockade. *Exp Physiol.* 82, 469-483, 1997.

Butera D, Tesoriere L, Di Gaudio F, Bongiorno A, Allegra M, Pintaudi AM, Kohen R & Livrea MA. Antioxidant activities of sicilian prickly pear (Opuntia ficus indica) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin. J Agric Food Chem 50(23), 6895-6901, 2002.

Cain DF, Infante AA, Davies RE. Chemistry of muscle contraction. Adenosine triphosphate and phosphorylcreatine as energy supplies for single contractions of working muscle. *Nature* 196: 214-217, 1962

Cain DF, Davies RE. Breakdown of adenosine triphosphate during a single contraction of working muscle. *Biochem Biophys Res Commun* 8: 361-366, 1962

Callahan LA, Nethery D, Stofan D, DiMarco A, Supinski G. Free radical-induced contractile protein dysfunction in endotoxin-induced sepsis. *Am J Respir Cell Mo*l Biol. 24: 210–217, 2001.

Callahan MJ, Parr EB, Hawley JA, Burke LM. Single and combined effects of beetroot crystals and sodium bicarbonate on 4-km cycling time trial performance. *Int J Sport Nutr Exerc Metab.* 27(3): 271-278, 2017.

Campos HO, Drummond LR, Rodrigues QT, Machado FSM, Pires W, Wanner SP, Coimbra CC. Nitrate supplementation improves physical performance specifically in non-athletes during prolonged open-ended tests: a systematic review and meta-analysis. *Br J Nutr.* 119(6): 636-657, 2018.

Cannon DT, Bimson WE, Hampson SA, Bowen TS, Murgatriyd SR, Marwood S, Kemp GJ, Rossiter HB. Skeletal muscle ATP turnover by 31P magnetic resonance spectroscopy during moderate and heavy bilateral knee extension. *J Physiol.* 592: 5287–5300, 2014.

Canton M, Neverova I, Menabo R, Van EJ, Di LF. Evidence of myofibrillar protein oxidation induced by postischemic reperfusion in isolated rat hearts. *Am J Physiol Heart Circ Physiol* 286: H870–H877, 2004.

Carlsson S, Wiklund NP, Engstrand L, Weitzberg E, Lundberg JO. Effects of pH, nitrite, and ascorbic acid on nonenzymatic nitric oxide generation and bacterial growth in urine. *Nitric Oxide* 5: 580–586 2001.

Carlström M, Larsen FJ, Nyström T, Hezel M, Borniquel S, Weitzberg E, Lundberg JO. Dietary inorganic nitrate reverses features of metabolic syndrome in endothelial nitric oxide synthase-deficient mice. *Proc Natl Acad Sci USA.* 107(41): 17716-17720, 2010.

Carriker CR, Vaughan RA, VanDusseldorp TA, Johnson KE, Beltz NM, McCormick JJ, Cole NH, Gibson AL. Nitrate-containing beetroot juice reduces oxygen consumption during submaximal exercise in low but not high aerobically fit male runners. *J Exerc Nutrition Biochem.* 20(4): 27-34, 2016.

Carpentier A, Stragier S, Bréjeon C, Poortmans JR. Nitrate supplementation, exercise, and kidney function: are there detrimental effects? *Med Sci Sports Exerc.* 47(7): 1519-1522, 2015.

Castello PR, David PS, McClure T, Crook Z, & Poyton RO. Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab, 3*(4), 277-287, 2006.

Cassens, R. Use of sodium nitrite in cured meats today. *Food Technol.* 49: 72–81, 1995.

Casey A, Constantin-Teodosiu D, Howell S, Hultman E, Greenhaff PL. Metabolic responses of type I and II muscle fibres during repeated bouts of maximal exercise in humans. *Am J Physiol*. 271: E38-E43, 1996.

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

Cermak NM, Resp P, Stinkens R, Lundberg JO, Gibala MJ, van Loon LJ. No improvement in endurance performance after a single dose of beetroot juice. *Int J Sport Nutr Exerc Metab.* 22(6): 470-478, 2012b.

Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J Biol Chem.* 217: 383–93, 1955.

Chasiotis D, Sahlin K, Hultman E. Regulation of muscle glycogenolysis at rest and during exercise. *J Appl Physiol.* 53: 852–877, 1982.

Chaves AA, Joshi MS, Coyle CM, Brady JE, Dech SJ, Schanbacher BL, Baliga R, Basuray A, Bauer JA. Vasoprotective endothelial effects of a standardized grape product in humans. *Vascul Pharmacol.* 50(1-2): 20-6, 2009.

Cheetham ME, Boobis LH, Brooks S, Williams C. Human muscle metabolism during sprint running. *J Appl Physiol.* 61: 54-60, 1986.

Chidnok W, DiMenna FJ, Fulford J, Bailey SJ, Skiba PF, Vanhatalo A, Jones AM. Muscle metabolic responses during high-intensity intermittent exercise measured by ^{31}P- MRS: relationship to the critical power concept. *Am J Physiol Regul Integr Comp Physiol.* 305: R1085–R1092, 2013.

Christensen EH, Hansen O. Arbeitsf€ahigkeit und ern€ahrung. *Skand Arch Physiol.* 81: 160– 171, 1939.

Christensen PM, Nyberg M, Bangsbo J. Influence of nitrate supplementation on VO2 kinetics and endurance of elite cyclists. *Scand J Med Sci Sports.* 23(1): e21-e31, 2013.

Christensen PM, Petersen NK, Friis SN, Weitzberg E, Nybo L. Effects of nitrate supplementation in trained and untrained muscle are modest with initial high plasma nitrite levels. *Scand J Med Sci Sports.* 27(12): 1616-1626, 2017.

Chin ER, Allen DG. Effects of reduced muscle glycogen concentration on force, Ca2+ release and contractile protein function in intact mouse skeletal muscle. *J Physiol.* 498: 17–29, 1997.

Clanton TL. Hypoxia-induced reactive oxygen species formation in skeletal muscle. *J Appl Physiol.* 102: 2379-2388, 2007.

Clausen T. Na+-K+ pump regulation and skeletal muscle contractility. *Physiol Rev*. 83: 1269–1324, 2003.

Clausen T, Overgaard K, Nielsen OB. Evidence that the Na+-K+ leak/pump ratio contributes to the difference in endurance be- tween fast- and slow-twitch muscles. *Acta Physiol Scand.* 180: 209–216, 2004.

Cleeter MW, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AH. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett. 345*:50-54, 1994.

Clifford T, Howatson G, West DJ, Stevenson EJ. The potential benefits of red beetroot supplementation in health and disease. *Nutrients.* 7: 2801-2822, 2015.

Clifford T, Contantinou CM, Keane KM, West DJ, Howatson G, Stevenson EJ. The plasma bioavailability of nitrate and betanin from Beta vulgaris

rubra in humans. *Eur J Nutr.* 56: 1245-1254, 2017.

Cobley JN, McGlory C, Morton JP, Close GL. N-acetylcysteine's attenuation of fatigue after repeated bouts of intermittent exercise: practice implications for tournament situations. *Int J Sport Nutr Exerc Metab.* 21(6): 451-461, 2011.

Commoner B, Townsend J, Pake GE. Free radicals in biological materials. *Nature*. 174, 689–691, 1954.

Cooke R, Franks K, Luciani GB, Pate E. The inhibition of rabbit skeletal muscle contraction by hydrogen ions and phosphate. *J Physiol.* 395, 77–97, 1988.

Coggan AR, Broadstreet SR, Mikhalkova D, Bole I, Leibowitz JL, Kadkhodayan A, Park S, Thomas DP, Theis D, Peterson LR. Dietary nitrate-induced increases in human muscle power: high versus low responders. *Physiol Rep.* 6(2): e13575, 2018.

Coggan AR, Leibowitz JL, Kadkhodayan A, Thomas DP, Ramamurthy S, Spearie CA, Waller S, Farmer M, Peterson LR. Effect of acute dietary nitrate intake on maximal knee extensor speed and power in healthy men and women. *Nitric Oxide* 1(48):16–21, 2015.

Coggan AR, Peterson LR. Dietary nitrate enhances the contractile properties of human skeletal muscle. *Exerc Sport Sci Rev.* 46(4): 254-261, 2018.

Constables SH, Favier RJ, McLane JA, Fell RD, Chen M, Holloszy JO. Energy metabolism in contracting rat skeletal muscle: adaptation to exercisie training. *Am J Physiol.* 253: C316-C322, 1987.

Cook MD, Myers SD, Gault ML, Willems MET. Blackcurrant alters physiological responses and femoral artery diameter during sustained isometric contraction. *Nutrients.* 9(6): E556, 2017.

Corn SD, Barstow TJ. Effects of oral N-acetylcysteine on fatigue, critical power, and W' in exercising humans. *Resp Phys Neurol.* 178: 261-268, 2011.

Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon RO 3rd, Gladwin MT. Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat. Med.* 9:1498–505, 2003.

Costello JT, Bieuzen F, Bleakley CM. Where are all the female participants in Sports and Exercise Medicine research?. *Eur J Sport Sci.* 14(8): 847-

851, 2014.

Coyle EF, Costill DL, Lesmes GR. Leg extension power and muscle fiber composition. *Med Sci Sports Exerc.* 11:12–15, 1979.

Coyle EF, Hamilton MT, Alonso JG, Montain SJ, Ivy JL. Carbohydrate metabolism during intense exercise when hyperglycemic. *J Appl Physiol.* 70(2): 834-840, 1991.

Coyle EF, Sidossis LS, Horowitz JF, Beltz JD. Cycling efficiency is related to the percentage of type I muscle fibers. *Med Sci Sports Exerc.* 24(7): 782-788, 1992.

Craig JC, Colburn TD, Hirai DM, Musch TI, Poole DC. Sexual dimorphism in the control of skeletal muscle interstitial PO2 of heart failure rats: Effects of dietary nitrate supplementation. *J Appl Physiol.* [Epub ahead of print]. doi: 10.1152/japplphysiol.01004.2018.

Craig JC, Colburn TD, Hirai DM, Schettler MJ, Musch TI, Poole DC. Sex and nitric oxide bioavailability interact to modulate interstitial PO2 in healthy rat skeletal muscle. *J Appl Physiol.* 1(6): 1558-1566, 2018.

Crawford JH, Isbell TS, Huang Z, Shiva S, Chacko BK, Schechter AN, Darley-Usmar VM, Kerby JD, Lang JD Jr, Kraus D, Ho C, Gladwin MT, Patel RP. Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood*. 107: 566–574, 2006.

Crow MT, Kushmerick MJ. Chemical energetics of slow- and fast-twitch muscles of the mouse. *J Gen Physiol.* 79(1): 147-166, 1982a.

Crow MT, Kushmerick MJ. Myosin light chain phosphorylation is associated with a decrease in the energy cost for contraction in fast twitch mouse muscle. *J Biol Chem*. 257: 2121–2124, 1982b.

Crow MT, Kushmerick MJ. Phosphorylation of myosin light chains in mouse fast-twitch muscle associated with reduced actomyosin turnover rate. *Science.* 217(4562): 835-837, 1982c.

Crowder MS, Cooke R. The effect of myosin sulphydryl modification on the mechanics of fibre contraction. *J Muscle Res Cell Motil*. 5(2): 131–146, 1984.

Crum EM, Barnes MJ, Stannard SR. Multiday pomegranate extract supplementation decreases oxygen uptake during submaximal cycling exercise, but cosupplementation with N-Acetylcysteine negates the effect. *Int J Sport Nutr Exerc Metab.* [epub ahead of print], 2018.

Crum EM, Muhamed AMC, Barnes MJ, Stannard SR. The effect of acute pomegranate extract supplementation on oxygen uptake in highly-trained cyclists during high-intensity exercise in a high altitude environment. *J Int Soc Sports Nutr.* 14: 14, 2017.

Dahlstedt AJ, Katz A, Westerblad H. Role of myoplasmic phosphate in contractile function of skeletal muscle: studies on creatine kinase-deficient mice. *J Physiol.* 533: 379–388, 2001.

Dalle-Donne I, Rossi R, Colombo G, Giustarin D, Milzani A. Protein S-glutathionylation: a regulatory device from bacteria to humans. *Trends Biochem Sci.* 34(2): 85–96, 2009.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta.* 329(1): 23-38, 2003.

Darques JL, Decherchi P, Jammes Y. Mechanisms of fatigue-induced activation of group IV muscle afferents: the roles played by lactic acid and inflammatory mediators. *Neurosci Lett*. 257: 109–112, 1998.

Darques JL, Jammes Y. Fatigue-induced changes in group IV muscle afferent activity: differences between high- and low-frequency electrically induced fatigues. *Brain Res*. 750: 147–154, 1997

Danieli-Betto D, Germinario E, Esposito A, Biral D, Betto R. Effects of fatigue on sarcoplasmic reticulum and myofibrillar prop- erties of rat single muscle fibers. *J Appl Physiol.* 89: 891–898, 2000.

Davies KJ, Quintanilha AT, Brooks GA, Packer L. Free radicals and tissue damage produced by exercise. *Biochem Biophys Res Commun.* 107: 1198–1205, 1982.

de Castro TF, Manoel FA, Fiqueiredo DH, Fiqueiredo DH, Machado FA. Effect of beetroot juice supplementation on 10-km performance in recreational runners. *Appl Physiol Nutr Metab.* 44(1): 90-94, 2019.

Debold EP. Potential molecular mechanisms underlying muscle fatigue mediated by reactive oxygen and nitrogen species. Front Physiol 2015; 6: 239, 2015.

Dejam A, Hunter CJ, Tremonti C, Pluta RM, Hon YY, Grimes G, Partovi K, Pelletier MM, Oldfield EH, Cannon RO 3rd, Schechter AN, Gladwin MT. Nitrite infusion in humans and nonhuman primates: endocrine effects, pharmacokinetics, and tolerance formation. *Circulation*. 116:

1821–31, 2007.

De Luca CJ. The use of surface electromyography in biomechanics. *J Appl Biomech.* 13: 135-163, 1993.

Delliaux S, Brerro-Saby C, Steinberg JG, Jammes Y. Reactive oxygen species activate the group IV muscle afferents in resting and exercising muscle in rats*. Pflugers Arch.* 459: 143–150, 2009.

Delp MP. Effects of exercise training on endothelium-dependent peripheral vascular responsiveness. *Med Sci Sports Exerc.* 27(8): 1152-1157, 1995.

Dempsey JA, Romer L, Rodman J, Miller J, Smith C. Consequences of exercise-induced respiratory muscle work. *Resp Physiol Neurobiol*. 151(2–3): 242–250, 2006.

Deneke SM. Thiol-based antioxidants. *Curr Top Cell Regul.* 36: 151-180, 2000.

Dewhurst-Trigg R, Yeates T, Blackwell JR, Thompson C, Linoby A, Morgan PT, Clarke I, Connolly LJ, Wylie LJ, Winyard PG, Jones AM, Bailey SJ. Lowering of blood pressure after nitrate-rich vegetable consumption is abolished with the co-ingestion of thiocyanate-rich vegetables in healthy normotensive males. *Nitric Oxide.* 74: 39-46, 2018.

Diaz PT, She ZW, Davis WB, Clanton TL. Hydroxylation of salicylate by the in vitro diaphragm: evidence for hydroxyl radical production during fatigue. *J Appl Physiol.* 75: 540–545, 1993.

Dillard CJ, Litov RE, Savin WM, Dumelin EE, Tappel AL. Effects of exercise, vitamin E, ozone on pulmonary function and lipid peroxidation*. J Appl Physiol*. 45:927–932, 1978.

Doel JJ, Benjamin N, Hector MP, Rogers M, Allaker RP. Evaluation of bacterial nitrate reduction in the human oral cavity. *Eur J Oral Sci.* 113:14 –19, 2005.

Dominelli PB, Archiz B, Ramsook AH, Mitchell RA, Peters CM, Molgat-Seon Y, Henderson WR, Koehle MS, Boushel R, Sheel AW. Effects of respiratory muscle work on respiratory and locomotor blood flow during exercise. *Exp Physiol.* 102: 1535-1547, 2017.

Doyle MP, Hoekstra JW. Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J Inorg Biochem*. 14: 351–358, 1981.

Dreissigacker U, Wendt, Wittke T, Tsikas D, Maassen N. Positive correlation between plasma nitrite and performance during high-intensive exercise

but not oxidative stress in healthy men. *Nitric Oxide.* 23(2): 128-135, 2010.

Duhamel TA, Green HJ, Perco JG & Ouyang J. Comparative effects of a low-carbohydrate diet and exercise plus a low-carbohydrate diet on muscle sarcoplasmic reticulum responses in males. *Am J Physiol Cell Physiol.* 291: C607–C617, 2006a.

Duhamel TA, Perco JG, Green HJ. Manipulation of dietary carbohydrates after prolonged effort modifies muscle sarcoplasmic reticulum responses in exercising males. *Am J Physiol Regul Integr Comp Physiol*. 291: R1100–R1110, 2006.

Duthie GG, Robertson JD, Maughan RJ, Morrice PC. Blood antioxidant status and erythrocyte lipid peroxidation following distance running. *Arch Biochem Biophys*. 282:78–83, 1990.

Duncan C, Dougall H, Johnston P, Green S, Brogan R, Leifert C, Smith L, Golden M, Benjamin N. Chemical generation of nitric oxide in the mouth from the enterosalivary circulation of dietary nitrate. *Nat Med.* 1(6): 546-551, 1995.

Echtay KS, Roussel D, St-Pierre J, Jekabsons MB, Cadenas S, Stuart JA, Harper JA, Roebuck SJ, Morrison A, Pickering S, Clapham JC, Brand MD. Superoxide activates mitochondrial uncoupling proteins. Nature 415, 96–9, 2002.

Edwards DA, Fletcher K, Rowlands EN. Antagonism between perchlorate, iodide, thiocyanate, and nitrate for secretion in human saliva; analogy with the iodide trap of the thyroid. *Lancet.* 266(6810): 498-499., 1954.

Endo MY, Kobayakawa M, Kinugasa R, Kuno S, Akima H, Rossiter HB, Miura A, Fukuba Y. Thigh muscle activation distribution and pulmonary VO2 kinetics during moderate, heavy, and very heavy intensity cycling exercise in humans. *Am J Physiol Regul Integr Comp Physiol.* 293: R812-820, 2007.

Engler MB, Engler MM, Chen CY, Malloy MJ, Browne A, Chiu EY, Kwak HK, Milbury P, Paul SM, Blumberg J, Mietus-Snyder ML. Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults. *J Am Coll Nutr.* 23(3): 197-204, 2004.

