

THE UNIVERSITY OF HULL

**The cardiovascular effects of watercress on human health and
performance**

being a thesis submitted for the degree of Doctor of Philosophy
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by

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Abstract

The purpose of this thesis is to assess an alternative dietary nitrate supplement as a possible ergogenic aid and to evaluate its therapeutic potential upon cardiovascular markers in healthy young and old age populations. The first experimental chapter of this thesis (Chapter 4) aimed to investigate the efficacy of an alternative dietary nitrate supplement. Watercress juice was compared to beetroot juice, a well-established, commercially available, dietary nitrate supplement, to sodium nitrate and to water, acting as control. The sodium nitrate condition would enable further insights into the mechanism of nitrate rich supplements. The first study aimed to investigate the acute effects of nitrate supplements on cardiovascular and oxidative/nitrosylative stress parameters at rest and following a 16.1 km cycling time trial on a SRM cycle ergometer (16.1 km TT). Participants (n=9) were randomly assigned to consume nitrate matched supplements and water. Plasma nitrate concentration significantly increased at 120 mins post-ingestion for the dietary nitrate conditions, ($P < 0.05$). However, plasma nitrite concentrations did not significantly increase, ($P > 0.05$). Watercress supplementation demonstrated a 1.4% performance improvement compared to the water trial and the fastest time (1655.1 (110.2) secs). Compared with beetroot juice (1673.8 (96.0) secs), sodium nitrate (1685 (102.3) secs) and water (1677.9 (129.8) secs). Interestingly, sodium nitrate observed the slowest time. The difference between trials were not significantly different ($P = 0.31$). However, a critical improvement in performance has been reported small as 0.5 - 1.5 % (Paton & Hopkins, 2006). Using this criterion, it is possible that our findings may be of real-world relevance. Watercress supplementation was the only supplement to indicate an increase in forearm blood flow (+0.18 (0.03) %/min), from baseline to 120 mins post-ingestion but this was not significant ($P = 0.73$). Beetroot decreased but was equivocal (-0.88 (0.30) %/min) ($P = 0.25$). Sodium nitrate tended to decrease (-0.60 (0.04) %/min) ($P = 0.07$) and water significantly decreased (-0.61 (0.01) %/min) ($P = 0.03$). These results suggest that there is potentially a synergistic effect between the nitrate content and other bioactive components in watercress or there is an effective bioactive component that is not nitrate which may have enhanced cycling performance in a young healthy physically active population.

Assessing nitrate supplementation in a cardiovascular compromised population, rather than young, physically active volunteers may provide further mechanistic insights. Therefore, the aim of the second experimental Chapter 5 was to investigate the efficacy

of watercress supplementation on cardiovascular and oxidative/nitrosylative stress markers in a normoxic and hypoxic setting in a healthy, physically active, aged population. Participants (n=9) were randomly assigned to consume watercress supplement and water (control) in normoxic and normobaric hypoxic environment. Oxygen saturation significantly decreased ($P < 0.05$) in hypoxic environments; the control condition at 30 mins (-6 (2.4) %) and 120 mins post-ingestion (-6 (3.2) %) and watercress at 30 mins (-4 (2.4) %) and 120 mins (-2 (2.3) %) post-ingestion. Plasma nitrate concentration significantly increased ($P < 0.05$) in the watercress normoxic condition at the 30 mins (+14.2 (6.7) $\mu\text{mol/L}$) and 120 mins post-ingestion (+13.8 (5.7) $\mu\text{mol/L}$); and in the watercress hypoxic condition at the 30 mins (+12.3 (5.9) $\mu\text{mol/L}$) and 120 mins post-ingestion (+8.4 (9.7) $\mu\text{mol/L}$). However, plasma nitrite concentration did not change ($P > 0.05$). There were no differences in vasodilatory or oxidative/nitrosylative stress measures. Acute watercress supplementation containing 400 mg of nitrate does not improve vascular function or attenuate oxidative stress biomarkers in a healthy aged population at rest under normoxia or hypoxia.

With the substantial variation seen in a range of measures in Chapters 4 and 5. The purpose of the third experiment, Chapter 6, was to assess the error of measurement to support the interpretation of the previous findings. Therefore, the aim of this work was to investigate the repeatability of forearm blood flow, 16.1 km cycling TT performance on a SRM cycle ergometer (one week apart) and the change in oxidative/nitrosylative stress markers at baseline and after the TT, in a healthy, physically active, male cohort in normoxic conditions. There was limited difference between 16.1 km TT performance times and the coefficient of variation was low 1% (90% CI; 0.7% -1.9%). Forearm blood flow observed a high coefficient of variation of 39.3% (90% CI; 31.6 – 99.5%). Baseline and post TT for both oxidative/nitrosylative stress markers reported a large variation. For example, baseline protein carbonyls concentration across the two visits showed a high coefficient of variation of 65.5% (90% CI; 43.6 - 136.7%). Therefore, the primary findings from this investigation suggest that a 16.1 km TT, on a SRM cycle ergometer in a physically active, male cohort, is highly repeatable. The low CV suggests that this test can enable sports scientists and trainers to better comprehend factors (e.g. ergogenic aids) that may influence cycling performance. In contrast, the repeatability of forearm blood flow was high and oxidative/nitrosylative stress markers, one week apart, demonstrated a high variability. Therefore, using these markers in a repeated measures research design should be used with caution.

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I dedicate this thesis to my family and Tom.

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Preface

Conference Presentations

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List of Abbreviations and Symbols

ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
BP	Blood pressure
BH ₄	Tetrahydrobiopterin
BMI	Body mass index
cGMP	Cyclic guanylate monophosphate
CI	Confidence Interval
cm	Centimetre
COV	Coefficient of Variation
CO ₂	Carbon dioxide
CVD	Cardiovascular disease
dL	Per decilitre
DNPH	Dinitrophenylhydrazine
EDTA	Di-potassium ethylene diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPR	Electron paramagnetic resonance
FAD	Flavin adenine dinucleotide
FMD	Flow-mediated dilation
FOX-1	Ferrous oxidation-xylenol orange
g	Grams
HR	Heart rate
iNOS	Inducible nitric oxide synthase
kg	Kilograms
Km	Kilometre
M	Molar
Mg	Milligrams
Min	Minute
ml	Millilitre
mmHg	Millilitre of mercury
mmol	Millimole
n	Number of participants
NADPH	Nicotinamide adenine dinucleotide phosphate
ng	Nanograms
nM	Nanomolar
nNOS	Nitric oxide synthase
NO ₃	Nitrate
NO ₂	Nitrite
NO	Nitric Oxide
NOS	Nitric oxide synthase
O ₂	Oxygen
PBS	Phosphate-buffered saline
PCr	Phosphocreatine concentration