Enoka RM, Stuart DG. Neurobiology of muscle fatigue. *J Appl Physiol.* 72: 1631–1648, 1992.

Enoka RM, Duchateau J. Muscle fatigue: what, why and how it influences muscle function. *J Physiol.* 586: 11–23, 2008.

Esatbeyoglu T, Wagner AE, Motafakkerazad R, Nakajima Y, Matsugo S, Rimbach G. Free radical scavenging and antioxidant activity of betanin: Electron spin resonance spectroscopy studies and studies in cultured cells. *Food Chem Toxicol.* 73: 119–126, 2014.

Esatbeyoglu T, Wagner AE, Schini-Kerth VB, Rimbach G. Betanin–A food colorant with biological activity. *Mol Nutr Food Res.* 59: 36-47, 2015.

Escribano MA, Pedrenp F, Garcia-Carmona, Munoz R. Characterization of the antiradical activity of betalains from Beta vulgaris L. roots. *Phytochem Anal.* 9(3): 124-127, 1998.

Esen O, Nicholas C, Morris M, Bailey SJ. No effect of beetroot juice supplementation on 100-m and 200-m swimming performance in moderately-trained swimmers. *Int J Sports Physiol Perform.* 14: 1-19, 2018.

Fan J, Bourdillon N, Meyer P, Kayser B. Oral nitrate supplementation differentially modulates cerebral artery blood velocity and prefrontal tissue oxygenation during 15 km time-trial cycling in normoxia but not in hypoxia. *Front Physiol,* 9: 869, 2018.

Febbraio MA, Lambert DL, Starkie RL, Proietto J, Hargreaves M. Effect of epinephrine on muscle glycogenolysis during exercise in trained men. *J Appl Physiol.* 1998: 84: 465–470.

Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, Poole DC. Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in rats*. J Physiol.* 591: 547-557. 2013

Ferguson SK, Holdsworth CT, Wright JL, Fees AJ, Allen JD, Jones AM, Musch TI, Poole DC. Microvascular oxygen pressures in muscles comprised of different fiber types: impact of dietary nitrate supplementation. *Nitric Oxide.* 48: 38-43, 2015.

Fernström M, Bakkman L, Tonkonogi M, Shabalina IG, Rozhdestvenskaya Z, Mattsson CM, Enqvist JK, Ekblom B, Sahlin K. Reduced efficiency, but increased fat oxidation, in mitochondria from human skeletal muscle

after 24-h ultraendurance exercise. *J Appl Physiol.* 102: 1844-1849, 2007.

Ferreira LF, Campbell KS, Reid MB. N-Acetylcysteine in handgrip exercise: plasma thiols and adverse reactions. *Int J Sport Nutr Exerc Metab.* 21(2): 146-154, 2011.

Ferreira LF, Reid MB. Muscle-derived ROS and thiol regulation in muscle fatigue. *J Appl Physiol (1985).* 104(3): 853-860, 2008.

Flanagan SD, Looney DP, Miller MJ, DuPont WH, Pryor L, Creighton BC, Sterczala AJ Azivak TK, Hooper DR, Maresh CM, VOlek JS, Ellis LA, Kraemer WJ. The effects of nitrate-rich supplementation on neuromuscular efficiency during heavy resistance exercise. *J Am Coll Nutr.* 35(2): 100-107, 2016.

Flueck JL, Bogdanova A, Mettler S, Perret C. Is beetroot juice more effect than sodium nitrate? The effects of equimolar nitrate dosages of nitrate-rich beetroot juice and sodium nitrate on oxygen consumption during exercise. *Appl Physiol Nutr Metab.* 41(4): 421-429, 2016.

Fowles JR, Green HJ, Tupling R, O'Brien S, Roy BD. Human neuromuscular fatigue is associated with altered Na+-K+-ATPase activity following isometric exercise. *J Appl Physiol* 92: 1585–1593, 2002.

Fitts RH. Cellular mechanisms of muscle fatigue. *Physiol Rev.* 74, 49–94, 1994.

Fleischer S, Inui M. Biochemistry and biophysics of excitation-contraction coupling. *Ann Rev Biophys Biophys Chem.* 18: 333-364, 1989.

Flueck JL, Bogdanova A, Mettler S, Perret C. Is beetroot juice more effect than sodium nitrate? The effects of equimolar nitrate dosages of nitrate-rich beetroot juice and sodium nitrate on oxygen consumption during exercise. *Appl Physiol Nutr Metab.* 41(4): 421-429, 2016.

Förstermann U. Nitric oxide and oxidative stress in vascular disease. *Pflugers Arch.* 459(6): 923-939, 2010.

Förstermann U, Pollock JS, Schmidt HH, Heller M, Murad F. Calmodulin-dependent endothelium-derived relaxing factor/nitric oxide synthase activity is present in the particulate and cytosolic fractions of bovine aortic endothelial cells. *Proc Natl Acad Sci USA*> 88: 1788-1792, 1991.

Fraser SF, Li JL, Carey MF,Wang XN, Sangkabutra T, Sostaric S, Selig SE, Kjeldsen K, McKenna MJ. Fatigue depresses maximal in vitro skeletal muscle Na+,K+-ATPase activity in untrained and trained individuals. *J Appl Physiol.* 93, 1650–1659, 2002.

Fuchs F, Reddy Y, Briggs FN. The interaction of cations with the calcium-binding site of troponin. *Biochim Biophys Acta.* 221: 407–409, 1970.

Fukuba Y, Miura A, Endo M, Kan A, Yanagawa K, Whipp BJ. The curvature constant parameter of the power-duration curve for varied-power exercise. *Med Sci Sports Exerc*. 35: 1413–1418, 2003.

Fulford J, Winyard PG, Vanhatalo A, Bailey SJ, Blackwell JR, Jones AM. Influence of dietary nitrate supplementation on human skeletal muscle metabolism and force production during maximum voluntary contractions. *Pflugers Arch.* 465(4): 517-528, 2013.

Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetycholine. *Nature.* 288(5789): 373-376, 1980.

Gago B, Lundberg JO, Barbosa RM, Laranjinha J. Red wine-dependent reduction of nitrite to nitric oxide in the stomach. *Free Rad Biol Med.* 43: 1233-1242, 2007.

Galler S, Hilber K, Göbesberger A. Effects of nitric oxide on force-generating proteins of skeletal muscle. *Pflügers Arch* 434: 242–245, 1997.

Gandevia SC. Spinal and supraspinal factors in human muscle fatigue. *Physiol Rev.* 81(4): 1725-1789, 2001.

Gangolli SD, van den Brandt PA, Feron VJ, Janzowsky C, Koeman JH, Speijers GJ, Spiegelhalder B, Walker, R, Wisnok JS. Nitrate, nitrite and *N*-nitroso compounds. *Eur J Pharmacol.* 292: 1–38, 1994.

Garland SJ, Enoka RM, Serrano LP, Robinson GA. Behavior of motor units in human biceps brachii during a submaximal fatiguing contraction. *J Appl Physiol.* 76: 2411–2419, 1994

Garthwaite J. Concepts of neural nitric oxide-mediated transmission. *Eur J Neurosci.* 27(11): 2783-2802, 2008.

Gaesser GA, Poole DC. The slow component of oxygen uptake kinetics in humans. *Exerc Sport Sci Rev.* 24: 35-71, 1996.

Georgiev VG, Weber J, Kneschke E-M, Denev PN, Bley T, Pavlov AI. Antioxidant activity and phenolic content of beta- lain extracts from intact plants and hairy root cultures of the red beetroot Beta vulgaris cv. Detroit dark red. *Plant Foods Hum Nutr.* 65(2):105–111, 2010.

Ghiarone T, Ataide-Silva T, Bertuzzi R, McConnell GK, Lima-Silva AE. Effect of acute nitrate ingestion on VO2 response at different exercise intensity

domains. *Appl Physiol Nutr Metab.* 42(11): 1127-1134, 2017.

Gheibi S, Jeddi S, Carlström M, Gholami H, Ghasemi A. Effects of long-term nitrate supplementation on carbohydrate metabolism, lipid profiles, oxidative stress, and inflammation in male obese type 2 diabetic rats. *Nitric Oxide.* 75: 27-41, 2018.

Gladwin MT, Schechter AN, Kim-Shapiro DB, Patel RP, Hogg N, Shiva S, Cannon RO 3rd, Kelm M, Wink DA, Espey MG, Oldfield EH, Pluta RM, Freeman BA, Lancaster JR Jr, Feelisch M, Lundberg JO. The emerging biology of the nitrite anion. *Nat Chem Biol.* 1(6): 308-314, 2005.

Glaister M, Pattison JR, Muniz-Pumares D, Patterson SD, Foley P. Effects of dietary nitrate, caffeine, and their combination on 20-km cycling time trial performance. *J Strength Cond Res.* 29(1): 165-174, 2015.

Gliszczyńska-Świgło A, Szymusiak H, Malinowska P. Betanin, the main pigment of red-beet molecular origin of its exceptionally high free radical scavenging activity. *Food Addit Contam.* 23(11): 1079-1087, 2006.

Gollnick PD, Piehl K, Saltin B. Selective glycogen depletion pattern in human muscle fibres after exercise of varying intensity and varying pedalling rates. *J Physiol.* 241: 45-57, 1974.

Goldfarb AH. Nutritional antioxidants as therapeutic and preventive modalities in exercise-induced muscle damage. *Can J Appl Physiol.* 24: 249–266, 1999.

Gong B, Legault D, Miki T, Seino S, Renaud JM. KATP channels depress force by reducing action potential amplitude in mouse EDL and soleus muscle. *Am J Physiol Cell Physiol*. 285, C1464-C1474, 2003.

Goulding RP, Roche DM, Marwood S. Prior exercise speeds pulmonary oxygen uptake kinetics and increases critical power during supine but not upright cycling. *Exp Physiol.* 102(9): 1158-1176, 2017.

Govoni M, Jansson EA, Weitzberg E, Lundberg JO. The increase in plasma nitrite after a dietary nitrate load is markedly attenuated by an antibacterial mouthwash. *Nitric Oxide* 19:333–37, 2008.

Gilchrist M, Shore AC, Benjamin N. Inorganic nitrate and nitrite and control of blood pressure. *Cardiovas Res.* 89(3): 492-498, 2011.

Gilliard CN, Lam JK, Cassel KS, Park JW, Schechter AN, Piknova B. Effect of dietary nitrate levels on nitrate fluxes in rat skeletal muscle and liver. *Nitric Oxide,* 75: 1-7, 2018.

Gladwin MT, Shelhamer JH, Schechter AN, Pease-Fye ME, Waclawiw MA, Panza JA, Ognibene FP, Cannon RO 3rd. Role of circulating nitrite and S-nitrosohemoglobin in the regulation of regional blood flow in humans. *Proc Natl Acad Sci U S A*. 97(21):11482-11487, 2000.

Gomez-Cabrera MC, Salvador-Pascual A, Cabo H, Ferrando B, Vina J. Redox modulation of mitochondriogenesis in exercise. Does antioxidant supple- mentation blunt the benefits of exercise training? *Free Radic Biol Med*. 86: 37–46, 2015.

Gomez-Cabrera MC, Borrás C, Pallardó FC, Sastre J, Ji LL, Viña J. Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. *J Physiol.* 567(Pt 1): 113-120, 2005.

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

Giraldez RR, Panda A, Xia Y, Sanders SP, Zweier JL. Decreased nitric-oxide synthase activity causes impaired endothelium-dependent relaxation in the postischemic heart. *J Biol Chem.* 272: 21420–21426, 1997.

Grassi B. Regulation of oxygen consumption at exercise onset: is it really controversial? *Exerc Sport Sci Rev.* 29: 134-138, 2001.

Grassi B, Rossiter HB, Hogan MC, Howlett RA, Harris JE, Goodwin ML, Dobson JL, Gladden LB. Faster O(2) uptake kinetics in canine skeletal muscle in situ after acute creatine kinase inhibition. *J Physiol*. 589: 221–233, 2011.

Grassi B, Rossiter HB, Zoladz JA. Skeletal muscle fatigue and decreased efficiency: two sides of the same coin? *Exerc Sport Sci Rev.* 43(2): 75-83, 2015.

Grassi B, Poole DC, Richardson RS, Knight DR, Erickson BK, Wagner PD. Muscle O2 uptake kinetics in humans: implications for metabolic control. *J Appl Physiol (1985).* 80(3): 988-998, 1996.

Greenhaff PL, Nevill ME, Soderlund K, Bodin K, Boobis LH, Williams C, Hultman E. The metabolic responses of human type I and II muscle fibres during maximal treadmill sprinting. *J Physiol.* 478:149–155, 1994.

Greenhaff PL, Gleeson M, Maughan RJ. The effects of dietary manipulation

on blood acid-base status and the performance of high intensity exercise. *Eur J Appl Physiol Occup Physiol.* 56(3): 331-337, 1987.

Gregory CM, Bickel CS. Recruitment patterns in human skeletal muscle during electrical stimulation. *Phys Ther*. 85:358–364, 2005.

Groen BH, Berden JA, van Dam K. Differentiation between leaks and slips in oxidative phosphorylation. *Biochim Biophys Acta*. 1019: 121–127, 1990.

Gross WL, Bak MI, Ingwall JS, Arstall MA, Smith TW, Balligand JL, Kelly RA. Nitric oxide inhibits creatine kinase and regulates rat heart contractile reserve. *Proc Natl Acad Sci USA.* 93(11): 5604-5609, 1996.

Grotto, D., **Santa Maria, L., Boeira, S., Valentini, J., Charao, M., Moro, A, Nascimento, P., Pomblum, V., & Garcia, S.** Rapid quantification of malondialdehyde in plasma by high performance liquid chromatography-visible detection. *J Pharm Biomed Anal.* 43: 619-624, 2007.

Grover AK, Samson SE, Misquitta CM. Sarco(endo)plasmic reticulum Ca2+ pump isoform SERCA3 is more resistant than SERCA2b to peroxide. *Am J Physiol*. 273: C420–C430, 1997.

Gutierrez-Martin Y, Martin-Romero FJ, Inesta-Vaquera FA, Gutierrez-Merino C, Henao F. Modulation of sarcoplasmic reticulum Ca2+-ATPase by chronic and acute exposure to peroxynitrite. *Eur J Biochem.* 271:2647–2657, 2004.

Hagan RD, Weis SE, Raven PB. Effect of pedal rate on cardiorespiratory responses during continuous exercise. *Med Sci Sports Exerc.* 24: 1088–1095, 1992.

Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 alpha. *Science.* 302: 1975–1978, 2003.

Hagberg JM, Mullin JP, Nagle FJ. Oxygen consumption during constant-load exercise. *J Appl Physiol*. 45: 381–384, 1978.

Hagen T, Taylor CT, Lam F, Moncada S. Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1 alpha. *Science.* 302: 1975–1978, 2003.

Haider G, Folland JP. Nitrate supplementation enhances the contractile properties of human skeletal muscle. *Med Sci Sports Exerc.* 46(12):2234-2243, 2014.

Haizlip KM, Harrison BC, Leinwand LA. Sex-based differences in skeletal muscle kinetics and fibre-type composition. *Physiol(Bethesda).* 30(1): 30-39, 2015.

Hakim TS, Sugimori K, Camporesi EM, Anderson G. Half-life of nitric oxide in aqueous solutions with and without haemoglobin. *Physiol Meas.* 17(4): 267-277, 1996.

Hall DT, Ma JF, Marco SD, Gallouzi IE. Inducible nitric oxide synthase (iNOS) in muscle wasting syndrome, sarcopenia, and cachexia. *Aging (Albany NY).* 3(8): 702-715, 2011.

Halliwell B. Free radicals and antioxidants: a personal view. *Nutr Rev.* 52: 253-265, 1994.

Halliwell B, Gutteridge J. Free Radicals in Biology and Medicine. Oxford, UK: Oxford Univ. Press; 2007.

Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine. New York: Oxford Univ. Press, 1998.

Halliwell B, Grootved M. The measurement of free radical reaction in humans. *FEBS Lett.* 213: 9-14, 1987.

Handzlik MK, Gleeson M. Likely additive ergogenic effects of combined preexercise dietary nitrate and caffeine ingestion in trained cyclists. *ISRN Nutr.* 2013: 396581, 2013.

Haouzi P, Hill JM, Lewis BK, Kaufman MP. Responses of group III and IV muscle afferents to distension of the peripheral vascular bed. *J Appl Physiol.* 87:545–553, 1999.

Hargreaves M, Richter EA. Regulation of skeletal muscle glycogenolysis during exercise. *Can J Sport Sci*. 1988 Dec;13(4):197-203.

Harms CA, Babcock MA, McClaran SR, Pegelow DF, Nickele GA, Nelson WB, Dempsey JA. Respiratory muscle work compromises leg blood flow during maximal exercise. *J Appl Physiol.* 82(5):1573–1583, 1997.

Haseler LJ, Hogan MC, Richardson RS. Skeletal muscle phosphocreatine recovery in exercise-trained humans is dependent on O2 availability. *J Appl Physiol*. 86: 2013-2018, 1999.

Hayes SG, McCord JL, Koba S, Kaufman MP. Gadolinium inhibits group III but not group IV muscle afferent responses to dynamic exercise. *J Physiol*. 587: 873–882, 2009.

Helander I, Westerblad H, Katz A. Effects of glucose on contractile function, [Ca2?]i and glycogen in isolated mouse skeletal muscle. *Am J Physiol Cell Physiol.* 282: C1306–C1312, 2002.

Henneman E, Somjen G, Carpenter DO. Excitability and inhibitability of motorneurons of different sizes. *J Neurophysiol.* 28: 599-620, 1965.

Hepple RT, Howlett RA, Kindig CA, Stary CM, Hogan MC. The O2 cost of the tension-time integral in isolated single myocytes during fatigue. *Am J Physiol Regul Integr Comp Physiol.* 298: R983–R988, 2010.

Hermansen L, Hultman E, Saltin B. Muscle glycogen during prolonged severe exercise. *Acta Physiol Scand*. 71: 129–139, 1967.

Hernández A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E, Westerblad H. Dietary nitrate increases tetanic $[Ca^{2+}]_i$ and contractile force in mouse fast-twitch muscle. *J Physiol.* 590(15): 3575-3583, 2012.

Heunks LM, Cody MJ, Geiger PC, Dekhuijzen PN, Sieck GC. Nitric oxide impairs Ca2+ activation and slows cross-bridge cycling kinetics in skeletal muscle. *J Appl Physiol* 91: 2233–2239, 2001.