Pi	Inorganic phosphate
PPO	Peak power output
RONs	Reactive oxygen species and reactive nitrogen species
RPE	Rate of perceived exertion
s	Seconds
SD	Standard deviation
SRM	Schoberer Rad Mebtechnik (Cycle Ergometer)
SST	Serum separation tubes
TBARS	Thiobarbituric reactive substances
TT	Time Trial
VO ₂	Oxygen uptake
VOP	Venous occlusion plethysmography
VO _{2peak}	Peak oxygen uptake
V _s	Stroke volume
W	Watt
Yrs	years
μl	microlitre
μM	micromolar
°C	degrees Celsius
%	Percentage
\dot{Q}	Cardiac output

Chapter 1: Introduction

1.1. Introduction

Cardiovascular diseases (CVD) remain the leading cause of death worldwide (Fleg, Aronow, & Frishman, 2011; Heidenreich et al., 2011). Ageing constitutes for the most significant risk factor of CVD (Go et al., 2013). Suggesting that most forms of CVDs are pathologies associated with age and the ageing process results in dysfunctional arteries leading to an increased risk of CVD (Go et al., 2013; Lakatta, 2002; Lonn et al., 2010; Najjar, Scuteri, & Lakatta, 2005; North & Sinclair, 2012). By 2030 almost 20 % of the populace will be aged 65 or older (Statistics, 2017). This means 40 % of deaths in this age group will be the result of CVDs, ranking CVD as the leading cause of mortality (Statistics, 2017). Additionally, the cost to treat cardiovascular disease will triple in that time (Fleg, Aronow, & Frishman, 2011; Heidenreich et al., 2011).

The beginning and progression of CVD is heavily dependent on the dynamic changes in vascular biology (Deanfield et al., 2007). The endothelium is a key regulator in this process and precedes the atherosclerotic development and incidence of lesions and later clinical complications (Ross, 1993). The two most significant alterations, the stiffness of larger elastic arteries (the carotid and aorta) and vascular endothelial dysfunction, contribute to one and other, with insufficient nitric oxide being a critical feature linking both (Soucy et al., 2006; Wilkinson et al., 2004).

Nitric oxide plays a pertinent role in the regulation of cardiovascular function; by reducing blood pressure, improving blood flow, enhancing mitochondrial function, diminishing cellular vascular adhesion, improving contractility of muscle, neurotransmission, and the homeostasis of glucose and calcium (Bryan, 2006; Dejam, Hunter, Schechter, & Gladwin, 2004; Kunz, 2000; Larsen et al., 2011; Stamler & Meissner, 2001; Webb et al., 2008). Paradoxically, nitric oxide is a highly reactive free radical that can be reduced or oxidised to produce various nitrogen species (Mayer &

Hemmens, 1997). Therein lies the complexity of nitric oxide functions, with the potential to react with other biomolecules (Rodriguez-Manas et al., 2009). Despite this, nitric oxide plays an essential protective role in the cardiovascular system by maintaining vessel wall homeostasis, with research reporting a decrease in nitric oxide availability contributing to pathologies including diabetes, hypertension, kidney failure or chronic heart failure (Vanhoutte, Shimokawa, Tang, & Feletou, 2009).

During the ageing process nitric oxide synthesis via the endogenous nitric oxide synthase (NOS) pathway is reported to be reduced (Delp, Behnke, Spier, Wu, & Muller-Delp, 2008; Sindler et al., 2011). This is attributed collectively to the lower availability of L-arginine and specifically the NOS cofactor tetrahydrobiopterin (BH₄), and the decrease in plasma nitrate and nitrite levels in older populations (Delp, Behnke, Spier, Wu, & Muller-Delp, 2008; Sindler et al., 2011). Moreover, the increased production of superoxide with ageing lowers nitric oxide bioavailability, this is due to the swift reaction between superoxide and nitric oxide to form peroxynitrite (Kang, Reyes, & Muller-Delp, 2009).

Initially, it was assumed NOS was the only pathway for nitric oxide synthesis *in vivo* and was considered a one-way reaction, with nitrate and nitrite assumed to be end-products of the nitric oxide metabolism. It has been recently discovered dietary nitrate acts as a precursor to nitric oxide independent of the L-arginine NOS pathway (Lundberg et al., 2009). This discovery has received a great amount of attention over the past few years, especially since it has become apparent nitrite and nitrate are physiologically recycled in blood and tissue to form nitric oxide and other bioactive nitrogen oxides (Celermajer et al., 1994; Fagard, 2002; Ignarro et al., 1999; Lakatta & Levy, 2003; Lauer et al., 2001; Lundberg & Govoni, 2004).

Nitrate, abundant in our everyday diet, has transpired as an option to replenish nitric oxide bioavailability through the reduction from nitrate to nitrite to nitric oxide (Rammos et al.,

2016; Siervo, et al., 2018). Research has demonstrated nitrate supplementation to increase blood flow, reduce blood pressure, reduce the oxygen cost of exercise and improve exercise tolerance, inhibit platelet aggregation, and reduce thrombotic events (Bailey et al., 2009; Larsen et al., 2007; Lidder & Webb, 2013; Park et al., 2013; Richardson et al., 2002; Webb et al., 2008). Partaking in physical activity and diet are amongst many factors that can affect a person's quality of life and health. As a result of this, a substantial amount of contemporary research is concentrated on the use of nutritional interventions to enhance health and exercise capacity. This discovery of dietary nitrate offers a feasible and cost-effective method to increase nitric oxide bioavailability and to prevent/attenuate cardiovascular dysfunction (Lutz et al., 2008; Rammos et al., 2016).

The majority of positive nitrate evidence stems from complex sources such as, beetroot juice, which is full of active constituents (fibre, antioxidants and minerals) (Bailey et al., 2009; Bailey et al., 2010; Cermak, Gibala, & van Loon, 2012; Lansley et al., 2011; Larsen et al., 2007; Masscheliem et al., 2012). Beetroot has garnered considerable attention due to its beneficial effects on vascular function and physical performance enhancement (Bailey et al., 2009; Bailey et al., 2010; Cermak, Gibala, & van Loon, 2012; Lansley et al., 2011; Larsen et al., 2007; Masscheliem et al., 2012). However, green leafy vegetables are also rich sources of nitrate, such as rocket, watercress and spinach (> 250 mg nitrate/100 g) (Hord et al., 2009; Lidder & Webb, 2013). Watercress is high in nitrate and has been reported to have anti-genotoxic and anti-inflammatory properties due its high content of bioactive compounds, thus making it an appealing potential nitrate supplement (Boyd et al., 2006; Gill et al., 2007; Fogarty et al., 2013).

1.2. Aims

The purpose of this thesis is to provide novel insight into the efficacy of an alternative nitrate-rich supplement; watercress juice, compared to a well-established, commercially available, dietary nitrate supplement; beetroot juice, both compared to sodium nitrate in order to gain mechanistic insights into the of action of nitrate food sources. This thesis was designed to assess the acute consumption of an alternative nitrate supplement as a possible ergogenic aid and to evaluate its therapeutic potential upon cardiovascular markers in healthy young and old age populations.