Hezel MP, Liu M, Schiffer TA, Larsen FJ, Checa A, Wheelock CE, Carlström M, Lundberg JO, Weitzberg E. Effects of long-term dietary nitrate supplementation in mice. *Redox Biol.* 5: 234-242, 2015.

Hidalgo C, Sánchez G, Barrientos G & Aracena-Parks P. A transverse tubule NADPH oxidase activity stimulates calcium release from isolated triads via ryanodine receptor type 1 *S*-glutathionylation. *J Biol Chem.* 281: 26473–26482, 2006.

Hinckson EA, Hopkins WG. Reliability of time to exhaustion analyzed with critical-power and log-log modeling. *Med Sci Sports Exerc.* 37(4): 696–701, 2005.

Hinkle PC. P/O ratios of mitochondrial oxidative phosphorylation. *Biochim Biophys Acta.* 1706(1-2): 1-22, 2005.

Hinshaw DB, Burger JM, Beals TF, Armstrong BC, Hyslop PA. Actin polymerization in cellular oxidant injury. *Arch Biochem Biophys*. 288: 311–316, 1991.

Hill CA, Thompson MW, Ruell PA, Thom JM, White MJ. Sarcoplasmic reticulum function and muscle contractile character following fatiguing exercise in humans. *J Physiol.* 531: 871–878, 2001.

Hodgkin AL, Horowicz P. Movements of Na and K in single muscle fibres. *J Physiol*. 145: 405–432, 1959.

Hogan MC, Richardson RS, Haseler LJ. Human muscle performrance and PCr hydrolysis with varied inspired oxygen fractions: a 31P-MRS study. *J Appl Physiol (1985).* 86(4): 1367-1373, 1999.

Hogg N. The biochemistry and physiology of S-nitrosothiols. *Annu Rev Pharmacol Toxicol.* 42: 585-600, 2002.

Hoon MW, Johnson NA, Chapman PG, Burke LM. The effect of nitrate supplementation on exercise performance in healthy individuals: a systematic review and meta-analysis. *Int J Sport Nutr Exerc Metab*. 23(5): 522, 2013.

Hooper L, Kay C, Abdelhamid A, Kroon PA, Cohn JS, Rimm EB, Cassidy A. Effects of chocolate, cocoa, and flavan-3-ols on cardiovascular health: a systematic review and meta-analysis of randomized trials. *Am J Clin Nutr.* 95(3): 740-751, 2012.

Holliss BA, Fulford J, Vanhatalo A, Pedlar CR, Jones AM. Influence of intermittent hypoxic training on muscle energetics and exercise tolerance. *J Appl Physiol.* 114, 611-619, 2013.

Holloszy JO, Coyle EF. Adaptations of skeletal muscle to endurance exercise and their metabolic consequences. *J Appl Physiol.* 56: 831-838, 1984.

Hoon MW, Hopkins WG, Jones AM, Martin DT, Halson SL, West NP, Johnson NA, Burke LM. Nitrate supplementation and high-intensity performance in competitive cyclists. *Appl Physiol Nutr Metab.* 39(9): 1043-1049, 2014a.

Hoon MW, Jones AM, Johnson NA, Blackwell JR, Broad EM, Lundy B, Rice AJ, Burke LM. The effect of variable doses of inorganic nitrate-rich beetroot juice on simulated 2,000-m rowing performance in trained athletes. *Int J Sports Physiol Perform.* 9(4): 615-620, 2014b.

Hoon MW, Fornusek C, Chapman PG, Johnson NA. The effect of nitrate supplementation on muscle contraction in healthy adults. *Eur J Sport Sci.* 15(8): 712-719, 2015.

Hopker JG, O'Grady C, Pageaux B. Prolonged constant load cycling exercise is associated with reduced gross efficiency and increased muscle oxygen uptake. *Scand J Med Sci Sports.* 27(4): 408-417, 2017.

Hopker J, Passfield L, Coleman D, Jobson S, Edwards L, Carter H . The effects of training on gross efficiency in cycling: a review. *Int J Sports Med.* 30: 845-850, 2009.

Hopkins WG, Hawley JA, Burke LM. Design and analysis of research on sport performance enhancement. *Med Sci Sports Exerc.* 31: 472– 485, 1999.

Hopkins WG, Schabort EJ, Hawley JA. Reliability of power in physical performance tests. *Sports Med.* 31:211–34, 2001.

Hord NG, Tang Y, Bryan NS. Food sources of nitrates and nitrites: the physiologic context for potential health benefits. *Am J Clin Nutr.* 90:1–10, 2009.

Horowitz JF, Sidossis LS, Coyle EF. High efficiency of type I muscle fibres improves performance. *Int J Sports Med.* 15(3): 152-157, 1994.

Howlett RA, Parolin ML, Dyck DJ, Hultman E, Jones NL, Heigenhauser GJF, Spriet LL. Regulation of skeletal muscle glycogen phosphorylase and PDH at varying exercise power outputs. *Am J Physiol.* 275: R418–R425, 1998.

Hultmann E, Bergstrom M, Spriet LL, Soderlund K. Energy metabolism and fatigue. *Biochem Ex VII.* 21: 73-92, 1990.

Husmann F, Bruhn S, Mittlmeier T, Zschorlich V, Behrens M. Dietary nitrate supplementation improves exercise tolerance by reducing muscle fatigue and perceptual responses. *Front Physiol.* 10: 404, 2019.

Hwang ES, Kim GH. Biomarkers for oxidative stress status of DNA, lipids, proteins in vitro and in vivod cancer research. *Toxicol.* 229: 1-10, 2007.

Hyde ER, Andrade F, Vaksman Z, Parthasarathy K, Jiang H, Parthasarathy DK, Torregrossa AC, Tribble G, Kaplan HB, Petrosino JF, Bryan NS. Metagenomic analysis of nitrate-reducing bacteria in the oral cavity: Implications for nitric oxide homeostasis. PLoS ONE. 9(3): e88645, 2014.

Iaia FM, Thomassen M, Kolding H, Gunnarsson T, Wendell J, Rostaard T, Nordsborg N, Krustrup P, Nybo L, Hellsten Y, Bangsbo J. Reduced volume but increased training intensity elevates muscle Na1-K1 pump alpha1-subunit and NHE1 expression as well as short-term work capacity in humans. *Am J Physiol Regul Integr Comp Physiol.* 294: R966-R974, 2008.

Ichinose M, Saito M, Fujii N, Ogawa T, Hayashi K, Kondo N, Nishiyasu T. Modulation of the control of muscle sympathetic nerve activity during incremental leg cycling. *J Physiol* 586(11):2753–2766, 2008.

Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci USA.* 90: 8103–8107, 1993.

Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol. 53*, 503–514, 2002.

Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci USA, 84(24): 9265-9629, 1987.

Ignarro LJ, Lippton H, Edwards JC, Baricos WH, Hyman AL, Kadowitz PJ, Gruetter CA. Mechanisms of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside, and nitric oxide: evidence for the involvement of s-nitrosothiols as active intermediates. *J Pharmacol Exp Ther.* 218: 739-749, 1981.

Imagawa TJ, Smith JS, Coronado R, Campbell KP. Purified ryanodine receptor from skeletal muscle sarcoplasmic reticulum is the Ca2+-permeable pore of the calcium release channel. *J Biol Chem*. 262: 16636-16643, 1987.

Ishii T, Sunami O, Saitoh N, Nishio H, Takeuchi T, Hata F. Inhibition of skeletal muscle sarcoplasmic reticulum Ca2+-ATPase by nitric oxide. *FEBS Lett.* 440(1-2): 218-222, 1998.

Ivarsson N, Schiffer TA, Hernández A, Lanner JT, Weitzberg E, Lundberg JO, Westerblad H. Dietary nitrate markedly improves voluntary running in mice. *Physiol Behav.* 168: 55-61, 2017.

Ivy JL, Costill DL, Maxwell BD. Skeletal muscle determinants of maximum aerobic power in men. *Eur J Appl Physiol Occup Physiol.* 44(1): 1–8, 1980.

Jackson MJ. Redox regulation of adaptive responses in skeletal muscle to contractile activity. *Free Radic Biol Med*. 47: 1267–1275, 2009.

Jaimes EA, Sweeney C, Raij L. Effects of reactive oxygen species hydrogen peroxide and hypochlorite on endothelial nitric oxide production. *Hypertension.* 38(4):877–883, 2001.

Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Rad Biol Med.* 9: 515-540, 1990.

Jannig PR, Alves CRR, Voltarelli VA, Bozi LHM, Vieira JS, Brum PC, Bechara LRG. Effects of N-acetylcysteine on isolated skeletal muscle contractile properties after an acute bout of aerobic exercise. *Life Sci.* 191: 46-51, 2017.

Jeukendrup AE, Currell K. Should time trial performance be predicted from three serial time-to-exhaustion tests? *Med Sci Sports Exerc*. 37(10):1820, 2005.

Jeukendrup A, Saris WH, Brouns F, Kester AD. A new validated endurance performance test. *Med Sci Sports Exerc.* 28(2): 266–70, 1996.

Ji LL, Fu R, Mitchell EW. Glutathione and antioxidant enzymes in skeletal muscle: effects of fiber type and exercise intensity. *J Appl Physiol.* 73: 1854–1859, 1992.

Jia L, Bonaventura C, Bonaventura J, Stamler JS. S-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control. *Nature.* 380(6571): 221-226, 1996.

Jiang H, Torregrossa AC, Potts A, Pierini D, Aranke M, Garg HK, Bryan NS. Dietary nitrite improves insulin signaling through GLUT4 translocation. *Free Radic Biol Med.* 67: 51-57, 2014.

Jo E, Fischer M, Auslander AT, Beigarten A, Daggy B, Hansen K, Kessler L, Osmond A, Wang H, Wes R. The effects of multi-day vs. single pre-exercise nitrate supplement dosing on simulated cycling time trial performance and skeletal muscle oxygenation. *J Strength Cond Res.* 33(1): 217-224, 2019.

Johnson MA, Polgar J, Weightman D, Appleton D. Data on the distribution of fibre types in thirty-six human muscles. An autopsy study. *J Neurol Sci.* 18: 111Y29, 1973.

Jones AM. Influence of dietary nitrate on the physiological determinants of exercise performance: a critical review. *Appl Physiol Nutr Metab.* 39(9): 1019-1028, 2014.

Jones AM. Dietary nitrate supplementation and exercise performance. *Sports Med.* 44(Suppl 1): S35-S45, 2014.

Jones AM, Berger NJ, Wilkerson DP, Roberts CL. Effects of "priming" exercise on pulmonary O2 uptake and muscle deoxygenation kinetics during heavy-intensity cycling in the supine and upright positions. *J Appl Physiol.* 101(5): 1432-1441, 2006.

Jones AM, Burnley M. Oxygen uptake kinetics: an underappreciated determinant of exercise performance. *Int J Sports Physiol Perform*. 4:524–532, 2009.

Jones AM, Ferguson SK, Bailey SJ, Vanhatalo A, Poole DC. Fiber type-specific effects of dietary nitrate. *Exerc Sport Sci Rev.* 44(2): 53-60, 2016.

Jones AM, Grassi B, Christensen PM, Krustrup P, Bangsbo J, Poole DC. Slow component of VO_2 kinetics: mechanistic bases and practical applications. *Med Sci Sports Exerc*. 43(11): 2046-2062, 2011.

Jones AM, Poole DC. Oxygen uptake dynamics: from muscle to mouth–an introduction to the symposium. *Med Sci Sports Exerc* 37: 1542–1550, 2005.

Jones AM, Thompson C, Wylie LJ, & Vanhatalo A. Dietary nitrate and physical performance. *Ann Rev Nutr.* 38: 303-328, 2018.

Jones AM, Wilkerson DP, DiMenna F, Fulford J, Poole DC. Muscle metabolic responses to exercise above and below the "critical power" assessed using 31P-MRS. *Am J Physiol Regul Integr Comp Physiol.* 294: R585-R593, 2008.

Jones AM, Wilkerson DP, Berger NJ, Fulford J. Influence of endurance training on muscle [PCr] kinetics during high-intensity exercise. *Am J Physiol Regul Integr Comp Physiol.* 293(1): R392-401, 2007.

Jones DP. Redefining oxidative stress. *Antioxid Redox Signal.* 8: 1865–1879, 2006.

Jonvik KL, Nyakayiru J, Pinckaers PJ, Senden JM, van Loon LJ, Verdijk LB. Nitrate-rich vegetables increase plasma nitrate and nitrite concentrations and lower blood pressure in healthy adults. *J Nutr* 146: 986–993, 2016.

Jonvik KL, Nyakayiru J, van Loon LJ, Verdijk LB. Can elite athletes benefit from dietary nitrate supplementation? *J Appl Physiol (1985).* 119: 759–761, 2015.

Joyner MJ, Coyle EF. Endurance exercise performance: the physiology of

champions. *J Physiol.* 586(Pt 1): 35-44, 2008.

Joyner MJ, Tschakosky ME. Nitric oxide and physiologic vasodilation in human limbs: where do we go from here? *Can J Appl Physiol.* 28(3): 475-490, 2003.

Jubrias SA, Vollestad NK, Gronka RK, Kushmerick MJ. Contraction coupling efficiency of human first dorsal interosseous muscle. *J Physiol*. 586(7): 1993-2002, 2008.

Juel C, Pilegaard H, Nielsen JJ, Bangsbo J. Interstitial K+ in human skeletal muscle during and after dynamic graded exercise determined by microdialysis. *Am J Physiol Regul Integr Comp Physiol.* 278: R400–R406, 2000.

Juel C. Muscle action potential propagation velocity changes during activity. *Muscle Nerve* 11: 714–719, 1988.

Juel C. Nitric oxide and Na,K-ATPase activity in rat skeletal muscle. *Acta Physiol(Oxf).* 216: 447-453, 2016.

Jungersten L, Ambring A, Wall B, Wennmalm A. Both physical fitness and acute exercise regulate nitric oxide formation in healthy humans. *J Appl Physiol*. 82(3):760–64, 1997.

Kalis JK, Freund BJ, Joyner MJ, Jilka SM, Nittolo J, Wilmore JH. Effect of beta-blockade on the drift in O2 consumption during prolonged exercise. *J Appl Physiol.* 64: 753-758, 1988.

Kanner J, Harel S, Granit R. Betalains–a new class of dietary cationized antioxidants. *J Agric Food Chem*. 49(11):5178–5185, 2001.

Kapil V, Milsom A, Okorie M, Maleki-Toyserkani S, Akram F, Rehman F, Arghandawi S, Pearl V, Benjamin N, Loukogeorgakis S, Macallister R, Hobbs AJ, Webb AJ, Ahluwalia A. Inorganic nitrate supplementation lowers blood pressure in humans: role for nitrite-derived NO. *Hypertension.* 56(2): 274-281, 2010.

Karlsson J, Saltin B. Diet, muscle glycogen, and endurance performance. *J Appl Physiol.* 31: 203–206, 1971.

Kay CD, Hooper L, Kroon PA, Rimm EB, Cassidy A. Relative impact of flavonoid composition, dose and structure on vascular function: a systematic review of randomised controlled trials of flavonoid-rich food products. *Mol Nutr Food Res.* 56(11): 1605-1616, 2012.

Keane KM, Bailey SJ, Vanhatalo A, Jones AM, Howatson G. Effects of

montmorency tart cherry (L. Prunus Cerasus) consumption on nitric oxide biomarkers and exercise performance. *Scand J Med Sci Sports.* 28(7): 1746-1756, 2018.

Keane KM, George TW, Constantinou CL, Brown MA, Clifford T, Howatson G. Effect of montmorency tart cherry (prunus Cerasus L.) consumption on vascular function in men with early hypertension. *Am J Clin Nutr.* 103: 1531-1539, 2016.

Kelly J, Vanhatalo A, Wilkerson DP, Wylie LJ, Jones AM. Effects of nitrate on the power-duration relationship for severe-intensity exercise. *Med Sci Sports Exerc.* 45: 1798–1806, 2013.

Kelly J, Vanhatalo A, Bailey SJ, Wylie LJ, Tucker C, List S, Winyard PG, Jones AM. Dietary nitrate supplementation: effects on plasma nitrite and pulmonary O_2 uptake dynamics during exercise in hypoxia and normoxia. *Am J Physiol Regul Integr Comp Physiol.* 307(7): R920-R930, 2014.

Kelly MK, Wicker RJ, Barstow TJ, Harms CA. Effects of N-acetylcysteine on respiratory muscle fatigue during heavy exercise. *Resp Phys Neurol.* 165: 67-72, 2009.

Kemp GJ, Taylor DJ, Radda GK. Control of phosphocreatine resynthesis during recovery from exercise in human skeletal muscle. *NMR Biomed.* 6: 66-72, 1993.

Kennedy DS, McNeil CJ, Gandevia SC. Firing of antagonist small-diameter muscle afferents reduces voluntary activation and torque of elbow flexors*. J Physiol.* 591:3591–3604, 2013.

Kennedy DS, McNeil CJ, Gandevia SC, Taylor JL. Fatigue-related firing of distal muscle nociceptors reduces voluntary activation of proximal muscles of the same limb. J Appl Physiol 116:385–394, 2014.

Kent GL, Dawson B, Cox GR, Burke LM, Eastwood A, Croft KD, Peeling P. Dietary nitrate supplementation does not improve cycling time-trial performance in the heat. *J Sports Sci.* 36(11): 1204-1211, 2018.

Keszler A, Piknova B, Schechter AN, Hogg N. The reaction between nitrite and oxyhemoglobin, a mechanistic study. *J Biol Chem*. 283: 9615–9622, 2008.

Khatri J, Mills CE, Maskell P, Odongerel C, Webb AJ. It is rocket science – why dietary nitrate is hard to 'beet'! Part I: twists and turns in the

realization of the nitrate–nitrite–NO pathway. *Br J Clin Pharmacol*. *83*: 129–139, 2017.

Khawli FA, Reid MB. N-acetylcysteine depresses contractile function and inhibits fatigue of diaphragm in vitro. *J Appl Physiol.* 77: 317-324, 1994.

Kobzik L, Reid MB, Bredt DS, Stamler JS. Nitric oxide in skeletal muscle. *Nature.* 372, 546–548, 1994.

Kocoloski GM, Crecelius AR. Effects of single-dose dietary nitrate on oxygen consumption during and after maximual and submaximal exercise in healthy humans: a pilot study. *Int J Exerc Sci.* 11(3): 214-225, 2018.