Thus, the overarching objective of this thesis was to investigate whether acute nitrate supplementation, can enhance cardiovascular parameters and alter nitrosylative and oxidative stress markers. Specifically, this thesis addressed:

Study 1. Can nitrate-rich supplements enhance time trial performance, vasodilatory variables and alter oxidative/nitrosylative stress markers, in comparison to sodium nitrate or water in a young, healthy, physically active, male cohort?

Study 2. Can watercress juice enhance vasodilatory variables, alter oxidative/nitrosylative stress biomarkers in normoxic or hypoxic environment, in healthy, physically active, older cohort?

Study 3. What is the repeatability of measures used in this thesis in order to understand the impact of inter and intra-individual variation in the studies in this thesis including; forearm blood flow prior to exercise using venous occlusion plethysmography, 16.1 km TT performance and the baseline and change in oxidative/nitrosylative stress markers after TT performance, in a young, healthy, physically active, male cohort?

Chapter 2: Literature Review

2.1. Ageing

In broad terms, ageing is the progressive manifestation of a variety of changes at the molecular and macromolecular level over time (Santos, Sinha, & Lindner, 2018). It is a process characterised by a gradual decline in the regulation of cellular function and integrity, consequently leading to a build-up of damaged by-products (Santos et al., 2018; Siervo et al., 2018). The course of ageing is complex as it has an accumulation of several confounding variables, such as socioeconomic status, physical characteristics, environmental and lifestyle factors (Davalli, Mitic, Caporali, Lauriola, & D'Arca, 2016). The biological mechanisms contributing to the ageing process is not fully understood. However, the most common stressors to cause damage include oxidative stress and inflammation (Lopez-Otin, Blasco, Partridge, Serrano, & Kroemer, 2013).

In industrialised countries, there is a diminishing rate of birth and mortality with a simultaneous growth in the elderly population (Davalli et al., 2016). For example, in 2006 in the United Kingdom, 15.9% of the population constituted in adults aged 65 years and above (Statistics, 2017). It is projected by 2046 this percentage will increase to 24% (Statistics, 2017). With the rise in the number of people over 65, there is a concomitant rise with age-related health issues, and a simultaneous rise in medical costs and economic pressure on governments and care systems (Age, 2017). In this growing age group, cardiovascular disease (CVD) will continue to be the primary cause of mortality, resulting in 40% of all deaths (North & Sinclair, 2012).

In all tissues, ageing is associated with functional and structural changes (Siervo, Lara, Ogbonmwan, & Mathers, 2013). These types of alterations increase risk of CVD regardless of other risk factors such as diabetes, hypertension or hypercholesterolaemia (Siervo et al., 2018). Ageing constitutes the most significant risk factor of CVD (Go et al., 2013). Suggesting that most forms of CVDs are pathologies associated with age and

the ageing process and results in dysfunctional arteries leading to an increased risk of CVD (Go et al., 2013; Edward G Lakatta, 2002; Lonn et al., 2010; Najjar et al., 2005; North & Sinclair, 2012).

Given the projected increase in the number of older adults and the associated increase in CVD, it is imperative to understand the mechanisms by which these alterations are mediated. From this, research can focus on strategies that may attenuate an ageing vascular system. Theoretically, this could reduce the CVD risk, improve quality of life of the elderly and alleviate pressure on healthcare systems.

2.1.1. Cardiovascular disease

CVD circumscribes all diseases of the cardiovascular system and is characterised by a number of pathogenesis including genetics, lifestyle, physical activity and diet (Rehabilitation, 2012; Siervo et al., 2018). The basic tenet of CVD encompasses damage and dysfunction of blood vessels that can obstruct blood flow and oxygen, vital to the organs and tissues (Seals, Jablonski & Donato, 2011). This occurs secondary to the thickening of the blood vessel wall and development of plaque causing narrowing and stiffening of arteries, also known as atherosclerosis (Gimbrone & García-Cardena, 2016).

Over the last 50 years, research has demonstrated that the beginning and progression of the CVD is heavily dependent on the dynamic changes in vascular biology (Deanfield, Halcox, & Rabelink, 2007). During the ageing process many changes take place to the arteries contributing to the risk of CVD (Lakatta, 2002). The two main significant alterations are; the stiffening of the large elastic arteries (the carotid and aorta) and the progression of endothelial dysfunction (Lakatta & Levy, 2003; Oliver & Webb, 2003; Seals et al., 2011; Widlansky, Gokce, Keaney, & Vita, 2003). The reduction and degradation of nitric oxide bioavailability is a critical feature linked to these alterations

during the process of ageing (Soucy et al., 2006; Wilkinson, Franklin, & Cockcroft, 2004).

2.1.2. Large elastic artery stiffness

The stiffening of large elastic arteries is due to both structural and functional influences (Lakatta & Levy, 2003; Najjar et al., 2005; Wilkinson et al., 2004). Research suggests, structurally, there is an increase in collagen that contributes to arterial stiffness and a decrease in elastin, a highly elastic protein (Lakatta & Levy, 2003). It is postulated that cytokine growth factor-beta may play a significant part in signalling collagen synthesis with ageing and therefore increasing the risk of CVD (Rodriguez-Manas et al., 2009). Furthermore, with age there is an increase in glucose derived molecules that cross-link with structural proteins in the arterial wall adding to arterial stiffness, called advanced glycation end products (Basta, Schmidt, & De Caterina, 2004; Greenwald, 2007). Arterial stiffness is functionally affected by factors that modify vascular smooth muscle tone, including paracrine molecules, of which nitric oxide synthesis from the endothelium is among the utmost vital (Soucy et al., 2006; Weiss & Ellis, 1933; Wilkinson et al., 2004).

2.1.3. The endothelium

The endothelium is paramount in regulating the health and function of vascular biology. Under basal conditions, the endothelium preserves the vessel in a moderately neutral state, facilitating dilation over vasoconstriction. It consists of a single layer of cells lining the interior wall of blood vessels, dividing the circulating blood from the smooth muscle cells (Siervo et al., 2018). The function of the vascular endothelium is beyond a conservational barricade, it is now recognised for its vital paracrine, autocrine and endocrine functions (Sena, Pereira, & Seica, 2013). Maintaining a haemostatic balance between anticoagulation and thrombosis and regulation of vascular tone and homeostasis (Brandes, Fleming, & Busse, 2005; Hobbs, George, & Lovegrove, 2013). This monolayer

of cells contributes to the inhibition of platelet aggregation and leukocyte adhesion, mediated by nitric oxide (Galley & Webster, 2004; Hoak, Czervionke, Fry, Haycraft, & Brotherton, 1981; Hobbs, George, et al., 2013). Vascular tone is regulated by the generation of vasodilators (endothelium-derived hyperpolarising factor, acetylcholine-induced, nitric oxide and prostacyclin), and vasoconstrictors (PGH₂, thromboxane A₂, and endothelin 1), circulated in response to nearby physical stimuli to provide adequate perfusion pressure to specific organs (Hobbs, George, et al., 2013). The endothelium dependent dilation is primarily adjusted proportionate to shear stress by nitric oxide (Siervo et al., 2018). Nitric oxide is synthesised by the L-arginine -eNOS pathway leading to the production of intracellular cGMP (Siervo et al., 2018). If compromised it is thought vasodilation can be mediated by cytochrome-derived factors, natriuretic peptide, and prostacyclin I₂ (Siervo et al., 2018). Other endothelium functions include; fibrosis, governance of angiogenesis, wound healing and smooth muscle cell proliferation (Widmer & Lerman, 2014). Factors that constructively affect the endothelium function are favourable lifestyle habits such as increased exercise, a diet incorporating anti-oxidant and anti-inflammatory foods, and pharmaceutical agents.

When endothelial dysfunction is present, the ability to perform one or more of these functions becomes challenging (Rajendran et al., 2013; Sindler, DeVan, Fleenor, & Seals, 2014). Endothelial dysfunction associated with ageing, is classically signified by impaired endothelial dependent dilatation and is mediated by a decrease or degradation in nitric oxide. (Lüscher & Barton, 1997; Seals, Jablonski, & Donato, 2011; Taddei et al., 2001). Endothelial dysfunction can be defined as any functional alteration from the normal phenotype of the endothelium (Deanfield et al., 2007; Endemann & Schiffrin, 2004). It is broadly characterised as a disruption in the anticoagulant, anti-proliferative, anti-inflammatory and vasodilatory function of the endothelium. Defects in endothelium function usually result from a reduction in nitric oxide bioavailability due to increased

oxidative or nitrosative stress and decreased antioxidant enzyme activity (Puddu, Cravero, Arnone, Muscari, & Puddu, 2005). Endothelial dysfunction augments the adhesion of leucocytes and platelets to the vessel wall (Drexler & Hornig, 1999). It also activates cytokines enhancing the permeability of the wall to inflammation mediators and oxidised lipoproteins (Drexler & Hornig, 1999). This causes damage of the vessel wall with atherosclerotic plaque generation and smooth muscle cell proliferation (Drexler & Hornig, 1999).

Endothelial dysfunction is a central mediator in the process of atherosclerosis and precedes atherosclerotic plaques or cardiovascular events (Deanfield et al., 2007; Seals et al., 2011). It is detected in a number of forms of CVD and is associated with major CVD risk factors (*i.e.* age, hypertension, diabetes, chronic inflammation, obesity, smoking and hypercholesterolaemia) (Bonetti, Lerman, & Lerman, 2003; Vita & Keaney 2002; Widlansky et al., 2003). It is recognised as a significant antecedent of clinical arterial diseases and serves as a marker for assessing the risk of CVD (Widlansky et al., 2003). Considering its fundamental role in the development of CVDs (cerebrovascular, coronary and peripheral artery diseases), endothelial dysfunction is regarded as a crucial therapeutic target for reducing risk of CVDs and mortality (Widlansky et al., 2003).

2.1.4. Clinical assessment of endothelial function

Endothelial dysfunction associated with ageing, is classically signified by impaired endothelial dependent dilatation and is mediated by a decrease in nitric oxide bioavailability (Lüscher & Barton, 1997; Seals et al., 2011; Taddei et al., 2001). There are numerous techniques to assess endothelial function *in vivo* (Donald et al., 2006) and different methods examine different vascular beds and have their own advantages and disadvantages (Donald et al., 2006). However, the general principle is similar across all practices: healthy arteries vasodilate in response to reactive hyperaemia or after the

infusion of a pharmacological agonist such as acetylcholine. In disease conditions, the endothelium-dependent dilatation is decreased or absent.

Endothelial function can be measured in the coronary circulation by angiography, this technique assesses the change in the diameter in reaction to the intracoronary infusion of acetylcholine (Ludmer et al., 1986). In healthy vessels, the acetylcholine causes endothelium-dependent vasodilation by increasing nitric oxide production. However, if endothelial dysfunction is present this process is blunted (Ludmer et al., 1986). This method is invasive, expensive, requires expertise and is overall inaccessible. To overcome this, non-invasive measurements assessing endothelial function have been developed.

Flow-mediated dilation (FMD), involves measuring the brachial artery's diameter before and after reactive hyperaemia using non-invasive ultrasound. The reactive hyperaemia increases shear stress, signifying arterial endothelial nitric oxide release (Celermajer et al., 1992). A relationship is shown between FMD measures and measures of coronary endothelial dysfunction (Anderson et al., 1995). While this measure has been widely used, there are reservations concerning the repeatability and reproducibility, as the technique requires expertise and the equipment is expensive (Anderson et al., 1995).

Recently, finger plethysmography has gained increasing attention (Kuvin et al., 2003). Endothelial function is measured using peripheral arterial tonometry, similar to strain gauge venous occlusion plethysmography (VOP), endothelium-mediated changes in vascular tone after occlusion of the brachial artery are reflected by a subsequent hyperaemic response, this determines arterial endothelial function. The device can measure observer independent pulsatile arterial volume changes by finger plethysmographic pressure alterations caused by the arterial pulse and this translates to peripheral arterial tone (Kuvin et al., 2003). A main advantage in this technique is the control for simultaneous non-endothelium-dependent changes in vascular tone by measuring the opposite arm. Although this index is validated, the augmented pulse

amplitude response to reactive hyperaemia is complex and is only partly dependent on nitric oxide (Nohria et al., 2006). As the device reflects adjustment in flow, along with micro vessel dilatation (Nohria et al., 2006). Results for both plethysmography techniques are difficult to standardise due to testing protocols and set-up differing between research studies (Deanfield et al., 2007).

Another technique assessing endothelial dysfunction is called laser Doppler imaging with iontophoresis. This technique measures the peripheral microcirculation, with the amount of endothelial dysfunction present in the microcirculation to be comparable to that occurring in the coronary arteries (Stehouwer, 1999). Two different types of vasoactive agents (acetylcholine – endothelium-dependent vasodilator and sodium nitroprusside – endothelium-independent vasodilator) are transdermally delivered to measure the reaction of the cutaneous blood vessels by iontophoresis. A decreased local vasodilatory response to acetylcholine indicates endothelial dysfunction (Ramsay, Ferrell, Greer, & Sattar, 2002). This technique is advantageous as it is less technically demanding compared with the FMD.

A key alteration in ageing and CVD is arterial stiffness, which in part depends on smooth muscle tone. Pulse wave velocity measures arterial stiffness directly by calculating sequential recordings of pressure waveforms from the femoral and carotid arteries using applanation tonometry. The arterial pressure waveform shape indicates a quantification of arterial stiffness and is assessed non-invasively by pulse wave analysis (O'Rourke & Gallagher, 1996). Arterial stiffness has been indicated to be associated to brachial artery FMD, signifying a role of arterial stiffness in the quantification of endothelial function (Nigam, Mitchell, Lambert, & Tardif, 2003). Although arterial stiffness and endothelial function represent separate aspects of CVD, it is probable that the pathophysiological processes are interconnected (Hobbs, George, et al., 2013).