Koga S, Poole DC, Shiojiri T, Kondo N, Fukuba Y, Miura A, Barstow TJ. Comparison of oxygen uptake kinetics during knee extension and cycle exercise. *Am J Physiol Regul Integr Comp Physiol.* 288(1): R212-20, 2005.

Koga S, Shiojiri T, Kondo N, Barstow T. Effect of increased muscle temperature on oxygen uptake kinetics during exercise. *J Appl Physiol.* 83(4): 1333-1338, 1997.

Koga S, Wüst R, Walsh B, Kindig C, Rossiter H, Hogan M. Increasing temperature speeds intracellular PO2 kinetics during contractions in single Xenopus skeletal muscle fibres. *Am J Physiol Regul Integr Comp Physiol.* 304(1): R59-R66, 2012.

Konczol F, Lorinczy D, Belagyi J. Effect of oxygen free radicals on myosin in muscle fibres. *FEBS Lett.* 427: 341–344, 1998.

Koren A, Schara M, Sentjurc M. EPR measurements of free radicals during tetanic contraction of frog skeletal muscle. Period Biol. 82: 399-401, 1980.

Kourie JI. Interaction of reactive oxygen species with ion transport mechanisms. *Am J Physiol Cell Physiol*. 275: C1–C24, 1998.

Kozlov AV, Staniek K, Nohl H. Nitrite reductase activity is a novel function of mammalian mitochondria. *FEBS Lett.* 454: 127–130, 1999.

Kozlov AV, Dietrich B, Nohl H. Various intracellular compartments cooperate in the release of nitric oxide from glycerol trinitrate in liver. *Br J Pharmacol.* 139: 989–997, 2003.

Krogh A, Lindhard J. The regulation of respiration and circulation during the initial stages of muscular work. *J Physiol.* 47: 112-136, 1913.

Krustrup P, Ferguson RA, Kiaer M, Bangsbo J. ATP and heat production in

human skeletal muscle during dynamic exercise: higher efficiency of anaerobic than aerobic ATP resynthesis. *J Physiol.* 549(Pt 1): 255-269, 2003.

Krustrup P, Söderlund K, Mohr M, Bangsbo J. Slow-twitch fiber glycogen depletion elevates moderate-exercise fast-twitch fiber activity and O_2 uptake. *Med Sci Sports Exerc*. 36(6):973-82, 2004.

Krustrup P. Muscle oxygen uptake and energy turnover during dynamic exercise in humans. [PhD thesis]. Copenhagen (Denmark): University of Copenhagen. ISBN: 87-89361-87-3: 1-241, 2004.

Kukreja RC, Weaver AB, Hess ML. Sarcolemmal Na+-K+-ATPase: inactivation by neutrophil-derived free radicals and oxidants. *Am J Physiol Heart Circ Physiol.* 259: H1330–H1336, 1990.

Lamarra N, Whipp BJ, Ward SA, Wasserman K. Effect of interbreath fluctuations on characterising exercise gas exchange kinetics. *J Appl Physiol.* 62: 2003–2012, 1987.

Lane SC, Hawley JA, Desbrow B, Jones AM, Blackwell JR, Ross ML, Zemski AJ, Burke LM. Single and combined effects of beetroot juice and caffeine supplementation on cycling time trial performance. *Appl Physiol Nutr Metab.* 39(9): 1050-1057, 2014.

Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Acute dietary nitrate supplementation improves cycling time trial performance. *Med Sci Sports Exerc.* 43(6): 1125-31, 2011a.

Lansley KE, Winyard PG, Fulford J, Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Gilchrist M, Benjamin N, Jones AM. Dietary nitrate supplementation reduces the O2 cost of walking and running: a place-controlled study. *J Appl Physiol.* 110(3): 591-600, 2011b.

Larsen FJ, Schiffer TA, Borniquel S, Sahlin K, Ekblom B, Lundberg JO, Weitzberg E. Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab*. 13(2): 149-159, 2011.

Larsen FJ, Schiffer TA, Ekbom B, Mattsson MP, Checa A, Wheelock CE, Nyström T, Lundberg JO, Weitzberg E. Dietary nitrate reduces resting metabolic rate: a randomized, crossover study in humans. *Am J Clin Nutr.* 99(4): 843-850, 2014.

Larsen FJ, Schiffer TA, Weitzberg E, Lundberg JO. Regulation of mitochondrial function and energetics by reactive nitrogen oxides. *Free Radic Biol Med.* 53(1): 1919-1928, 2012.

Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol (Oxf).* 191(1): 59-66, 2007.

Larsen FJ, Weitzberg E, Lundberg JO, Ekblom B. Dietary nitrate reduces maximal oxygen consumption while maintaining work performance in maximal exercise. *Free Rad Biol Med.* 48(2): 342-347, 2010.

Lauer T, Heiss C, Balzer J, Kehmeier E, Mangold S, Leyendecker T, Rottler J, Meyer C, Merx MW, Kelm M, Rassaf T. Age-dependent endothelial dysfunction is associated with failure to increase plasma nitrite in response to exercise. Basic Res Cardiol. 103(3): 291-297, 2008.

Laurent D, Reutenauer H, Payen JF, Favre-Junvin A, Eterrdossi J, Lebas JF, Rossi A. Muscle bioenergetics in skiers: studies using NMR spectroscopy. *Int J Sports Med.* 13: S150-S152, 1992.

Laurin J, Pertici V, Dousset E, Marqueste T, Decherchi P. Group III and IV muscle afferents: role on central motor drive and clinical implications. *Neuroscience.* 290: 543-551, 2015.

Lawler JM, Cline CC, Hu Z, Coast JR. Effect of oxidant challenge on contractile function of the aging rat diaphragm. *Am J Physiol*. 272, E201–E210, 1997.

Leelarungrayb D, Khansuwan R, Pothongsunun P, Klaphajone J. N-Acetylcysteine supplementation controls total antioxidant capacity, creatine kinase, lactate, and tumor necrotic factor-alpha against oxidative stress induced by graded exercise in sedentary men. *Oxid Med Cell Longev.* 329643, 2011.

Leeuwenburgh C, Hollander J, Leichtweis S, Griffiths M, Gore M, Ji LL. Adaptations of glutathione antioxidant system to endurance training are tissue and muscle fiber specific. *Am J Physiol Regul Integr Comp Physiol.* 272:R363–R369, 1997.

Lepers R, Hausswirth C, Maffiuletti N, Brisswalter J, van Hoecke J. Evidence of neuromuscular fatigue after prolonged cycling exercise. *Med Sci Sports Exerc.* I32(11): 1880-1886, 2000.

Lepers R, Maffiuletti NA, Rochette L, Brugniaux J, Millet GY. Neuromuscular fatigue during long-duration cycling exercise. *J Appl Physiol (1985).* 92(4): 1487-1493, 2002.

Leppik JA, Aughey RJ, Medved I, Fairweather I, Carey MF, McKenna MJ. Prolonged exercise to fatigue in humans impairs skeletal muscle Na+-K+-ATPase activity, sarcoplasmic reticulum Ca2+ release, Ca2+ uptake. *J Appl Physiol.* 97: 1414–1423, 2004.

Leung AM, Braverman LE, Pearce EN. History of U.S. iodine fortification and supplementation. *Nutrients.* 4: 1740e1746, 2012.

Li CY, Liu JZ, Wu LP, Li B, Chen LF. Effects of hypobaric hypoxia on adenine nucleotide pools, adenine nucleotide transporter activity and protein expression in rat liver. *World J Gastroenterol.* 12: 2120-2124, 2006.

Li JL, Wang XN, Fraser SF, Carey MF, Wrigley TV, McKenna MJ. Effects of fatigue and training on sarcoplasmic reticulum Ca2+ regulation in human skeletal muscle. *J Appl Physiol* 92: 912–922, 2002.

Li H, Cui H, Kundu TK, Alzawahra W, Zweier JL. Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase. *J Biol Chem.* 283: 17855–63, 2008.

Li SH, Tian HB, Zhao HJ, Chen LH, Cui LQ. The acute effects of grape polyphenols supplementation on endothelial function in adults: meta-analyses of controlled trials. *PLoS One.* 8(7): e69818, 2013.

Li YP, Atkins CM, Sweatt JD, Reid MB. Mitochondria mediate tumor necrosis factor-alpha/NF-kappaB signaling in skeletal muscle myotubes. *Antiox Redox Signal*. 1: 97– 104, 1999.

Lidder S, Webb AJ. Vascular effects of dietary nitrate (as found in green leafy vegetables and beetroot) via the nitrate-nitrite-nitric oxide pathway. *Br J Clin Pharmacol*. 75(3):677–96, 2013.

Linnarsson D. Dynamics of pulmonary gas exchange and heart rate changes at start and end of exercise. *Acta Physiologica Scandinavica.* 415: 1-68.

Liu X, Miller MJS, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster Jr JR. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem.* 273: 18709-18713, 1998.

Lo Conte M, Carroll KS. The redox biochemistry of protein sulfenylation and sulfinylation. *J Biol Chem.* 288: 26480–26488, 2013.

Lowings S, Shannon OM, Deighton K, Matu J, Barlow MJ. Effect of dietary nitrate supplementation on swimming performance in trained swimmers. *Int J Sport Nutr Exerc Metab.* 27(4): 377-384, 2017.

Lundberg JO, Weitzberg E, Gladwin MT. The nitrate–nitrite–nitric oxide pathway in physiology and therapeutics. *Nat Rev Drug Discov*. *7*: 156–167, 2008.

Lundberg JO, Govoni M. Inorganic nitrate is a possible source for systemic generation of nitric oxide. *Free Radic Biol Med*. 37:395 – 400, 2004.

Lundberg JO, Weitzberg E. NO generation from inorganic nitrate and nitrite: role in physiology, nutrition and therapeutics. *Arch Pharmacol Res.* 32:1119–26, 2009.

Lundberg JO, Weitzberg E, Lundberg JM, Alving K. Intragastric nitric oxide production in humans: measurements in expelled air. *Gut* 35:1543–46, 1994.

Lundberg JO, Weitzberg E. NO-synthase independent NO generation in mammals. *Biochem Biophys Res Commun*. 396: 39e45, 2010.

MacDonald WA, Stephenson DG. Effects of ADP on sarcoplasmic reticulum function in mechanically skinned skeletal muscle fibres of the rat. *J Physiol.* 532: 499–508, 2001.

MacLeod KE, Nugent SF, Barr SI, Koehle MS, Sporer BC, MacInnis MJ. Acute beetroot juice supplementation does not improve cycling performance in normoxia or moderate hypoxia. *Int J Sport Nutr Exerc Metab.* 25(4): 359-366, 2015.

MacIntosh BR, Holash RJ, Renaud JM. Skeletal muscle fatigue regulation of excitation-contraction coupling to avoid metabolic catstrophe. *J Cell Sci.* 125: 2105-2114, 2012.

Mador MJ, Acevedo FA. Effect of respiratory muscle fatigue on subsequent exercise performance. *J Appl Physiol.* 70: 2059–2065, 1991.

Mahler M. First-order kinetics of muscle oxygen consumption, and equivalent proportionality between QO_2 and phosphorylcreatine level. Implications for the control of respiration. *J Gen Physiol.* 86: 135–65, 1985.

Mason SA, Morrison D, McConell GK, Wadley GD. Muscle redox signalling pathways in exercise. Role of antioxidants. *Free Rad Biol Med.* 98: 29-45, 2016.

Maréchel G, Gailly P. Effects of nitric oxide on the contraction of skeletal muscle. *Cell Mol Life Sci.* 55(8-9): 1088-1102, 1999.

Marozkina NV, Gaston B. S-Nitrosylation signaling regulates cellular protein interactions. *Biochim Biophys Acta*. 1820: 722–729, 2012.

Martyn DA, Gordon AM. Force and stiffness in glycerinated rabbit psoas fibers. Effects of calcium and elevated phosphate*. J Gen Physiol.* 99: 795–816, 1992.

Mason SA, Morrison D, McConell GK, Wadley GD. Muscle redox signalling pathways in exercise. Role of antioxidants. *Free Radic Biol Med.* 98: 29-45, 2016.

Matuszczak Y, Arbogast S, Reid MB. Allopurinol mitigates muscle contractile dysfunction caused by hindlimb unloading in mice. *Aviat Space Environ Med*. 75, 581–588, 2005.

Matuszczak Y, Farid M, Jones J, Lansdowne S, Smith MA, Taylor AA, Reid MB. Effects of N-acetylcysteine on glutathione oxidation and fatigue during handgrip exercise. *Muscle Nerve*. 32: 633-638, 2005.

Maughan RJ, Burke LM, Dvorak J, Larson-Meyer DE, Peeling P, Phillips SM, Rawson ES, Walsh NP, Garthe I, Geyer H, Meeusen R, van Loon L, Shirreffs SM, Spriet LL, Stuart M, Vernec A, Currell K, Ali VM, Budgett RGM, Ljungqvist A, Mountjoy M, Pitsiladis Y, Soligard T, Erdener U, Engebretsen L. IOC consensus statement: dietary supplements and the high-performance athlete. *Int J Sport Nutr Exerc Metab.* 28(2): 104-125, 2018.

Maughan RJ, Poole DC. The effects of a glycogen-loading regimen on the capacity to perform anaerobic exercise. *Eur J Appl Physiol*. 46: 211-219, 1981.

Maughan RJ, Greenhaff PL, Leiper JB, Ball D, Lambert CP, Gleeson M. Diet composition and the performance of high-intensity exercise. *J Sport Sci.* 15: 265-275, 1997.

McCully KK, Boden BP, Tuchler M, Fountain MR, Chance B. Wrist flexor muscles of elite rowers measured with magnetic resonance spectroscopy. *J Appl Physiol.* 67: 926-932, 1989.

McDonagh ST, Wylie LJ, Winyard PG, Vanhatalo A, Jones AM. The effects of chronic nitrate supplementation and the use of strong and weak antibacterial agents on plasma nitrite concentration and exercise blood

pressure. *Int J Sports Med.* 36(14): 1177-1185, 2015.

McIlvenna LC, Monaghan C, Liddle L, Fernandez BO, Feelisch M, Muggeridge DJ, Easton C. Beetroot juice versus chard gel: A pharmacokinetic and pharmacodynamics comparison of nitrate bioavailability. *Nitric Oxide.* 64: 61-67, 2017.

McLellan TM, Cheung SS, Jacobs I. Variability of time to exhaustion during submaximal exercise. *Can J Appl Physiol*. 20: 39–51, 1995.

McKenna MJ. The roles of ionic processes in muscular fatigue during intense exercise. *Sports Med.* 13: 134-145, 1992.

McKenna MJ, Medved I, Goodman CA, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X. N-acetylcysteine attenuates the decline in muscle Na^+ K^+-pump activity and delays fatigue during prolonged exercise in humans. *J Physiol.* 576: 279-288, 2006.

McMahon NF, Leveritt MD, Pavey TG. The effect of dietary nitrate supplementation on endurance exercise performance in healthy adults: a systematic review and meta-analysis. *Sports Med.* 47(4): 735-756, 2017.

McMorrow C, Fredsted A, Carberry J, O'Connell RA, Bradford A, Jones JF, O'Halloran KD. Chronic hypoxia increases rat diaphragm muscle endurance and sodium-potassium ATPase pump content. *Eur Respir J.* 37: 1474-1481, 2011.

McKnight GM, Smith LM, Drummond RS, Duncan CW, Golden M, Benjamin N. Chemical synthesis of nitric oxide in the stomach from dietary nitrate in humans. *Gut.* 40: 211–214, 1997.

McQuillan JA, Dulson DK, Laursen PB, Kilding AE. Dietary nitrate fails to improve 1 and 4 km cycling performance in highly trained cyclists. *Int J Sport Nutr Exerc Metab.* 27(3): 255-263, 2017a.

McQuillan JA, Dulson DK, Laursen PB, Kilding AE. The effect of dietary nitrate supplementation on physiology and performance in trained cyclists. *Int J Sports Physiol Perform.* 12(5): 684-689, 2017b.

McQuillan JA, Casadio JR, Dulson DK, Laursen PB, Kilding AE. The effect of nitrate supplementation on cycling performance in the heat in well-trained cyclists. *Int J Sports Physiol Perform.* 13(1): 50-56, 2018.

Medved I, Brown MJ, Bjorksten AR, Leppik JA, Sostaric C, McKenna MJ. N-acetylcysteine infusion alters blood redox status but not time to fatigue during intense exercise in humans. *J Appl Physiol.* 94: 1572-1582, 2003.

Medved I, Brown MJ, Bjorksten AR, McKenna MJ. Effects of intravenous N-acetylcysteine infusion on time to fatigue and potassium regulation during prolonged cycling exercise. *J Appl Physiol.* 96: 211-217, 2004a.

Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X, McKenna MJ. N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. *J Appl Physiol (1985).* 97(4): 1477-1485, 2004b.

Meister A, Anderson ME. Glutathione. *Annu Rev Biochem.* 52: 711–760, 1983.

Mensinga TT, Speijers GJ, Meulenbelt J. Health implications of exposure to environmental nitrogenous compounds. *Toxicol Rev.* 22:41–51, 2003.

Meissner G, Darling E, Eveleth J. Kinetics of rapid Ca2+ release by sarcoplasmic reticulum. Effects of Ca2+, Mg2+, adenine nucleotides. *Biochem.* 25: 236–244, 1986.

Merry TL, Lynch GS, McConell GK. Downstream mechanisms of nitric oxide-mediated skeletal muscle glucose uptake during contraction. *Am J Physiol Regul Integr Comp Physiol.* 299(6): R1656-1665, 2010.

Merry TL, McConell GK. Do reactive oxygen species regulate skeletal muscle glucose uptake during contraction? *Exerc Sport Sci Rev.* 40(2): 102-105, 2012.

Merry TL, Wadley GD, Stathis CG, Garnham AP, Rattigan S, Hargreaves M, McConell GK. N-Acetylcysteine infusion does not affect glucose disposal during prolonged moderate-intensity exercise in humans. *J Physiol.* 588: 1623-1634, 2010.

Meszaros LG, Minarovic I, Zahradnikova A. Inhibition of the skeletal muscle ryanodine receptor calcium release channel by nitric oxide. *FEBS Lett.* 380: 49-52, 1996.

Millar TM, Stevens CR, Benjamin N, Eisenthal R, Harrison R, Blake DR. Xanthine oxidoreductase catalyses the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. *FEBS Lett.* 427(2): 225-228, 1998.

Millar NC, Homsher E. The effect of phosphate and calcium on force generation in glycerinated rabbit skeletal muscle fibers: a steady-state and transient kinetic study. *J Biol Chem.* 265: 20234– 20240, 1990.

Millet GY, Lepers R. Alterations of neuromuscular function after prolonged

running, cycling, and skiing exercise. *Sports Med.* 34: 105-116, 2004.

Mitchell JH, Kaufman MP, Iwamoto GA. The exercise pressor reflex: its cardiovascular effects, afferent mechanisms and central pathways. *Annu Rev Physiol.* 45: 229-242, 1983.

Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature.* 191: 144-148, 1961.

Modin A, Björne H, Herulf M, Alving K, Weitzberg E, Lundberg JO. Nitrite-derived nitric oxide: a possible mediator of "acidic–metabolic vasodilation. *Acta Physiol Scand.* 171: 9–16, 2001.

Mogensen M, Bagger M, Pedersen PK, Fernstrom M, Sahlin K. Cycling efficiency in humans is related to low UCP3 content and to type I fibres but not to mitochondrial efficiency. *J Physiol.* 571: 669–681, 2006.

Moncada S, Higgs A. The L-Arginine-Nitric Oxide Pathway. *N Engl J Med*. 329: 2002–2012, 1993.

Moncada S, Palmer RMJ, Higgs EA. Biosynthesis of nitric oxide from L-arginine. *Biochem Pharmacol.* 38:1709–15, 1989.

Montenegro CF, Kwong DA, Minow ZA, Davis BA, Lozada CF, Casazza GA. Betalain-rich concentrate supplementation improves exercise performance and recovery in competitive triathletes. *Appl Physiol Nutr Metab.* 42(2): 166-172, 2017.

Moonpanar TR, Allen DG. Reactive oxygen species reduce myofibrillar Ca^{2+} sensitivity in fatiguing mouse skeletal muscle at 37 degrees C. *J Physiol*. 564, 189–199, 2005.

Morrison RJ, Miller CC, Reid MB. Nitric oxide effects on shortening velocity and power production in the rat diaphragm. *J Appl Physiol.* 80: 1065-1069, 1996.

Moseley L, Achten J, Martin J, Jeukendrup A. No differences in cycling efficiency between world-class and recreational cyclists. *Int J Sports Med.* 25: 374–379, 2004.

Moselhy HF, Reid RG, Yousef S, Boyle SP. A specific, accurate, and sensitive measure of total plasma malondialdehyde by HPLC. *J Lipid Research.* 54(3): 852-8, 2013.

Mosher SL, Gough LA, Deb S, Saunderes B, Mc Naughton LR, Brown DR, Sparks SA. High does nitrate ingestion does not improve 40 km cycling time trial performance in trained cyclists. *Res Sports Med.* 8:1-9, 2019.

Moylan JS, Reid MB. Oxidative stress, chronic disease, and muscle wasting. *Muscle Nerve.* 35(4): 411-429, 2007.

Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, Easton C. The effects of a single dose of concentrated beetroot juice on performance in trained flatwater kayakers. *Int J Sport Nutr Exerc Metab.* 23(5): 498-506, 2013.

Muggeridge DJ, Howe CC, Spendiff O, Pedlar C, James PE, Easton C. A single dose of beetroot juice enhances cycling performance in simulated altitude. *Med Sci Sports Exerc.* 46(1): 143-150, 2014.

Muggeridge DJ, Sculthorpe N, Grace FM, Willis G, Thornhill L, Weller RB, James PE, Easton C. Acute whole body UVA irradiation combined with nitrate ingestion enhances time trial performance in trained cyclists. *Nitric Oxide.* 48: 3-9, 2015.

Mumford PW, Kephard WC, Romero MA, Haun CT, Mobley CB, Osburn SC, Healy JC, Moore AN, Pascoe DD, Ruffin WC, Beck DT, Martin JS, Roberts MD, Young KC. Effect of 1-week betalain-rich beetroot concentrate supplementation on cycling performance and select physiological parameters. *Eur J Appl Physiol.* 118(11): 2465-2476, 2018.

Murad F. Nitric oxide: the coming of the second messenger. *Rambam Maimonides Med J.* 2(2): e0038, 2011.

Murad F, Mittal CK, Arnold WP, Katsuki S, Kimura H. Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv Cyclic Nucleotide Res.* 9: 145-158, 1978.

Murgatroyd SR, Wylde LA. The power-duration relationship of high-intensity exercise: from mathematical parameters to physiological mechanisms. *J Physiol*. 589: 2443Y5, 2011.

Murphy M, Eliot K, Heuertz RM, Weiss E. Whole beetroot consumption acutely improves running performance*. J Acad Nutr Diet.* 112: 548–552, 2012.

Nakane M, Schmidt HH, Pollock JS, Forstermann U, Murad F. Cloned human brain nitric oxide synthase is highly expressed in skeletal muscle. *FEBS Lett.* 316(2): 175-180, 1993.

Nagababu E, Ramasamy S, Abernethy DR, Rifkind JM. Active nitric oxide produced in the red cell under hypoxic conditions by deoxyhemoglobin-

mediated nitrite reduction. *J Biol Chem.* 278: 46349–46356, 2003.

Newton GL, Dorian R, Fahey RC. Analysis of biological thiols: derivatization with monobromobimane and separation by reverse-phase high-performance liquid chromatography. *Analytical Biochem.* 114: 383-387, 1981.

Nielsen J, Schroder HD, Rix CG, Ortenblad N. Distinct effects of subcellular glycogen localization on tetanic relaxation time and endurance in mechanically skinned rat skeletal muscle fibres. *J Physiol.* 587: 3679–3690, 2009.

Nielsen J, Holmberg HC, Schrøder HD, Saltin B, Ortenblad N. Human skeletal muscle glycogen utilization in exhaustive exercise: role of subcellular localization and fibre type. *J Physiol.* 589(Pt 11): 2871-2885, 2011.

Nevill ME, Boobis LH, Brooks S, Williams C. Effect of training on muscle metabolism during treamill sprinting. *J Appl Physiol.* 67: 2376-2382, 1989.

Nielsen JJ, Mohr M, Klarskov C, Kristensen M, Krustrup P, Juel C, Bangsbo J. Effects of high-intensity intermittent training on potassium kinetics and performance in human skeletal muscle. *J Physiol.* 554: 857-870, 2004.

Ninfali P, Angelino D. Nutritional and functional potential of Beta vulgaris cicla and ruba. *Fiterapia.* 89: 188-199, 2013.

Nyakayiru JM, Jonvik KL, Pinckaers PJ, Senden J, van Loon LJ, Verdijk LB. Beetroot juice supplementation improves high-intensity intermittent type exercise performance in trained soccer players. *Int J Sport Nutr Exerc Metab.* 27(1): 11-17, 2017a.

Nyakayiru J, Kouw IWK, Cermak NM, Senden JM, van Loon LJC, Verdijk LB. Sodium nitrate ingestion increases skeletal muscle nitrate content in humans. *J Appl Physiol*. 123: 637–644, 2017b.

Nyakayiru JM, Jonvik KL, Pinckaers PJ, Senden J, van Loon LJ, Verdijk LB. No effect of acute and 6-day nitrate supplementation on VO2 and time-trial performance in highly trained cyclists. *Int J Sport Nutr Exerc Metab.* 27(1): 11-17, 2017c.

Nybäck L, Glännerud C, Larsson G, Weitzberg E, Shannon OM, McGawley K. Physiological and performance effects of nitrate supplementation

during roller-skiing in normoxia and normobaric hypoxia. *Nitric Oxide.* 70: 1-8, 2017.

Oba T, Koshita M, Yamaguchi M. H2O2 modulates twitch tension and increases Po of Ca2+ release channel in frog skeletal muscle. *Am J Physiol*. 63: 460–468, 1996.

Oestergaard L, Stankevicius E, Andersen MR, Eskildsen-Helmond Y, Ledet T, Mulvany MJ, Simonsen U. Diminished NO release in chronic hypoxic human endothelial cells. *Am J Physiol Heart Circ Physiol.* 293: H2894–H2903, 2007.

Olea F, Parras P. Determination of serum levels of dietary thiocyanate. *J Anal Toxicol*. 16: 258–260, 1992.

Ørtenblad N, Nielsen J, Saltin B, Holmberg HC. Role of glycogen availability in sarcoplasmic reticulum Ca2+ kinetics in human skeletal muscle. *J Physiol.* 589(Pt 3): 711-725, 2011.

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

Özyener F, Rossiter HB, Ward SA, Whipp BJ. Influence of exercise intensity on the on- and off-transient kinetics of pulmonary oxygen uptake in humans. *J Physiol.* 533: 891-902, 2001.

Paganini AT, Foley JM, Meyer RA. Linear dependence of muscle phosphocreatine kinetics on oxidative capacity. *Am J Physiol.* 272(Cell Physiol 41): C501-C510, 1997.

Palmer GS, Borghouts LB, Noakes TD, Hawley JA. Metabolic and performance responses to constant-load vs. variable-intensity exercise in trained cyclists. *J Appl Physiol.* 87: 1186–1196, 1999.

Park CS, Park R, Krishna G. Constitutive expression and structural diversity of inducible isoform of nitric oxide synthase in human tissues. *Life Sci.* 59: 219-225, 1996.

Parker N, Affourtit C, Vidal-Puig A, Brand MD. Energization-dependent endogenous activation of proton conductance in skeletal muscle mitochondria. *Biochem J*. 412: 131–139, 2008.

Paris HL, Fulton TJ, Chapman RF, Fly AD, Koceja DM, Mickleborough TD. Effect of carbohydrate ingestion on central fatigue during prolonged running exercise in moderate hypoxia. *J Appl Physiol.* 126: 141-151, 2019.

Parolin ML, Chesley A, Matsos MP, Spriet LL, Jones NL, Heigenhauser GJF. Regulation of skeletal muscle glycogen phosphorylase and PDH during maximal intermittent exercise. *Am J Physiol.* 277: E890-E900, 1999.

Pattwell DM, McArdle A, Morgan JE, Patridge TA, Jackson MJ. Release of reactive oxygen and nitrogen species from contracting skeletal muscle cells. *Free Radic Biol Med*. 37: 1064– 1072, 2004.

Passfield L, Doust JH. Changes in cycling efficiency and performance after endurance exercise. *Med Sci Sports Exerc.* 32(11): 1935-1941, 2000.

Pawlak-Chaouch M, Boissierèere J, Gamelin FX, Cuvelier G, Berthoin S, Aucouturier J. Effect of dietary nitrate supplementation on metabolic rate during rest and exercise in human: A systematic review and a meta-analysis. *Nitric Oxide.* 53: 65-76, 2016.

Peacock O, Tjønna AE, James P, Wisløff U, Welde B, Böhlke N, Smith A, Stokes K, Cook C, Sandbakk O. Dietary nitrate does not enhance running performance in elite cross-country skiers. *Med Sci Sports Exerc.* 44(11): 2213-2219, 2012.

Peeling P, Cox GR, Bullock N, Burke LM. Beetroot juice improves on-water 500 m time-trial performance, and laboratory-based paddling economy in national and international-level kayak athletes. *Int J Sport Nutr Exerc Metab*. 25(3): 278-284, 2015. doi: 10.1123/ijsnem.2014-0110.

Pennington J. Dietary exposure models for nitrates and nitrites. *Food Control.* 9: 385–395, 1998.

Percival JM, Anderson KN, Huang P, Adams ME, Froehner SC. Golgi and sarcolemmal neuronal NOS differentially regulate contraction-induced fatigue and vasoconstriction in exercising mouse skeletal muscle. *J Clin Investig.* 120: 816–26, 2010.

Peri L, Pietraforte D, Scorza G, Napolitano A, Fogliano V, Minetti M. Apples increase nitric oxide production by human saliva at the acidic pH of the stomach a new biological function for polyphenols with a catechol group? *Free Radic Biol Med.* 39(5): 668-681, 2005.

Periasamy M, Kalyanasundaram A. SERCA pump isoforms: their role in calcium transport and disease. *Musc Nerv.* 35: 430-442, 2007.

Pessah IN, Feng W. Functional role of hyperreactive sulfhydryl moieties within the ryanodine receptor complex. *Antiox Redox Signal*. 2: 17–26, 2000.

Petersson J, Jädert C, Phillipson M, Borniquel S, Lundberg JO, Holm L. Physiological recycling of endogenous nitrate by oral bacteria regulates gastric mucus thickness. *Free Radic Biol Med*. 89: 241–247, 2015.

Piknova B, Park JW, Lam KK, Schechter AN. Nitrate as a source of nitrite and nitric oxide during exercise hyperemia in rat skeletal muscle. *Nitric Oxide.* 55-56: 54 –61, 2016.

Piknova B, Park JW, Swanson KM, Dey S, Noguchi CT, Schechter AN. Skeletal muscle as an endogenous nitrate reservoir. *Nitric Oxide.* 47: 10 –16, 2015.

Pirkmajer S, Chibalin AV. Na,K-ATPase regulation in skeletal muscle. *Am J Physiol Endocrinol Metab.* 311: E1-E31, 2016a.

Pirkmajer S, Chabalin AV. NO turns on Na,K-ATPase in skeletal muscle. *Acta Physiol(Oxf).* 216: 386-391, 2016b.

Polgar J, Johnson MA, Weightman D, Appleton D. Data on fibre size in thirty-six human muscles. An autopsy study. *J Neurol Sci.* 19: 307-318, 1973.

Poole DC, Jones AM. Oxygen uptake kinetics. *Compr Physiol.* 2: 933– 996, 2012.

Poole DC, Richardson RS. Determinants of oxygen uptake. Implications for exercise testing. *Sports Med.* 24: 308-320, 1997.

Poole DC, Ward SA, Gardner GW, Whipp BJ. Metabolic and respiratory profile of the upper limit for prolonged exercise in man. *Ergonomics.* 31: 1265-1279, 1988.

Porcelli S, Pugliese L, Rejc E, Pavei G, Bonato M, Montorsi M, La Torre A, Rasica L, Marzorati M. Effects of a short-term high-nitrate diet on exercise performance. *Nutrients.* 8(9): E534, 2016.

Porcelli S, Ramaglia M, Bellistri G, Pavei G, Pugliese L, Montorsi M, Rasica L, Mazorati M. Aerobic fitness affects the exercise performance responses to nitrate supplementation. *Med Sci Sports Exerc.* 47(8): 1643-1651, 2015.

Powers SK, Jackson MJ. Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. *Physiol Rev.* 88(4): 1243-1276, 2008.

Powers SK, Duarte J, Kavazis AN, Talbert EE. Reactive oxygen species are signalling molecules for skeletal muscle adaptation. *Exp Physiol.* 95(1): 1-9, 2010.

Powers SK, Talbert EE, Adhihetty PJ. Reactive oxygen and nitrogen species as intracellular signals in skeletal muscle. *J Physiol*. 589: 2129–2138, 2011.

Powers SK, Deruisseau KC, Quindry J, Hamilton KL. Dietary antioxidants and exercise. *J Sports Sci.* 22: 81–94, 2004.

Putkey JA, Dotson DG, Mouawad P. Formation of inter- and intramolecular disulfide bonds can activate cardiac troponin C. *J Biol Chem.* 268: 6827–6830, 1993.

Puype J, Ramaekers M, Van Thienen R, Deldicque L, Hespel P. No Effect of dietary nitrate supplementation on endurance training in hypoxia. *Scand J Med Sci Sports.* 25(2): 234-241, 2015.

Qin L, Liu X, Sun Q, Fan Z, Xia D, Ding G, Ong HL, Adams D, Gahl WA, Zheng C, Qi S, Jin L, Zhang C, Gu L, He J, Deng D, Ambudkar IS, Wang S. Sialin (SLC17A5) functions as a nitrate transporter in the plasma membrane. *Proc Natl Acad Sci USA.* 109(33): 13434–39, 2012.

Rassaf T, Flögel U, Drexhage C, Hendgen-Cotta U, Kelm M, Schrader J. Nitrite reductase function of deoxymyoglobin: oxygen sensor and regulator of cardiac energetics and function. *Circ Res.* 100: 1749–1754, 2007a.

Rassaf T, Lauer T, Heiss C, Balzer J, Mangold S, Leyendecker T, Rottler J, Drexhage C, Meyer C, Kelm M. Nitric oxide synthase-derived plasma nitrite predicts exercise capacity*. Br J Sports Med*. 41: 669–673, 2007b.

Reggiani C, Potma EJ, Bottinelli R, Canepari M, Pellegrino MA, Stienen GJ. Chemo-mechanical energy transduction in relation to myosin isoform composition in skeletal muscle fibres of the rat. *J Physiol.* 502(Pt 2): 449-460, 1997.

Regnier M, Lorenz RR, Sieck GC. Effects of oxygen radical scavengers on force production in single living frog skeletal muscle fibers. *FASEB J*. 6: A1819, 1992.

Reid MB, Haack KE, Franchek KM, Valberg PA, Kobzik L, West MS. Reactive oxygen in skeletal muscle. I. Intracellular oxidant kinetics and fatigue in vitro. *J Appl Physiol.* 73: 1797-1804, 1992.

Reid MB, Shoji T, Moody MR, Entman ML. Reactive oxygen in skeletal muscle. II. Extracellular release of free radicals*. J Appl Physiol.* 73:1805–1809, 1992.

Reid MB, Khawli FA, Moody MR. Reactive oxygen in skeletal muscle. III. Contractility of unfatigued muscle. *J Appl Physiol.* 75:1081–1087, 1993.

Reid MB, Stokic DS, Koch SM, Khawli FA, Leis, AA. N-acetylcysteine inhibits muscle fatigue in humans. *J Clin Invest.* 94(6): 2468–2474, 1994.

Reid MB, Kobzik L, Bredt DS, Stamler JS. Nitric oxide modulates excitation–contraction coupling in the diaphragm. *Comp Biochem Physiol.* 119: 211–218, 1998.

Reid MB. Reactive oxygen species as agents of fatigue. *Med Sci Sports Exerc.* 48(11): 2239-2246, 2016a.

Reid MB. Redox interventions to increase exercise performance. *J Physiol.* 594(18): 5125-5133, 2016b.

Reid MB. Role of nitric oxide in skeletal muscle: synthesis, distribution and functional importance. *Acta Physiol Scand*. 162(3):401-9, 1998.