Detection of forearm, calf and thigh blood flow can be measured by strain-gauge VOP, non-invasively. This method is practical and a low-cost tool to assess blood flow. The VOP quantifies the changes in forearm blood flow in response to agonists that stimulate nitric oxide production (Wilkinson & Webb, 2001). This method has been implemented in acute intervention studies when repeated measures are required (Wilkinson & Webb, 2001). The technique is suitable to quantify differences in blood flow to a variety of inhibitors or stimuli in an individual patient. However, due to differing arterial pressure, environmental influences, different sizes of the forearm and other elements, comparisons between groups have limited value (Benjamin et al., 1995). Repeatability is one of the major limitations with this technique (Benjamin et al., 1995). This technique was used throughout this thesis as it was safe, cheap and non-invasive (Wilkinson & Webb, 2001).

The capacity of these methods to detect endothelial dysfunction prior to CVD manifesting makes the techniques appealing clinical tools for prevention and rehabilitation. Endothelial function testing reflects important vascular biology, however, the current available tests are too expensive, difficult and variable for practice in a clinical setting.

2.1.5. Nitric oxide

Nitric oxide is a free radical, with a short half-life, and is involved in multifarious roles within the human body, including cell signalling and regulatory functions (Channon, 2006). Nitric oxide plays a pertinent role in the regulation of cardiovascular function, including, blood flow, mitochondrial respiration, contractility of muscle, neurotransmission, and the homeostasis of glucose and calcium (Bryan, 2006; Dejam, Hunter, Schechter, & Gladwin, 2004; Stamler & Meissner, 2001; Webb et al., 2008). Paradoxically, nitric oxide is a highly reactive free radical that can be reduced or oxidised to produce various nitrogen species (Mayer & Hemmens, 1997). Therein lies the complexity of nitric oxide functions, with the potential to react with other molecules (Rodriguez-Manas et al., 2009). Despite this, nitric oxide plays an essential protective

role in the cardiovascular system by maintaining vessel wall homeostasis, with research reporting a decrease in nitric oxide availability contributing to pathologies including diabetes, hypertension, kidney failure or chronic heart failure (Vanhoutte, Shimokawa, Tang, & Feletou, 2009).

L-arginine is oxidised in a reaction catalysed by a family of nitric oxide synthase (NOS) enzymes (Moncada & Higgs, 1993). There are three isoforms of this enzyme: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). The production of nitric oxide is triggered by mechanical stimulus of the vessel wall or release of agonists such as, acetylcholine and bradykinin (Deanfield et al., 2007). Endothelium derived nitric oxide is one of the most essential molecules produced to help maintain vascular homeostasis (Siervo et al., 2018). The NOS-dependent pathway is displayed in Figure 2.1 (Lundberg, Weitzberg, Lundberg, & Alving, 1994; Moncada & Higgs, 1993). The reaction requires a number of co-factors and substrates including BH₄, oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), haem, flavin adenine dinucleotide (FAD) and calmodulin (Alderton, Cooper, & Knowles, 2001). A decreased number of any of these substrates or co-factors can reduce nitric oxide production via this NOS specific pathway (Alderton et al., 2001; Crabtree, Tatham, Hale, Alp, & Channon, 2009). This reduction in bioavailability is associated with CVD, and a reduced tolerance to exercise (Seals et al., 2011; Wu & Meininger, 2009). Nitric oxide diffuses into vascular smooth muscle cells activating the nitric oxide- cGMP-protein kinase G signalling pathway initiating vascular relaxation and dilation (Forstermann, Mulsch, Bohme, & Busse, 1986).

Nitric oxide plays an essential role in the prevention of thrombosis by reducing platelet activation, adhesion and agonist induced secretion and prevents the binding of fibrinogen (Loscalzo, 2001). This is thought to be in part due to the cGMP-mediated mechanism (Loscalzo, 2001). Nitric oxide also inhibits leucocyte adhesion and prevents injury derived by intimal proliferation and migration of smooth muscle cells (Hobbs, George, et

al., 2013). When the bioavailability of nitric oxide is reduced, these favourable effects are lost and endothelial dysfunction is predominant (Hobbs, George, et al., 2013).

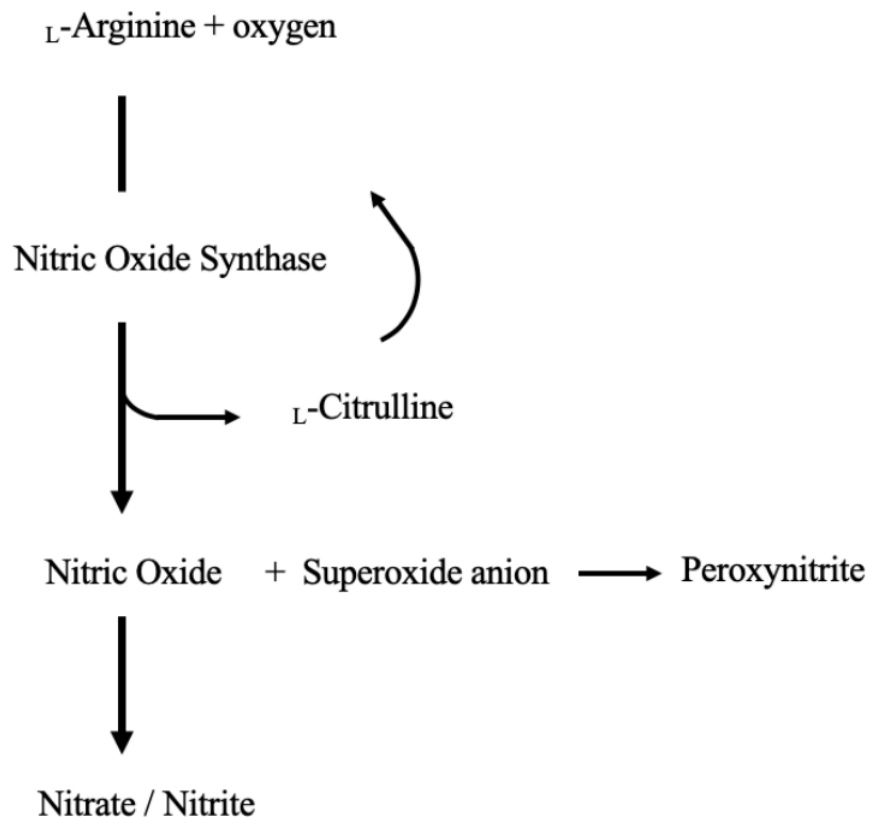


Figure 2.1.1. The NOS- dependent pathway (Sindler et al., 2013). L-arginine and oxygen produce nitric oxide in a reaction catalysed by the NOS enzymes. Note, L-citrulline is coproduced with nitric oxide in this reaction before being effectively recycled into L-arginine. After its production nitric oxide can be reduced to nitrite and nitrate.

2.2. Mechanisms of ageing-induced nitric oxide insufficiency

Research has demonstrated a reduction in nitric oxide bioavailability and eNOS expression with age (Chinellato et al., 1991; Csiszar et al., 2002; Tanabe et al., 2003; Tschudi et al., 1996). Various factors can alter nitric oxide synthesis and degradation, and in turn affect the endothelium function. In terms of ageing, oxidative stress is the primary mechanism for reduction in nitric oxide bioavailability (Eskurza, Monahan, Robinson, &

Seals, 2004; Seals et al., 2011; Siervo et al., 2018; Taddei et al., 2001). The increased oxidative stress stimulates pro-inflammatory signals altering cellular oxidant production contributing towards endothelial dysfunction (Ushio-Fukai et al., 1999).

2.2.1. Oxidative stress

Oxidative stress is defined as a disturbance in the balance between reactive oxygen species relative to antioxidants defences, leading to an extreme oxidising environment (MacRae & Mefferd, 2006). Reactive oxygen species and reactive nitrogen species (RONS) such as superoxide, hydroxyl radical, hydrogen peroxide and nitric oxide, play an important role in various processes such as cell signalling, immunological response, initiation of apoptosis, gene expression, triggering of antioxidant and repair processes (Baker, Hayden, & Ghosh, 2011; Knight, 2000; Powers, Nelson, & Hudson, 2011; Radak, Chung, & Goto, 2005; Vollaard, Shearman, & Cooper, 2005). However, RONS must be kept in balance as certain radical species may disrupt cellular homeostasis (Halliwell & Gutteridge, 2015). The cellular antioxidant defence system regulates RONS stringently by suppressing RONS harmful effects and maintaining the balance of oxidant species (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012). Key antioxidant enzymes include superoxide dismutase, glutathione peroxidase and catalase (Birben et al., 2012). However, if the production of RONS is greater than the antioxidant capacity this may cause oxidative stress (Birben et al., 2012). RONS can initiate cellular tissue injury by altering macromolecules such as lipids, proteins and DNA, resulting in oxidation of these macromolecules (Finaud, Lac, & Filaire, 2006; Sies, 2015; Sies, Schewe, Heiss, & Kelm, 2005). This compromises cell health by causing damage to cell components and initiating a number of cellular responses (Finkel & Holbrook, 2000; Lobo, Patil, Phatak, & Chandra, 2010). This disrupts homeostasis and threatens integrity in cellular, organic and systemic levels (Finkel & Holbrook, 2000; Lobo, Patil, Phatak, & Chandra, 2010). Factors that may influence the level of stress are; the species of RONS produced, cell type

and the duration of oxidant production (Finkel & Holbrook, 2000). RONS are involved in most human pathologies, such as CVD, neurodegenerative diseases and cancer and plays a role in the ageing process (Finkel & Holbrook, 2000; Halliwell & Gutteridge, 2015).

In terms of vascular ageing, numerous studies have reported an increased accumulation of superoxide in the vasculature and superoxide is recognised to promote impaired relaxation (Blackwell et al., 2004; Csiszar et al., 2002; Hamilton, Brosnan, McIntyre, Graham, & Dominiczak, 2001). As humans age, research has observed the cellular anti-oxidative defence system diminishes over time, in contrast to this, studies have shown free radical scavenging enzymes to improve endothelium dependent responses (Blackwell et al., 2004; Csiszar et al., 2002; Hamilton et al., 2001). Augmented production of superoxide by oxidant enzymes and decreased expression/action by endogenous antioxidant enzymes contribute to oxidative stress (Finkel & Holbrook, 2000). This stress reduces the bioavailability of nitric oxide via the excessive production of superoxide which reacts with nitric oxide to form peroxynitrite, leading to vascular dysfunction with ageing (Brandes et al., 2005; Griendling & Fitzgerald, 2003; Montezano & Touyz, 2012). Oxidised low density lipoprotein (LDL) and lipid peroxy radicals intercept and interact with the newly synthesised endothelial nitric oxide prior to reaching the smooth muscle cells, preventing dilation of the blood vessels (Sena et al., 2013).

A concurrent increase in superoxide and nitric oxide causes generation of the highly reactive radical peroxynitrite and the uncoupling of the eNOS (Csiszar et al., 2002; Christiaan Leeuwenburgh et al., 1997). Formation of nitric oxide is also reduced by uncoupling of eNOS due to the oxidation of BH₄, an essential cofactor for nitric oxide synthesis by endothelial NOS (Brocq, Leslie, Milliken, & Megson, 2008). Peroxynitrite oxidizes BH₄ to its inactive form BH₂, the decrease in BH₄ leads to the uncoupling of endothelial NOS, leading to more superoxide and a cyclic reduction of nitric oxide (Brocq

et al., 2008; Landmesser et al., 2003). The rise in the reactive radical peroxynitrite causes an unfavourable modification in protein function through nitrosylation of tyrosine residues (Siervo et al., 2018). Peroxynitrite causes tyrosine nitration producing nitrotyrosine, a cellular marker of oxidative stress (Leeuwenburgh et al., 1997; Reiter, Teng, & Beckman, 2000).

2.2.2. Methods to quantify oxidative stress

For direct detection and measurement of free radicals, the only method available to date, involves the use of electron paramagnetic resonance (EPR) spectroscopy. However, even with this technique it is difficult to detect RONS in biological samples due to their short half-life (Bailey et al., 2007). One ‘gold standard’ biomarker or one technique to quantify oxidative stress *in vivo* has yet to be established (Clifford et al., 2017; Powers, Duarte, Kavazis, & Talbert, 2010). To overcome this, there are a variety of indirect biomarkers of oxidative stress available and a number of techniques that can be applied to bodily fluids (saliva, urine and blood) and tissue to quantify the impact of oxidative stress (Di Meo & Venditti, 2001; Finaud et al., 2006; Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013; Leeuwenburgh & Heinecke, 2001; Margaritelis et al., 2014). RONS biomarkers have the potential not only to establish the magnitude of oxidative injury but also to calculate the possible efficacy of therapeutic approaches intended at decreasing the oxidative stress (Yazdanparast, Ardestani, & Jamshidi, 2007).

Reliable oxidative stress markers principally have the following qualities: chemically unique and detectable, sensitive to detect increase or decrease of oxidative stress, not influenced by other cellular processes and has a relatively long half-lives (Powers & Jackson, 2008). The most common oxidative stress biomarkers measure different cellular damage, such as proteins, DNA and lipids or endogenous antioxidant levels, see Table 2.2 (Powers et al., 2010; Vassalle, Pingitore, De, Vigna, & Bamonti, 2015). Typically, oxidative stress is measured in blood rather than other biological tissues owing to the

comparative ease of both the collection of the blood sample and the measurement of the marker (Margaritelis et al., 2014). There are many direct and indirect biomarkers for oxidative stress, which have been explored and critically analysed by Jackson (1999), Powers et al., (2010) and Finaud et al., (2006). This thesis will provide an overview of the biomarkers relevant to this thesis.