Reid MB, Durham WJ. Generation of reactive oxygen and nitrogen species in contracting skeletal muscle: potential impact on aging. *Ann NY Acad Sci.* 959: 108–116, 2002.

Richardson RS, Noyszewski EA, Kendrick KF, Leigh JS, Wagner PD. Myoglobin O_2 desaturation during exercise. Evidence of limited O_2 transport. *J Clin Investig.* 96:1916, 1995.

Rienks JN, Vanderwoude AA, Maas E, Blea ZM, Subudhi AW. Effect of beetroot juice on moderate-intensity exercise at a constant rating of perceived exertion. *Int J Exerc Sci.* 8(3): 277-286, 2015.

Rimer EG, Peterson LR, Coggan AR, Martin JC. Increase in maximal cycling power with acute dietary nitrate supplementation. *Int J Sports Physiol Perform.* 11(6): 715-720, 2016.

Rocha BS, Gago B, Barbosa RM, Laranjinha J. Dietary polyphenols generate nitric oxide from nitrite in the stomach and induce smooth muscle relaxation. *Toxicol.* 265(1-2): 41-48, 2009.

Rodriguez-Mateos A, Rendeiro C, Bergillos-Meca T, Tabatabaee S, George TW, Heiss C, **Spencer JP.** Intake and time dependence of blueberry flavonoid-induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic

insights into biological activity. *Am J Clin Nutr*. 98(5): 1179–91, 2013.

Roelofs EJ, Smith-Ryan AE, Trexler ET, Hirsch KR, Mock MG. Effects of pomegranate extract on blood flow and vessel diameter after high-intensity exercise in young, healthy adults. *Eur J Sport Sci.* 17(3): 317-324, 2017.

Rokkedal-Lausch T, Franch J, Poulsen MK, Thomsen LP, Weitzberg E, Kamavuako EN, Karbing DS, Larsen RG. Chronic high-dose beetroot juice supplementation improves time trial performance of well-trained cyclists in normoxia and hypoxia. *Nitric Oxide.* 85: 44-52, 2019.

Rolfe DF, Hulbert AJ, Brand MD. Characteristics of mitochondrial proton leak and control of oxidative phosphorylation in the major oxygen-consuming tissues of the rat. *Biochim Biophys Acta.* 1188(3): 405-416, 1994.

Romer LM, Polkey MI. Exercise-induced respiratory muscle fatigue: implications for performance. *J Appl Physiol (1985).* 104(3): 879-888, 2008.

Rosselli M, Imthurm B, Macas E, Keller PJ, Dubey R. Circulating nitrite/nitrate levels increase with follicular development: indirect evidence for esetradiol mediated NO release. *Biochem Biophys Res Commun.* 202*:* 1543-1552, 1994.

Rossiter HB, Ward SA, Kowalchuk JM, Howe FA, Griffiths JR & Whipp BJ. Effects of prior exercise on oxygen uptake and phosphocreatine kinetics during high-intensity knee-extension exercise in humans. *J Physiol.* 537: 291–303, 2001.

Rossiter HB, Ward SA, Kowalchuk JM, Howe FA, Griffiths JR, Whipp BJ. Dynamic asymmetry of phosphocreatine concentration and O2 uptake between the on- and off-transients of moderate- and high-intensity exercise in humans. *J Physiol*. 541: 991–1002, 2002.

Rotto DM, Kaufman MP. Effect of metabolic products of muscular contraction on discharge of group III and IV afferents. *J Appl Physiol*. 64(6): 2306-13, 1988.

Rossman MJ, Venturelli M, McDaniel J, Amann M, Richardson RS. Muscle mass and peripheral fatigue: a potential role for afferent feedback? *Acta Physiol.* 206: 242-250, 2012.

Ruffmann R, Wendel A. GSH rescue by N-acetylcysteine. *Klin Wochenschr.* 69: 857-862, 1991.

Sahlin K, Katz A, Broberg S. Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. *Am J Physiol Cell Physiol.* 259: C834–C841, 1990.

Sandbakk SB, Sandbakk Ø, Peacock O, James P, Welde B, Stokes K, Böhlke N, Tjønna AE. Effects of acute supplementation of L-arginine and nitrate on endurance and sprint performance in elite athletes. *Nitric Oxide.* 48: 10-15, 2015.

Sandiford SD, Green HJ, Duhamel TA, Perco JG, Schertzer JD & Ouyang J. Inactivation of human muscle Na+-K+-ATPase in vitro during prolonged exercise is increased with hypoxia. *J Appl Physiol.* 96: 1767–1775, 2004.

Sargeant AJ. Neuromuscular determinants of human performance. In B.J. Whipp and A. J. Sargeant (Eds) *Physiological determinants of exercise tolerance in humans.* Portland Press, London, pp. 13-28, 1999.

Schneider MF, Chandler WK. Voltage dependent charge movement in skeletal muscle: a possible step in excitation-contraction coupling. *Nature Lond.* 242: 244-246, 1973.

Scherer NM, Deamer DW. Oxidative stress impairs the function of sarcoplasmic reticulum by oxidation of sulfhydryl groups in the Ca2+-ATPase. *Arch Biochem Biophys.* 246: 589–601, 1986.

Sen CK, Packer L, Hanninen O. Handbook of Oxidants and Antioxidants in Exercise. Amsterdam: Elsevier. p. 1207, 2000.

Sen CK. Glutathione homeostasis in response to exercise training and nutritional supplements. *Mol Cell Biochem.* 196: 31–42, 1999.

Sen CK. Oxidants and antioxidants in exercise. *J Appl Physiol.* 79: 675–686, 1995a.

Sen CK. Nutritional biochemistry of cellular glutathione. *J Nutr Biochem*. 8: 660–672, 1997.

Sen CK, Kolosova I, Hanninen O, Orlov SN. Inward potassium transport systems in skeletal muscle derived cells are highly sensitive to oxidant exposure. *Free Radic Biol Med.* 18: 795–800, 1995b.

Sejersted OM, Sjøgaard G. Dynamics and consequences of potassium shifts in skeletal muscle and heart during exercise. *Physiol Rev*. 80: 1411–1481, 2000.

Shannon OM, Barlow MJ, Duckworth L, Williams E, Wort G, Woods D, Siervo M, O'Hara JP. Dietary nitrate supplementation enhances short but not longer duration running time-trial performance. *Eur J Appl Physiol.* 117(4): 775-785, 2016a.

Shannon OM, Duckworth L, Barlow MJ, Woods D, Lara J, Siervo M, O'Hara JP. Dietary nitrate supplementation enhances high-intensity running performance in moderate normobaric hypoxia, independent of aerobic fitness. *Nitric Oxide.* 59: 63-70, 2016b.

Sheel AW, Derchak PA, Morgan BJ, Pegelow DF, Jacques AJ, Dempsey JA. Fatiguing inspiratory muscle work causes reflex reduction in resting leg blood flow in humans. *J Physiol.* 537(Pt 1):277–289, 2001.

Shen X, Kolluru GK, Yuan S, Kevil C. Measurement of H2S in vivo and in vitro by the monobromobimane method. *Methods Enzymol.* 554: 31-45, 2015.

Shepherd AI, Gilchrist M, Winyard PG, Jones AM, Hallmann E, Kazimierczak R, Rembialkowska E, Benjamin N, Shore AC, Wilkerson DP. Effects of dietary nitrate supplementation on the oxygen cost of exercise and walking performance in individuals with type 2 diabetes: a randomized, double-blind, placebo-controlled crossover trial. *Free Radic Biol Med.* 86: 200-208, 2015.

Shindoh C, DiMarco A, Thomas A, Manubay P, Supinski G. Effect of N-acetylcysteine on diaphragm fatigue. *J Appl Physiol.* 68: 2107-2113, 1990.

Shinohara M, Moritani T. Increase in neuromuscular activity and oxygen uptake during heavy exercise. *Ann Physiol Anthropol*. 11(3): 257-262, 1992.

Shiva S, Huang Z, Grubina R, Sun J, Ringwood LA, MacArthur PH, Xu X, Murphy E, Darley-Usmar VM, Gladwin MT. Deoxymyoglobin is a nitrite reductase that generates nitric oxide and regulates mitochondrial respiration. *Circ Res.* 100: 654–661, 2007.

Shiva S, Wang X, Ringwood LA, Xu X, Yuditskaya S, Annavajjhala V, Miyajima H, Hogg N, Harris ZL, Gladwin MT. Ceruloplasmin is a NO oxidase and nitrite synthase that determines endocrine NO homeostasis. *Nat Chem Biol.* 2: 486–493, 2006.

Sidhu SK, Cresswell AG, Carroll TJ. Motor cortex excitability does not increase during sustained cycling exercise to volitional exhaustion. *J Appl Physiol.* 113: 401-409, 2012.

Simon DI, Stamler JS, Jaraki O, Keaney JF, Osborne JA, Francis SA. Antiplatelet properties of protein S-nitrosothiols derived from nitric oxide and endothelium-derived relaxing factor. *Arterioscler Thromb.* 13: 791-799, 1993.

Sjøgaard G, Adams RP, Saltin B. Water and ion shifts in skeletal muscle of humans with intense dynamic knee extension. *Am J Physiol Regul Integr Comp Physiol.* 248: R190–R196, 1985.

Slattery KM, Dascombe B, Wallace LK, Bentley DJ, Coutts AJ. Effect of N-acetylcysteine on cycling performance after intensified training. *Med Sci Sports Exerc.* 46(6):1114-23, 2014.

Smith BC, Marletta MA. Mechanisms of *S*-nitrosothiol formation and selectivity in nitric oxide signaling. *Curr Opin Chem Biol.* 16(5-6): 498-506, 2012.

Smith MA, Reid MB. Redox modulation of contractile function in respiratory and limb skeletal muscle. *Respir Physiol Neurobiol.* 151: 229–241, 2006.

Smith JR, Ade CJ, Broxterman RM, Skutnik BC, Barstow TJ, Wong BJ, Harms CA. Influence of exercise intensity on respiratory muscle fatigue and brachial artery blood flow during cycling exercise. *Eur J Appl Physiol.* 114(8): 1767-1777, 2014.

Smith JR, Broxterman RM, Ade CJ, Evans KK, Kurti SP, Hammer SM, Barstow TJ, Harms CA. Acute supplementation of N-acetylcysteine does not affect muscle blood flow and oxygenation characteristics during handgrip exercise. *Physiol Rep.* 4(7): e12748, 2016.

Söderlund K, Greenhaff PL, Hultman E. Energy metabolism in type I and type II human muscle fibres during short term electrical stimulation at different frequencies. *Acta Physiol Scand.* 144(1): 15-22, 1992.

Solianik R, Kreivėnaitė L, Streckis V, Mickevičienė D, Skurvydas A. Effects of age and sex on fatigability and recovery from sustained maximal isometric voluntary contraction. *J Electromyogr Kinesiol.* 32: 61-69, 2017.

Spriet LL, Heigenhauser GJF. Regulation of pyruvate dehydrogenase (PDH) activity in human skeletal muscle during exercise. *Exerc Sport Sci Rev*. 30: 91-5, 2002.

Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell.* 78(6): 931-936, 1994.

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

Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox activated forms. *Science.* 258: 1898–1903, 1992.

Stark-Leyva KN, Beck KC, Johnson BD. Influence of expiratory loading and hyperinflation on cardiac output during exercise. *J Appl Physiol.* 96: 1920–1927, 2004.

St Croix CM, Morgan BJ, Wetter TJ, Dempsey JA. Fatiguing inspiratory muscle work causes reflex sympathetic activation in humans. *J Physiol.* 529(Pt 2): 493–504, 2000.

Stoyanovsky D, Murphy T, Anno PR, Kim YM, Salama G. Nitric oxide activates skeletal and cardiac ryanodine receptors. *Cell Calcium.* 21: 19 –29, 1997.

St Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem.* 277: 44784–44790, 2002.

Sulova Z, Vyskocil F, Stankovicova T, Breier A. Ca2+-induced inhibition of sodium pump: effects on energetic metabolism of mouse diaphragm tissue. *Gen Physiol Biophys.* 17: 271–283, 1998.

Supinski GS, Nethery D, Stofan D, DiMarco A. Effect of free radical scavengers on diaphragmatic fatigue. *Am J Respir Crit Care Med.* 155(2): 622–629, 1997.

Sweeney HL, Bowman BF, Stull JT. Myosin light chain phosphorylation in vertebrate striated muscle: regulation and function. *Am J Physiol.* 264: C1085–C1090, 1993.

Szentesi P Zaremba R, van Mechelen W, Stienen GJ. ATP utilization for calcium uptake and force production in different types of human skeletal muscle fibres. *J Physiol.* 531: 393-403, 2001.

Tan R, Wylie LJ, Thompson C, Blackwell JR, Bailey SJ, Vanhatalo A, Jones AM. Beetroot juice ingestion during prolonged moderate-intensity exercise attenuates progressive rise in O_2 uptake. *J Appl Physiol.* 124(5): 1254-1263, 2018.

Taylor BJ, How SC, Romer LM. Exercise-induced abdominal muscle fatigue in healthy humans. *J Appl Physiol*. 100: 1554–1562, 2006.

Taylor JL, Amann M, Duchateau J, Meeusen R, Rice CL. Neural contributions to muscle fatigue: from the brain to the muscle and back again. *Med Sci Sports Exerc*. 48: 2294–2306, 2016.

Taylor BJ, Romer LM. Effect of expiratory muscle fatigue on exercise tolerance and locomotor muscle fatigue in healthy humans. *J Appl Physiol (1985).* 104(5): 1442-1451, 2008.

Tesch PA, Thorsson A, Kaiser P. Muscle capillary supply and fiber type characteristics in weight and power lifters. J. Appl. Physiol. *Respir Environ Exerc Physiol*. 56: 35Y8, 1984.

Tesoriere L, Butera D, D'Arpa D, Di Gaudio F, Allegra M, Gentile C, Livrea MA. Increased resistance to oxidation of betalain-enriched human low density lipoproteins. *Free Radic Res.* 37(6): 689-696, 2003.

Thompson KG, Turner L, Prichard J, Dodd F, Kennedy DO, Haskell, C, Blackwell JR, Jones AM. Influence of dietary nitrate supplementation on physiological and cognitive responses to incremental cycle exercise. *Respir Physiol Neurobiol*. 193: 11–20, 2014.

Thompson C, Wylie LJ, Fulford J, Kelly J, Black MI, McDonagh ST, Jeukendrup AE, Vanhatalo A, Jones AM. Dietary nitrate improves sprint performance and cognitive function during prolonged intermittent exercise. *Eur J Appl Physiol.* 115(9): 1825-1834, 2015.

Thompson C, Vanhatalo A, Jell H, Fulford J, Carter J, Nyman L, Bailey SJ, Jones AM. Dietary nitrate supplementation improves sprint and high-intensity intermittent running performance. *Nitric Oxide.* 61:55-61, 2016.

Thompson C, Wylie LJ, Blackwell JR, Fulford J, Black MI, Kelly J, McDonagh ST, Carter J, Bailey SJ, Vanhatalo A, Jones AM. Influence of dietary nitrate supplementation on physiological and muscle metabolic adaptations to sprint interval training. *J Appl Physiol (1985).* 122(3): 642-652, 2017.

Thompson C, Vanhatalo A, Kadach S, Wylie LJ, Fulford J, Ferguson SK, Blackwell JR, Bailey SJ, Jones AM. Discrete physiological effects of beetroot juice and potassium nitrate supplementation following 4-wk sprint interval training. *J Appl Physiol (1985).* 124(6): 1519-1528, 2018.

Tillin NA, Moudy S, Nourse KM, Tyler CJ. Nitrate supplement benefits contractile forces in fatigued but not unfatigued muscle. *Med Sci Sports Exerc.* 50(10): 2122-2131, 2018.

Tonkonogi M, Sahlin K. Physical exercise and mitochondrial function in human skeletal muscle. *Exerc Sport Sci Rev.* 30(3): 129-137, 2002.

Tonkonogi M, Walsh B, Tiivel T, Saks V, Sahlin K. Mitochondrial function in human skeletal muscle is not impaired by high intensity exercise. *Pflügers Arch.* 437: 562–568, 1999.

Totzeck M, Hendgen-Cotta UB, Rammos C, Frommke LM, Knackstedt C, Predel HG, Kelm M, Rassaf T. Higher endogenous nitrite levels are associated with superior exercise capacity in highly trained athletes. *Nitric Oxide.* 27: 75–81, 2012.

Taschakovsky ME, Hughson RL. Interaction of factors determining oxygen uptake at the onset of exercise. *J Appl Physiol.* 86: 1101-1113, 1999.

Travaline JM, Sudarshan S, Roy BG, Cordova F, Leyenson V, Criner GJ. Effect of N-acetylcysteine on human diaphragm strength and fatigability. *Am J Respir Crit Care Med. 156*: 1567-1571, 1997.

Trewin AJ, Petersen AC, Billaut F, McQuade LR, McInerney BV, Stephto NK. N-acetylcysteine alters substrate metabolism during high-intensity cycle exercise in well-trained humans. *Appl Physiol Nutr Metab.* 38: 1217-1227, 2013.

Tsintzas K, Williams C. Human muscle glycogen metabolism during exercise. Effect of carbohydrate supplementation. *Sports Med.* 25: 7-23, 1998.

Tsuge K, Kataoka M, Seto Y. Cyanide and thiocyanate levels in blood and saliva of healthy adult volunteers. *J Health Sci*. 46: 343e350, 2000.

Tsukiyama Y, Ito T, Nagaoka K, Eguchi E, Ogino K. Effects of exercise training on nitric oxide, blood pressure and antioxidant enzymes. *J Clin Biochem Nutr.* 60(3): 180-186, 2017.

Turrens JF. Mitochondrial formation of reactive oxygen species. *J Physiol.* 552: 335–344, 2003.

Valenzuela A. The biological significance of malondialdehyde determination in the assessment of tissue oxidative stress. *Life Sci*. 48: 301–309, 1991.

Van Hoorebeke JS, Trias CO, Davis BA, Lozada CF, Casazza GA. Betalain-rich concentrate supplementation improves exercise performance in competitive runners. *Sports (Basel).* 4(3): pii: E40, 2016.

Vanhatalo A, Bailey SJ, Blackwell JR, DiMenna FJ, Pavey TG, Wilkerson DP, Benjamin N, Winyard PG, Jones AM. Acute and chronic effects of dietary nitrate supplementation on blood pressure and the physiological responses to moderate-intensity and incremental exercise. *Am J Physiol Regul Integr Comp Physiol.* 299(4): R1121-1131, 2010a.