Table 2.1 Overview of oxidative stress biomarkers (Michalis G Nikolaidis et al., 2015; S. K. Powers et al., 2010).

Target	Biomarker
Protein	Protein Carbonyls Advanced oxidation products
Lipids	Lipid hydroperoxides Malondialdehyde Conjugated dienes F ₂ - isoprostanes
Antioxidant capacity	Oxidant radical absorbance capacity Trolox equivalent antioxidant capacity Ferric-ion-reducing antioxidant-power assay
Free radical	Electro paramagnetic resonance spectroscopy
	Glutathione peroxidase Glutathione S-transferase Glutathione reductase Superoxide dismutase Catalase
DNA	8-Hydroxydeoxyguanosine Comet assay

2.2.3. Lipid peroxidation

Lipid peroxidation is the oxidative degradation of lipids and is caused mainly by the effect of ROS (Ayala, Muñoz, & Argüelles, 2014). The cell membrane contains a large amount of lipids, specifically polyunsaturated fatty acids which are susceptible to oxidation and produce detectable primary and by-products (Finaud et al., 2006; Powers et al., 2010). Markers include hydroperoxides, F₂ isoprostanes, MDA and conjugated dienes (Finaud et al., 2006). The lipid peroxidation products can be extracted from biological samples (classically, muscle or blood) and contribute a value to help determine the degree of oxidative stress (Devasagayam, Bolor, & Ramasarma, 2003; Jackson, 1999). Lipid damage can be so substantial it can go on to initiate further oxidative injury to DNA and proteins (Hulbert, Pamplona, Buffenstein, & Buttemer, 2007).

MDA, a by-product of oxidising polyunsaturated fatty acids can be quantified by causing a colorimetric or fluorescent reaction with thiobarbituric reactive substances (TBARS). However, this reaction has been shown to lack specificity and this experimental data produced using this method should be considered carefully (Finaud et al., 2006). MDA is unstable in biological compounds, with poor reproducibility, lack specificity to lipid peroxidation and rapid degradation (Khoubnasabjafari, Ansarin, & Jouyban, 2015; Liu et al., 1999). To overcome these disadvantages to quantify MDA, the use of high-performance liquid chromatography (HPLC) has been shown to be precise and accurate, nevertheless, this method is expensive and time consuming (Khoubnasabjafari et al., 2015; Kil et al., 2014).

Halliwell (2009) found F₂- isoprostanes to be a reliable lipid peroxidation biomarker through use of a HPLC- mass spectrometry or immune assay kits offering accurate results. Both methods require a deal of expertise, sophisticated equipment and are expensive to run (Nikolaidis et al., 2007; Powers et al., 2010).

A method to quantify lipid peroxidation is with the colorimetric ferrous oxidation-xylenol orange (FOX) assay. This assay measures lipid peroxidation by quantification of lipid hydroperoxides oxidise ferrous to ferric ions followed by ferric ions binding to the xylenol orange dye which is ferric sensitive (Jiang, Hunt, & Wolff, 1992; Wolff, 1994). The FOX method is simple, sensitive and has the capacity to identify higher in the cascade products of lipid oxidation, this allows for early membrane-associated stress events to be measured (DeLong et al., 2002). Although the chemicals are inexpensive for this assay, the method requires a degree of expertise and a plate reader for analysis (Nikolaidis et al., 2015; Powers et al., 2010).

2.2.4. Protein carbonyls

A common approach to access overall oxidative stress is to determine end point protein oxidation through the use of the quantification of protein carbonyl assay with either dinitrophenylhydrazine (DNPH) or by with DNPH antibodies in immunochemical techniques, such as western blotting and enzyme linked immunosorbent assay (Buss, Chan, Sluis, Domigan, & Winterbourn, 1997; Shacter, Williams, Lim, & Levine, 1994). Although other methods are available, the carbonyl assay is widely used in laboratories and is considered a sensitive and reliable measure of oxidative stress induced modification to cellular proteins (Bowtell, Sumners, Dyer, Fox, & Mileva, 2011; Finkel & Holbrook, 2000; Fogarty, Hughes, Burke, Brown, & Davison, 2013; Levine, Wehr, Williams, Stadtman, & Shacter, 2000).

2.2.5. Nitrotyrosine

Amino acids such as tyrosine are targets of reactive nitrogen species, especially peroxynitrite. Peroxynitrite free radical can damage amino acids and proteins, yielding nitrotyrosine, thus, detection of nitrotyrosine in proteins is considered as a biomarker for endogenous peroxynitrite activity (Ahsan, 2013). The formation of nitrotyrosine in

human tissues is associated with a number of human diseases which involve a strong inflammatory component (Knight et al., 2018).

Various methods including gas chromatography/liquid chromatography or mass spectrometry or enzyme-linked immunosorbent assay (ELISA) can be used for evaluation of nitrotyrosine generated by oxidative or nitrative damage (Ahsan, 2013). Gas chromatography/liquid chromatography and mass spectrometry is a highly sensitive and accurate, however, is time consuming and expensive. While the ELISA kits offer an alternative means to detect nitrotyrosine, do not involve extensive sample preparation and are cost-effective. Issues of specificity must also be considered as antibodies might cross-react with other oxidative modifications present.

2.2.6. Inflammation

Under healthy conditions, inflammation is considered a constructive process. The activation of the immune system in the short term may cause swelling, redness, pain and infection and perhaps diminished function at the site of inflammation, however, inflammation maintains an inherent response to stimuli such as infection, other pathogens and trauma which may threaten to disrupt homeostasis (Monteiro & Azevedo, 2010; Ricciotti & FitzGerald, 2011; Yoon & Baek, 2005). Although, the long-term implications if unresolved are concerning (Yoon & Baek, 2005). Inflammation over a long period of time can advance ageing and contribute to age-associated pathology, such as, atherosclerosis (Chung et al., 2009; Shiva & Gladwin, 2009). Research has shown that inflammation is a determinant of unhealthy ageing and increases the risk of cardiovascular and non-cardiovascular mortality (Akbaraly et al., 2013). For example, the Whitehall II study observed in middle-aged adults an elevated level of IL-6 to half the odds of successful ageing after ten years (Akbaraly et al., 2013).

Cumulative evidence proposes there is an important link between inflammation and oxidative stress during the process of vascular ageing at the beginning of endothelial dysfunction, preceding atherosclerosis (Ungvari, Kaley, de Cabo, Sonntag, & Csiszar, 2010). RONS influence pro-inflammatory vicissitudes in the vascular endothelium, including, production of autocrine/paracrine factors, the increase in expression of cellular adhesion molecules and leucocyte-endothelial interaction (Herrera, Mingorance, Rodriguez-Rodriguez, & Alvarez de Sotomayor, 2010). Oxidative stress can induce redox-sensitive transcription factors including the activator protein and NF- κ B, increasing the expression of proinflammatory genes including cytokines (IL-1, TNF-alpha and IL-6), chemokines, and adhesion molecules (El Assar et al., 2012).

Ageing is linked with increased levels of cytokines (TNF-alpha, IL-1beta and IL-6), which trigger the acute phase of C-reactive protein (El Assar et al., 2012). This supports an advancing inflammatory environment and accelerates the progression of vascular dysfunction (El Assar et al., 2012). The Framingham heart study reported an inverse relationship between intracellular adhesion molecule inflammatory markers (IL-6, C-reactive protein) to flow mediated dilation among middle aged and older adults (Vita et al., 2004). In addition to this, when NF- κ B signalling was inhibited in a group of overweight and obese middle aged and elderly adults, the study observed a significantly improved vascular endothelial function (Pierce, Lesniewski, Lawson, Beske, & Seals, 2009). Showing that NF- κ B in part through stimulation of oxidative stress, plays a role in mediating endothelial dysfunction in overweight and obese, middle aged and elderly humans (Pierce et al., 2009).

An alternative age-dependent reduction in nitric oxide availability may be associated with a reduction in L-arginine and eNOS activity (Seals et al., 2011; Siervo et al., 2018). Asymmetric dimethyl L-arginine is a product of protein metabolism formed secondarily to methylation of L-arginine, this by-product inhibits the production of nitric oxide by

decreasing the expression of eNOS (Siervo et al., 2018). Increased asymmetric dimethyl L-arginine is found in many pathologies including hypertension, atherosclerosis, diabetes and heart failure (North & Sinclair, 2012).

In summary, with ageing there is crosstalk between oxidative stress and inflammation in the development of vascular inflammation adding to endothelial dysfunction (Csiszar, Toth, Peti-Peterdi, & Ungvari, 2007; Donato, Pierce, Lesniewski, & Seals, 2009; Vlachopoulos et al., 2005). The decrease in the production of vascular nitric oxide in part is due to the reduction by eNOS (Cau, Carneiro, & Tostes, 2012). Evidence suggests NOS activity is reduced with age due to post-translational alterations such as phosphorylation, nitrosylation or glycation (Cau et al., 2012). Moreover, this decrease in eNOS activity may be secondary to the shortage of cofactors required in the process of nitric oxide production (e.g. BH₄) (Seals et al., 2011). An age-related increase in arginase activity is also observed and contends for L-arginine with eNOS, further preventing nitric oxide production (Seals et al., 2011). Superoxide production and reduced antioxidant activity/expression are among the main factors that contribute to oxidative stress, reduced nitric oxide and vascular dysfunction with ageing (Sindler et al., 2014).

For the first time in history, it is forecast that over the next five years people over 65 years will outnumber children (< 5 years old) (Lutz, Sanderson, & Scherbov, 2008). With the increased prevalence of arterial ageing and risk of CVD, in accordance with the imminent economic burden on health care systems, there is an urgency to attenuate/treat age-associated arterial dysfunction, as this may in turn prevent age-associated CVD (Kirkland, 2013; Lunenfeld & Stratton, 2013; Lutz et al., 2008; Rae et al., 2010). From the known mechanisms and understanding of vascular ageing, hypothetically, a treatment that can increase and/or improve nitric oxide bioavailability while also attenuating oxidative stress and inflammation is likely to have considerable implications for the development of ageing and CVD.

2.3. The nitrate-nitrite-nitric oxide pathway

Until recently, it was thought nitric oxide was solely generated through oxidation of L-arginine catalysed by nitric NOS enzymes, endogenously producing inactive by-products nitrate and nitrite (Moncada & Higgs, 1993). It is now recognised, that nitrate and nitrite can be physiologically reduced in blood and tissue to form nitric oxide and bioactive nitrogen oxides *in vivo* (Larsen et al., 2011; Lundberg, Weitzberg, & Gladwin, 2008). This pathway presents a supplementary system to generate nitric oxide production following consumption of nitrate when nitric synthesis via the NOS pathway is diminished, which tends to become less effective in older age and in conditions where oxygen availability is limited. This complementary pathway is a key focus of the current research (Carlström et al., 2010).

Nitrate has emerged as an alternative to replenish nitric oxide bioavailability and to enhance vascular function (Rammos et al., 2016). Abundant in everyday diet, green leafy vegetables, such as watercress, lettuce rocket, and spinach and root vegetables such as beetroot, contain the highest nitrate content, with > 250 mg nitrate/100 g (Clements, Lee, & Bloomer, 2014; Hord, Tang, & Bryan, 2009).

The reduction of nitrate involves a complex inter-organ metabolic path, as seen in Figure 2.2.1. Nitrate peaks around sixty minutes post consumption with a plasma half-life of ~ 5 - 6 hours (McKnight et al., 1997). Nitrite plasma levels peak 2 – 3 hours post nitrate consumption and is an important biomarker of nitric oxide availability (Jones, 2014). The exact mechanism behind how nitrite enters the circulation and is reduced is still unclear (Clifford, Howatson, West, & Stevenson, 2015; Kapil et al., 2013). There are several pathways nitrite can further reduce to nitric oxide involving a variety of enzymes and proteins including, haemoglobin, myoglobin, xanthine oxidoreductase, ascorbate, polyphenols such as betalins, and protons (Carlsson, Wiklund, Engstrand, Weitzberg, &

Lundberg, 2001; Cosby et al., 2003; Gago, Lundberg, Barbosa, & Laranjinha, 2007; Lundberg et al., 1994; Sruti Shiva et al., 2007; Zhang et al., 1998). The formation of nitric oxide by these specific pathways is greatly enhanced in a hypoxic or acidic environment, enhancing the nitrite vasodilatory reduction to nitric oxide, .e.g. within the skeletal muscle during exercise and for facilitating nitric oxide to be formed wherever it is utmost essential (Lundberg et al., 2008; Modin et al., 2001). This capacity to vasodilate in a hypoxic environment is of great interest to researchers not only from an athletic point of view but also for aging or diseased populations.

It is important to note that endogenous nitrate can be taken up by commensal bacteria in your mouth acting as a pool of precursors for nitric oxide generation (Govoni, Jansson, Weitzberg, & Lundberg, 2008). Research has shown the use of antibacterial mouthwash and failure to swallow saliva attenuates the reduction nitrate to nitrite, highlighting the significance of the commensal bacteria in this pathway (Clifford et al., 2015; Govoni et al., 2008; Kapil et al., 2013; Webb et al., 2008). This study highlighted the importance of the nitrate reductase activity of oral bacteria (Govoni et al., 2008). From this, it is assumed the anti-septic and anti-bacterial mouthwash ablates the commensal bacteria in the oral cavity which has a significant role in supporting nitric oxide bioavailability (Govoni et al., 2008; Joshipura, Munoz-Torres, Morou-Bermudez, & Patel, 2017). Substantial efforts are presently being made to distinguish the specific human oral microbiome and to characterise the nitrate reducing species and its interplay with diet and exercise interventions (Doel, Benjamin, Hector, Rogers, & Allaker, 2005). This research may advance the progression of nitrate interventions by optimising the oral microbiome and maximising therapeutic and ergogenic benefits.