Vanhatalo A, Black MI, DiMenna FJ, Blackwell JR, Friis Schmidt J, Thompson C, Wylie LJ, Mohr M, Bangsbo J, Krustrup P, Jones AM. The mechanistic bases of the power-time relationship: muscle metabolic responses and relationships to muscle fibre type. *J Physiol.* 594(15): 4407-4423, 2016.

Vanhatalo A, Blackwell JR, L'Heureux JE, Williams DW, Smith A, van der Giezen M, Winyard PG, Kelly J, Jones AM. Nitrate-responsive oral microbiome modulates nitric oxide homeostasis and blood pressure in humans. *Free Rad Biol Med.* 124: 21-30, 2018.

Vanhatalo A, Fulford J, Bailey SJ, Blackwell JR, Winyard PG, Jones AM. Dietary nitrate reduces muscle metabolic perturbation and improves exercise tolerance in hypoxia. *J Physiol.* 589: 5517-5528, 2011a.

Vanhatalo A, Fulford J, DiMenna FJ, Jones AM. Influence of hyperoxia on muscle metabolic responses and the power-duration relationship during severe-intensity exercise in humans: a 31P magnetic resonance spectroscopy study. *Exp Physiol.* 95(4): 528-540, 2010b.

Vanhatalo A, Jones AM, Blackwell JR, Winyard PG, Fulford J. Dietary nitrate accelerates postexercise muscle metabolic recovery and O2 delivery in hypoxia. *J Appl Physiol.* 117: 1460-1470, 2014.

Vanhatalo A, Poole DC, DiMenna FJ, Bailey SJ, Jones AM. Muscle fiber recruitment and the slow component of O2 uptake: constant work rate vs. all-out sprint exercise. *Am J Physiol Regul Integr Comp Physiol.* 300(3): R700-707, 2011b.

Van De Walle GP, Vukovich MD. The effect of nitrate supplementation on exercise tolerance and performance: a systematic review and meta-analysis. *J Strength Cond Res.* 32(6): 1796-1808, 2018.

Veal EA, Day AM, Morgan BA. Hydrogen peroxide sensing and signaling. *Mol Cell.* 26: 1–14, 2007.

Velmurugan S, Gan JM, Rathod KS, Khambata RS, Ghosh SM, Hartley A, Van Eijl S, Sagi- Kiss V, Chowdhury TA, Curtis M, Kuhnle GG, Wade WG, Ahluwalia A. Dietary nitrate improves vascular function in patients with hypercholesterolemia: a randomized, double- blind, placebo-controlled study. *Am J Clin Nutr*. 103:25-38, 2016.

Vidal PJ, Lopez-Nicolas JM, Gandia-Herrero F, Garcia-Carmona F. Inactivation of lipoxygenase and cyclooxygenase by natural betalains

and semi-synthetic analogues. *Food Chem*. 154: 246–254, 2014.

Viner RI, Williams TD, Schöneich C. Nitric oxide-dependent modification of the sarcoplasmic reticulum Ca-ATPase: localization of cysteine target sites. *Free Radic Biol Med.* 29: 489–96, 2000.

Viner RI, Ferrington DA, Huhmer AFR, Bigelow DJ, Schoneic, C. Accumulation of nitrotyrosine on the SERCA2a isoform of SR Ca-ATPase of rat skeletal muscle during aging: a peroxynitrite-mediated process? *FEBS Lett.* 379: 286–290, 1996.

Vøllestad NK, Vaage O, Hermansen L. Muscle glycogen depletion patterns in type I and subgroups of type II fibres during prolonged severe exercise in man. *Acta Physiol Scand.* 122(4): 433-441, 1984.

Vøllestad NK, Wesche J, Sejersted OM. Gradual increase in leg oxygen uptake during repeated submaximal contractions in humans. *J Appl Physiol.* 68: 1150-1156, 1990.

Wagner DA, Schultz DS, Deen WM, Young VR, Tannenbaum SR. Metabolic fate of an oral dose of 15N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer Res*. 43:1921–25, 1983.

Walsh B, Tonkonogi M, Soderlund K, Hultman E, Saks V, Sahlin K. The role of phosphorylcreatine and creatine in the regulation of mitochondrial respiration in human skeletal muscle. *J Physiol.* 537: 971-978, 2001.

Watt MJ, Howlett KF, Febbraio MA, Spriet LL, Hargreaves M. Adrenaline increases skeletal muscle glycogenolysis, pyruvate dehydrogenase activation and carbohydrate oxidation during moderate exercise in humans. *J Physiol.* 534: 269–278, 2001.

Watt MJ, Spriet LL. Regulation and role of hormone-sensitive lipase activity in human skeletal muscle. *Proc Nutr Soc*. 63: 315-22, 2004.

Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension*. 51: 784 –790, 2008.

Webb A, Bond R, McLean P, Uppal R, Benjamin N, Ahluwalia A. Reduction of nitrite to nitric oxide during ischemia protects against myocardial ischemia-reperfusion damage. *Proc Natl Acad Sci USA*. 101: 13683–13688, 2004.

Webb AJ, Patel N, Loukogeorgakis S, Okorie M, Aboud Z, Misra S, Rashid R, Miall P, Deanfield J, Benjamin N, MacAllister R, Hobbs AJ, Ahluwalia A. Acute blood pressure lowering, vasoprotective, and antiplatelet properties of dietary nitrate via bioconversion to nitrite. *Hypertension.* 51(3): 784-790, 2008.

Weitzberg E, Lundberg JO. Nonenzymatic nitric oxide production in humans. *Nitric Oxide.* 2: 1–7, 1998.

Westerblad H, Allen DG. Cellular mechanisms of skeletal muscle fatigue. *Adv Exp Med Biol.* 538: 563–570, 2003.

Westerblad H, Allen DG, Lännergren J. Muscle fatigue: lactic acid or inorganic phosphate the major cause? *News Physiol Sci*. 17: 17–21, 2002.

Whipp BJ. The slow component of O_2 uptake kinetics during heavy exercise. *Med Sci Sports Exerc.* 26: 1319-1326, 1994.

Whipp BJ, Davis JA, Torres F, Wasserman K. A test to determine parameters of aerobic function during exercise. *J Appl Physiol* 50: 217– 221, 1981.

Whipp BJ, Wasserman K. Efficiency of muscular work. *J Appl Physiol.* 26: 644-648, 1969.

Whipp BJ, Wasserman K. Oxygen uptake kinetics for various intensities of constant-load work. *J Appl Physiol.* 33: 351–356, 1972.

Whipp BJ, Ward SA, Lamarra N, Davis JA, Wasserman K. Parameters of ventilator and gas exchange dynamics during exercise. *J Appl Physiol.* 52: 1506-1513, 1982.

Whitfield J, Ludzki A, Heigenhauser GJG, Senden JMG, Verdijk LB, van Loon LJC, Spriet LL, Holloway GP. Beetroot juice supplementation reduces whole body oxygen consumption but does not improve indices of mitochondrial efficiency in human skeletal muscle. *J Physiol.*(2): 421-435, 2016.

Whitfield J, Gamu D, Heigenhauser GJF, van Loon LJC, Spriet LL, Tupling AR, & Holloway GP. Beetroot juice increases human muscle force without changing Ca^{2+}-handling proteins. *Med Sci Sport Exerc.* 49(10): 2016-2024, 2017.

Wickham KA, McCarthy DG, Pereira JM, Cervone DT, Verdijk LB, van Loon LJC, Power GA, Spriet LL. No effect of beetroot juice supplementation

on exercise economy and performance in recreationally active females despite increase torque production. *Physiol Rep.* 7(2): e13982, 2019.

Wilkerson DP, Koppo K, Barstow TJ, Jones AM. Effect of work rate on the functional 'gain' of Phase II pulmonary O2 uptake response to exercise. *Respir Physiol Neurobiol.* 142: 211-223, 2004.

Wilkerson DP, Hayward GM, Bailey SJ, Vanhatalo A, Blackwell JR, Jones AM. Influence of acute dietary nitrate supplementation on 50 mile time trial performance in well-trained cyclists. *Eur J Appl Physiol.* 112: 4127–4134, 2012

Williams G, Brown T, Becker M, Prager M, Giroir BP. Cytokine-induced expression of nitric oxidie synthase in C2C12 skeletal muscle myocytes. *Am J Physiol.* 267: R1020-R1025, 1994.

Williams Jr DL, Swenson CA. Disulfide bridges in tropomyosin. *Eur J Biochem*. 127: 495–499, 1982.

Willis WT, Jackman MR. Mitochondrial function during heavy exercise. *Med Sci Sports Exerc.* 26(11): 1347-1353, 1994.

Wilson DF. Oxidative phosphorylation: regulation and role in cellular and tissue metabolism. *J Physiol*. 595(23): 7023-7038, 2017. doi: 10.1113/JP273839.

Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB. Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci*. 90: 9813–9817, 1993.

Witt JD, Guenette JA, Rupert JL, McKenzie DC, Sheel A. Inspiratory muscle training attenuates the human respiratory muscle metaboreflex. *J Physiol.* 584: 1019-1028, 2007.

Woledge RC. Possible effects of fatigue on muscle efficiency. *Acta Physiol Scand*. 162: 267–73, 1998.

Wootton-Beard PC, Ryan L. Combined use of multiple methodologies for the measurement of total antioxidant capacity in UK commercially available vegetable juices. *Plant Foods Hum Nutr*. 67: 142–147, 2012.

Woessner M, Smoliga JM, Tarzia B, Stabler T, Van Bruggen M, Allen JD. A stepwise reduction in plasma and salivary nitrite with increasing strengths of mouthwash following a dietary nitrate load. *Nitric Oxide.* 54: 1-7, 2016.

Wu, G, Meininger CJ. Nitric oxide and vascular insulin resistance. *Biofactors*.

35(1): 21-27, 2009.

Wüst RC, Morse CI, de Haan A, Jones DA, Degens H. Sex differences in contractile properties and fatigue resistance of human skeletal muscle. *Exp Physiol.* 93(7): 843-850, 2008.

Wüst RC, van der Laarse WJ, Rossiter HB. On-off asymmetries in oxygen consumption kinetics of single xenopus laevis skeletal muscle fibres suggest higher-order control . *J Physiol.* 591(3): 731-744, 2013.

Wylie LJ, Kelly J, Bailey SJ, Blackwell JR, Skiba PF, Winyard PG, Jeukendrup AE, Vanhatalo A, Jones AM. Beetroot juice and exercise: pharmacodynamics and dose-response relationships. *J Appl Physiol.* 15(3): 325-336, 2013a.

Wylie LJ, Mohr M, Krustrup P, Jackman SR, Ermidis G, Kelly J, Black MI, Bailey SJ, Vanhatalo A, Jones AM. Dietary nitrate supplementation improves team sport-specific intense intermittent exercise performance. *Eur J Appl Physiol.* 113(7): 1673-1684, 2013b.

Wylie LJ, Ortiz de Zevallos J, Isidore T, Nyman L, Vanhatalo A, Bailey SJ, Jones AM. Dose-dependent effects of dietary nitrate on the oxygen cost of moderate-intensity exercise: Acute vs. chronic supplementation. *Nitric Oxide.* 57*:* 30-39, 2016.

Wynia-Smith SL, Smith BC. Nitrosothiol formation and S-nitrosation signaling through nitric oxide synthases. *Nitric Oxide.* 63: 52-60, 2017.

Xu L, Eu JP, Meissner G, Stamler JS. Activation of the cardiac calcium release channel (ryanodine receptor) by poly-S-nitrosylation. *Science.* 279: 234-237, 1998.

Yamada T, Mishima T, Sakamoto M, Sugiyama M, Matsunaga S, Wada M. Oxidation of myosin heavy chain and reduction in force production in hyperthyroid rat soleus. *J Appl Physiol.* 100:1520–1526, 2006.

Yensen C, Matar W, Renaud JM. K+-induced twitch potentiation is not due to longer action potential. *Am J Physiol Cell Physiol*. 283: C169- C177, 2002.

Ysart G, Miller P, Barrett G, Farrington D, Lawrance P, Harrison N. Dietary exposures to nitrate in the UK. *Food Addit Contam.* 16: 521–3, 1999.

Zhang Z, Naughton DP, Blake DR, Benjamin N, Stevens CR, Winyard PG, Symons MC, Harrison R. Human xanthine oxidase converts nitrite ions into nitric oxide (NO). *Biochem Soc Trans.* 25(3): 524S, 1997.

Zergeroglu MA, McKenzie MJ, Shanely RA, VanGammeren D, DeRuisseau KC, Powers SK. Mechanical ventilation-induced oxidative stress in the diaphragm. *J Appl Physiol*. 95: 1116–1124, 2003.

Zoladz JA, Gladden BL, Hogan MC, Niecarz Z, Grassi B. Progressive recruitment of muscle fibers is not necessary for the slow component of $\dot{V}O_2$ kinetics. *J Appl Physiol.* 105: 575–580, 2008.

Zuo L, Christofi FL, Wright VP, Liu CY, Merola AJ, Berliner LJ, Clanton TL. Intra- and extracellular measurement of reactive oxygen species produced during heat stress in diaphragm muscle. *Am J Physiol Cell Physiol*. 279: C1058– C1060, 2000.

Zweier JL, Wang P, Samouilov A, Kuppusamy P. Enzyme-independent formation of nitric oxide in biological tissues. *Nat Med.* 1: 804-809, 1995.

Appendix I

Sport and Health Sciences
College of Life and Environmental Sciences
St Luke's Campus
Heavitree Road
Exeter
Devon
Telephone: +44 (0)1392 26
Email: sshs-school-office@ex.ac.uk
Web: www.ex.ac.uk/sshs

The effect of nitrate-depleted beetroot juice on the oxygen cost of moderate-intensity cycling exercise.

PARTICIPANT INFORMATION SHEET
Version one: 13/03/2019

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether you would like to participate. If you decide to participate, we thank you in advance for the time and effort you have decided to devote to our investigation. If you decide not to take part, there will be no disadvantage to you of any kind and we thank you for considering our request.

WHAT IS THE PURPOSE OF THE STUDY?
The purpose of this study is to find out if the polyphenol compounds in nitrate-depleted beetroot juice influences exercise economy compared to a control condition and nitrate-rich beetroot juice. The results of this study will give us a better understanding on the beneficial properties of beetroot juice ingestion.

WHO AND HOW MANY PEOPLE WILL BE IN THIS STUDY?
We plan to recruit and test 14 healthy male and female volunteers between 18 to 45 years of age. To take part in this study you should be a non-smoker, not a user of dietary supplements, and in good health and engage in recreational exercise but not highly trained. You should be free of any injury or health conditions which might make it difficult for you to exercise.

WHAT ARE MY RESPONSIBILITIES IF I PARTICIPATE IN THE STUDY?
Should you agree to participate, we will ask you to visit the testing venue on St. Luke's Campus on 7 occasions over a 4-week period. Your visits will be scheduled at the same time of day (± 2 hours). Aside from the supplementation regimen detailed below, we will ask you to maintain your usual dietary and exercise habits throughout this study. We will ask you to record and repeat these 24 hours prior to all exercise tests. Please remain hydrated prior to each visit, though you should not drink alcohol for at least 24 hours before all visits and you should avoid caffeine and food (aside from your breakfast for the morning of testing visits) for 6 hours before all exercise tests. Additionally, you must refrain from any antibacterial mouthwash throughout the duration of the study. You will have the opportunity to ask questions and clarify any issues you may be unsure of before deciding whether you want to take part.

VISIT #1: Participant Screening and Baseline Testing

You will be asked to read and sign the consent form and have your general health and readiness to undertake exercise tests assessed. Following initial screening, you will complete a maximal incremental exercise test on a cycle ergometer, on St. Luke's Campus. You will cycle on a protocol which increases in intensity gradually until you have reached exhaustion. This exercise protocol typically does not take longer than 15 minutes. In total, this visit will take no longer than 45 min.

SUPPLEMENTATION

You will undergo 4 days of supplementation for each of the three conditions:

a) 140 mL (2 x 70 mL 'shots') per day of nitrate-depleted beetroot juice
b) 140 mL (2 x 70 mL 'shots') per day of water
c) 140 mL (2 x 70 mL 'shots') per day of nitrate-rich beetroot juice

The nitrate-depleted and nitrate-rich beetroot juice looks, smells, and tastes the same. You will consume 1 x 70 mL shot in the morning, and 1 x 70 mL in the evening on days 1 and 2 of the supplementation period. On day 3 and 4 of each supplementation period, you will be asked to attend the laboratory for experimental testing. On these days you will be asked to consume 2 x 70 mL 2 hours prior to arrival at the laboratory. The specific times of supplement ingestion will be outlined to you prior to each visit.

A period of at least 5 days will take place in between each supplement condition.
Additionally, you will be required to complete a food and physical activity log for the 24 hours prior to visits #2 and you will be asked to replicate your diet and physical activity in the 24 hours preceding all following visits.

VISIT #2, 3, 4, 5, 6, and 7: Experimental testing sessions

Visit #2, 3, 4, 5, 6, and 7 will take place on day 3 and 4 of the 4-day supplementation period (outlined in section above) in each condition. These visits will serve as the testing sessions. You will arrive to St. Luke's Campus for all of testing. Upon arrival, two blood samples will be taken before the exercise begins. These blood samples will be used to measure the amount of important biomarkers. After the blood draw is taken, you will complete two 6- minute bouts of moderate-intensity cycling exercise, interspersed by a 10-minute period of recovery between each bout. You will be asked to wear a mouthpiece throughout the exercise test to determine how much oxygen you are using during the exercise.
These visits will take no longer than 45 minutes.

Table 1 shows a summary of measurements done during each visit.

Visit	Exercise test and measurement of VO_2	Blood sample (through a single venipuncture)
1	√	
2	√	√ x 2
3	√	√ x 2
4	√	√ x 2
5	√	√ x 2
6	√	√ x 2
7	√	√ x 2

BLOOD SAMPLES

Two blood draws (~6 ml each; equal to a small teaspoon) in the arm will be taken before each exercise test (visit #2, 3, 4, 5, 6, and 7).

A total of 12 blood samples (2 for each testing visit) will be drawn over the entire study. This equates to a total volume of ~72 mL over 4 weeks, which is significantly less than the amount you would provide when giving blood (around 500 mL in 1 day) and should not have any detrimental effect on a healthy individual. All samples will be taken by a trained research team member.

DESCRIPTION OF POTENTIAL RISKS AND DISCOMFORTS

The blood sampling may cause some temporary discomfort. However, these techniques are regularly used in exercise physiology testing. The investigators are trained and experienced in all aspects of these procedures to ensure that they are completed safely. The maximal exercise you are asked to undertake is likely to cause some discomfort. However, this discomfort will do no lasting harm and will simply represent the feelings of exertion that we normally experience when working very hard. The risks involved with maximal exercise testing in a controlled laboratory environment are minimal. Possible adverse effects include nausea, vomiting, and loss of balance. You may experience localised muscle pain, which may persist for more than 24 h. Every effort will be made to minimise the above risks by thorough instruction and observation of symptoms during sessions. Please be aware that any concerns you have about the degree of effort that you will be asked to expend will be addressed in full by the researchers prior to you giving your consent to participate. Furthermore, if at any time, you decide that you no longer want to take part in this project for any reason, you are at liberty to withdraw without giving any reason and without disadvantage to yourself of any kind.

When ingesting large doses of beetroot, it can cause beeturia, causing discolouration of urine and stool. This is a normal side effect and poses no harm.

WHAT ARE THE POSSIBLE BENEFITS FOR ME AND/OR FOR SOCIETY?

We cannot promise you any personal benefits to you from your participation in this study. However, you may gain valuable information regarding your fitness level. You will also be helping provide scientific research and knowledge towards the effects of dietary nitrate supplementation.

WHAT INFORMATION WILL BE KEPT PRIVATE?

The blood samples will be code labeled without showing your name and stored securely. These samples may be transported to other laboratories within the UK or overseas for further analyses. Samples will be disposed of immediately following analysis. Your anonymity will be protected in the case of publication of our findings. Any data collected during these exercise tests will be used to establish response profiles across the group of subjects involved and these cumulative scores may be made available for public inspection in research journals and/or at seminars and conferences. In addition, individual response profiles indicative of the typical response may also be presented. However, in all cases, anonymity will be strictly preserved. Therefore, while results of this project may be available for public inspection, any data displayed will in no way be linked to any specific individual participating in this investigation.

Upon completion of the study, the data collected will be securely stored in such a way that only the researchers involved in this investigation will be able to gain access to it. At this point, you are most welcome to request a copy of the results of the project should you wish and we will be available to explain and interpret your specific data.

CAN PARTICIPATION IN THE STUDY END EARLY?

If you volunteer to be in this study, you may withdraw at any time without providing any reason and without disadvantage to yourself of any kind. You also have the option of removing your data from the study. You may also refuse to answer any questions you don't want to answer and still remain in the study. The investigators may also withdraw you from the study if circumstances arise which warrant doing so.

IF I HAVE ANY QUESTIONS OR PROBLEMS, WHOM CAN I CALL?

If you have any questions about this research project now or later, you may contact any one of the investigators listed on the next page.
This study has been approved by the Sports and Health Science Ethics Committee.

Locally Responsible Investigator

Rachel Tan, B.Sc., M. Sc.
PhD Student, Sports and Health Sciences
Email: rt354@exeter.ac.uk

Appendix II

Sport and Health Sciences
College of Life and Environmental Sciences

St Luke's Campus
Heavitree Road
Exeter
Devon
Telephone: +44 (0)1392 26
Email: sshs-school-office@ex.ac.uk
Web: www.ex.ac.uk/sshs

Study: The effect of nitrate-depleted beetroot juice on the oxygen cost of moderate-intensity cycling exercise.
Principal Investigator: Rachel Tan
Researcher: Rachel Tan
Organisation: The University of Exeter
Version: #1 13/03/2019

Participant Identification Number:

Informed Consent Form for Participants

	Please initial box
1. I confirm that I have read and understand the information sheet version 1 dated 13/03/2019 for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason.	☐
3. I understand that any information given by me may be used in future reports, articles or presentations by the research team.	☐
4. I understand that my name will not appear in any reports, articles or presentations.	☐
5. I will be asked to provide 12 venous blood samples over the course of the study and I understand the blood sampling procedures.	☐
6. I understand blood samples may be transported to other laboratories within the UK or overseas for further analyses.	☐
7. I understand that I will consume 8 x 70 mL nitrate-depleted and 8 x 70 mL nitrate-rich beetroot juice shots (total of 16 supplements) over the course of the study.	☐
8. I agree to take part in the above study.	☐

Name of Participant: **Signature:** **Date:**

Researcher: Rachel Tan **Signature:**

Appendix III

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

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

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

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

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

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

If you answered YES to one or more questions

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

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

NO to all questions

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

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

DELAY BECOMING MUCH MORE ACTIVE:

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

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

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

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

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

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

NAME _____

SIGNATURE _____ DATE _____

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

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

CSEP | SCPE

College of Life and Environmental Sciences
SPORT AND HEALTH SCIENCES

St. Luke's Campus
University of Exeter
Heavitree Road
Exeter
EX1 2LU
United Kingdom

Certificate of Ethical Approval

Proposal Ref No: 171025/A/01

Title: The effect of dietary nitrate and N-acetylcysteine supplementation on exercise efficiency and tolerance during prolonged moderate-intensity exercise

Applicants: Rachel Tan, Andrew Jones, Anni Vanhatalo, Jamie Blackwell, Adam Linoby, Lee Wylie, Chris Thompson, Tuesday Platt, Felix Leonard-Fox, Joseph Home, Paul Morgan, Ida Clark

The proposal was reviewed by a Representative on the Committee.

Decision: This proposal has been approved until August 2022

Signature: Melvyn Hillsdon Date: 01/09/17

Name/Title of Ethics Committee Reviewer: Dr Melvyn Hillsdon

Your attention is drawn to the attached paper which reminds the researcher of information that needs to be observed when Ethics Committee approval is given.

Appendix VI

Sport and Health Sciences
College of Life and Environmental Sciences

St Luke's Campus
Heavitree Road
Exeter
Devon
Telephone: +44 (0)1392 26
Email: sshs-school-office@ex.ac.uk
Web: www.ex.ac.uk/sshs

Post-Procedure Care for your Biopsy Site

What are the immediate risks?

The most common problem immediately following a biopsy is pain after the local anaesthetic has worn off. This will likely wear off in a few hours to days. A small amount of bleeding may occur for 2-3 days following the procedure. Swelling and bruising may also be present, but in most cases fade in a couple of days.

What might happen later on?

Although very rare, there is always a chance of infection with any wound. This can slow the healing of the wound. However, infection can be easily prevented by following the information below. In extreme cases, numbness and swelling can persist at the site surrounding the scar.

How to care for my biopsy site

After the procedure, you will have the wound covered with sterile dressing and a bandage. The dressing should be kept in place for 3-5 days. Although waterproof, it is important that the dressing is kept as clean and dry as possible by avoiding bathing and swimming. Taking a shower is acceptable. If needed, the dressing can be changed after 3 days. You will be given extra waterproof dressing to apply yourself. Alternatively, you can return to the laboratory to have the wound re-dressed by a member of staff.
Ice will also be applied to prevent bruising and swelling. The ice should be kept on for 20-30 minutes following the procedure. Ice can be reapplied later in the day, but should be kept on for no longer than 20-30 minutes each time.
Ideally, the leg should be rested for at least 3 hours following the procedure. On your journey home you should avoid cycling and running. If possible, keep your leg raised when resting. 3-24 hours after, it is important to maintain mobility in your leg but try to avoid excessive exercise.

What If I have any questions about my wound

Please seek medical advice if you are concerned about your biopsy wound. If you are concerned about excessive pain, swelling or bleeding or have any other concerns, do not hesitate to contact the principle investigator or members of staff involved with the project you are enrolled on. Contact details should have been provided to you at the outset of the trial. Alternatively you could seek advice from your family doctor (GP), practice nurse or from your local Accident and Emergency department.

10 Appendix 2 (user and visitor safety checklist)

Magnetic environment screening form for Non-scanned Individuals

The MR system has a very strong magnetic field that may be hazardous to individuals entering the MR environment if they have certain metallic, electronic, magnetic or mechanical implants, devices or objects. Therefore all individuals are required to fill out this form before entering the scanner room. Be advised, the magnet is **always ON**.

Date:…… **Name:**……..
Contact Address:……..

1. Have you had prior surgery or an operation (e.g., arthroscopy, endoscopy, etc.) of any kind? ☐ No ☐ Yes

If yes, please indicate date and type of surgery:……

2. Have you had an injury to the eye involving a metallic object (e.g., slivers, foreign body)? ☐ No ☐ Yes

If yes, please describe:……

3. Have you ever been injured by a metallic object or foreign body (e.g. shrapnel, bullet etc.)? ☐ No ☐ Yes

If yes, please describe:……

4. Are you pregnant, or suspect that you may be pregnant? ☐ No ☐ Yes

WARNING: Certain implants, devices, or objects may be hazardous to you in the MR environment. Do not enter the scanning room if you have any question or concern regarding an implant, device or object.

5. Please indicate if you have any of the following:

☐ No ☐ Yes Aneurysm clip(s)
☐ No ☐ Yes Cardiac pacemaker
☐ No ☐ Yes Implanted cardioverter defibrillator (ICD)
☐ No ☐ Yes Electronic implant or device
☐ No ☐ Yes Magnetically-activated implant or device
☐ No ☐ Yes Neurostimulation system
☐ No ☐ Yes Spinal cord stimulator
☐ No ☐ Yes Cochlear implant or implanted hearing aid
☐ No ☐ Yes Insulin or infusion pump
☐ No ☐ Yes Implanted drug infusion pump
☐ No ☐ Yes Any type of prosthesis or implant
☐ No ☐ Yes Artificial or prosthetic limb
☐ No ☐ Yes Any metallic fragment of foreign body
☐ No ☐ Yes Any external or internal metallic object
☐ No ☐ Yes Hearing aid
☐ No ☐ Yes Other implant .....

Important Instructions

Remove all metallic objects before entering the scanner room including hearing aids, mobile phone, keys, glasses, hair pins, jewellery, watches, safety pins, paperclips, credit cards, magnetic strip cards, coins, pens, pocket knives, nail clippers, steel-toed boots/shoes and all tools. Loose metallic objects are especially prohibited within the MR environment.

I confirm the above information is correct to the best of my knowledge. I have read and understood the entire contents of this form and have had the opportunity to discuss its contents to my satisfaction.

Signature (person completing the form):…… Reviewed by:…… Signature:……

For repeated visits only:

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

Signature (person completing the form):…………………… Reviewed by:……………..…… Signature:………

11 Appendix 3 (Participant safety checklist)

Participant Safety Checklist

Name: ……..
Weight: ……..

Date of Birth: ……..
Study Name/Volunteer Number: ……

Please check the following list carefully, answering all appropriate questions.
Please do not hesitate to ask staff, if you have any queries regarding these questions.

1.Do you have a pacemaker, artificial heart valve or coronary stent? Yes☐ No☐

2.Have you ever had major surgery? Yes☐ No☐
If yes, please give brief details:

3.Do you have any aneurysm clips (clips put around blood vessels during surgery)? Yes☐ No☐

4.Do you have any implants in your body?

Yes☐ No☐ Joint replacements, pins or wires
Yes☐ No☐ Implanted cardioverter defibrillator (ICD)
Yes☐ No☐ Electronic implant or device
Yes☐ No☐ Magnetically-activated implant or device
Yes☐ No☐ Neurostimulation system
Yes☐ No☐ Spinal cord stimulator
Yes☐ No☐ Insulin or infusion pump
Yes☐ No☐ Implanted drug infusion pump
Yes☐ No☐ Internal electrodes or wires
Yes☐ No☐ Bone growth/bone fusion stimulator
Yes☐ No☐ Any type of prosthesis
Yes☐ No☐ Heart valve prosthesis
Yes☐ No☐ Eyelid spring or wire
Yes☐ No☐ Metallic stent, filter or coil
Yes☐ No☐ Shunt (spinal or intraventricular)
Yes☐ No☐ Vascular access port and/or catheter
Yes☐ No☐ Wire mesh implant
Yes☐ No☐ Bone/joint pin, screw, nail, wire, plate etc.
Yes☐ No☐ Other Implant ……..

5.Do you have an artificial limb, calliper or surgical corset? Yes☐ No☐

6.Do you have any shrapnel or metal fragments, for example from working in a machine tool shop? Yes☐ No☐

7.Do you have a cochlear implant? Yes☐ No☐

8.Do you wear dentures, plate or a hearing aid? Yes☐ No☐

9.Are you wearing a skin patch (e.g. anti-smoking medication), have any tattoos, body piercing, permanent makeup or coloured contact lenses? Yes☐ No☐

10.Are you aware of any metal objects present within or about your body, other than those described above? Yes☐ No☐

11.Are you susceptible to claustrophobia? Yes☐ No☐

12.Do you suffer from blackout, diabetes, epilepsy or fits? Yes☐ No☐

For women:

13.Are you pregnant or experiencing a late menstrual period? Yes☐ No☐

14.Do you have an intra-uterine contraceptive device fitted? Yes☐ No☐

15.Are you taking any type of fertility medication or having fertility treatment? Yes☐ No☐

Important Instructions

Remove all metallic objects before entering the scanner room including hearing aids, mobile phones, keys, glasses, hair pins, jewellery, watches, safety pins, paperclips, credit cards, magnetic strip cards, coins, pens, pocket knives, nail clippers, steel-toed boots/shoes and all tools. Loose metallic objects are especially prohibited within the MR environment.

I have understood the above questions and have marked the answers correctly.

Signature ..... Date …… MR Centre Staff Signature .....
(Participant/Parent/Guardian)

For repeated visits only:
Signature ..... Date …… MR Centre Staff Signature .....
(Participant/Parent/Guardian)

Signature ..... Date …… MR Centre Staff Signature .....
(Participant/Parent/Guardian)

Signature ..... Date …… MR Centre Staff Signature .....
(Participant/Parent/Guardian)

Study Instructions

Nitrate supplementation on muscle energetics

To ensure that we gain valid data, it is important that you follow the study procedures as described below. If you have any concerns about the following requests, please do not hesitate to contact the experimenter.

1. Throughout the duration of this study, please restrain from consuming foods high in dietary nitrate. These include; beetroot, lettuce, spinach, rocket and kale.

2. Please DO NOT use anti-bacterial mouthwash and avoid using chewing gum throughout the study duration.

3. Avoid caffeine up to 8 h and alcohol up to 24 h before all laboratory visits.

4. Please DO NOT participate in intense exercise for 24 h prior to each laboratory visit.

5. Please replicate your dietary intake and exercise habits in the 24 h preceding each laboratory visit. To help with this, you will be asked to complete a 24 h recall of your dietary intake and exercise habit. To allow the experimenter to confirm that you have replicated your diet and exercise appropriately, you will be asked to complete a further 24 h recall on all subsequent visits.

6. Your designated supplements will be provided to you in a sealed bag. Specific information on when you need to consume these supplements will be detailed on the outside of this bag. The experimenter will also explain there instructions to you verbally. If at any point you are confused about when you should consume each supplement, please do not hesitate to call the experimenter.

7. Please consume your designated supplement at the EXACT time detailed on your supplement bag.

8. Once consumed, please place your empty supplement bottle back into the supplement bag. Please bring this supplement bag with you to your next laboratory visit.

9. IMPORTANT: If at any point you experience an adverse reaction to the supplement, please contact the experimenter immediately.

Supplementation Log

The effect of dietary nitrate supplementation and N-acetylcysteine during prolonged exercise and exercise performance

Participant number ☐☐

Initials ☐☐☐

Day	Desired Time	Date	Supplementation (Tick the box)	Actual Time
1	08:00 20:00			: :
2	08:00 20:00			: :
3	08:00 20:00			: :
4	08:00 20:00			: :
5	08:00 20:00			: :
		Testing Day		
6	: :			: :

Arrive to CHERC for _____

Your exercise start time is _____

I confirm that the information above has been recorded accurately and honestly.

Signature of Participant..... Date ☐☐/☐☐/☐☐☐☐

UNIVERSITY OF EXETER

UNIVERSITY OF EXETER

Exercise record Log

The effect of dietary nitrate supplementation and N-acetylcysteine during prolonged exercise and exercise performance

Subject Number ☐☐ **Date** ☐☐/☐☐/☐☐☐☐

Date	Time	What Exercise Have You Done in the last 24 hours? (Please provide approximate intensity and duration)
		What? How Long? Intensity? Low / Moderate / High / Very high
		What? How Long? Intensity? Low / Moderate / High / Very high
		What? How Long? Intensity? Low / Moderate / High / Very high

I confirm that the exercise information above has been recorded accurately and honestly.

Name of Participant .....

Signature of Participant ..... Date ☐☐/☐☐/☐☐☐☐

Name of Researcher .....

Signature of Researcher ..... Date ☐☐/☐☐/☐☐☐☐

Appendix X

Dietary record Log

The effect of nitrate-depleted beetroot juice on the oxygen cost of moderate-intensity exercise

Subject Number ☐☐

24h before testing day

Please choose a meal you can easily replicate 24h prior to each lab visit

Date	Meal Breakfast(B) / Lunch(L) / Dinner(D) / Snack(S)	Time	What Was Consumed in the last 24 hours? (Please provide approximate weight)
	Breakfast		
	Lunch		
	Dinner		
	Snack		

I confirm that the dietary information above has been recorded accurately and honestly.

Name of Participant .....

Signature of Participant ..... Date ☐☐/☐☐/☐☐☐☐

Name of Researcher .....

Signature of Researcher ..... Date ☐☐/☐☐/☐☐☐☐

Appendix XI

Pre-test meeting: participant screening

The effect of nitrate-depleted beetroot juice on the oxygen cost of moderate-intensity exercise

Participant name .....

DOB / /

Age

Height . cm

Weight . kg

Would you consider yourself as highly-trained? Yes ☐ No ☐

If yes describe here:

Do you have any food allergies? Yes ☐ No ☐

If yes describe here:

Have you recently been using any dietary supplements regularly? Yes ☐ No ☐

If yes describe here:

Have you had, or do you have, a blood-borne infection, e.g. hepatitis/HIV? Yes ☐ No ☐

Appendix XI

Have you donated blood in the last 3 months?	Yes	☐	No	☐
Are you a smoker?	Yes	☐	No	☐
Are you currently participating in any other study?	Yes	☐	No	☐

If yes describe here:

Are you taking any hyper or hypotension medication?	Yes	☐	No	☐

If yes describe here:

Within the last 2 years have you taken any illegal substances?	Yes	☐	No	☐

If yes describe here: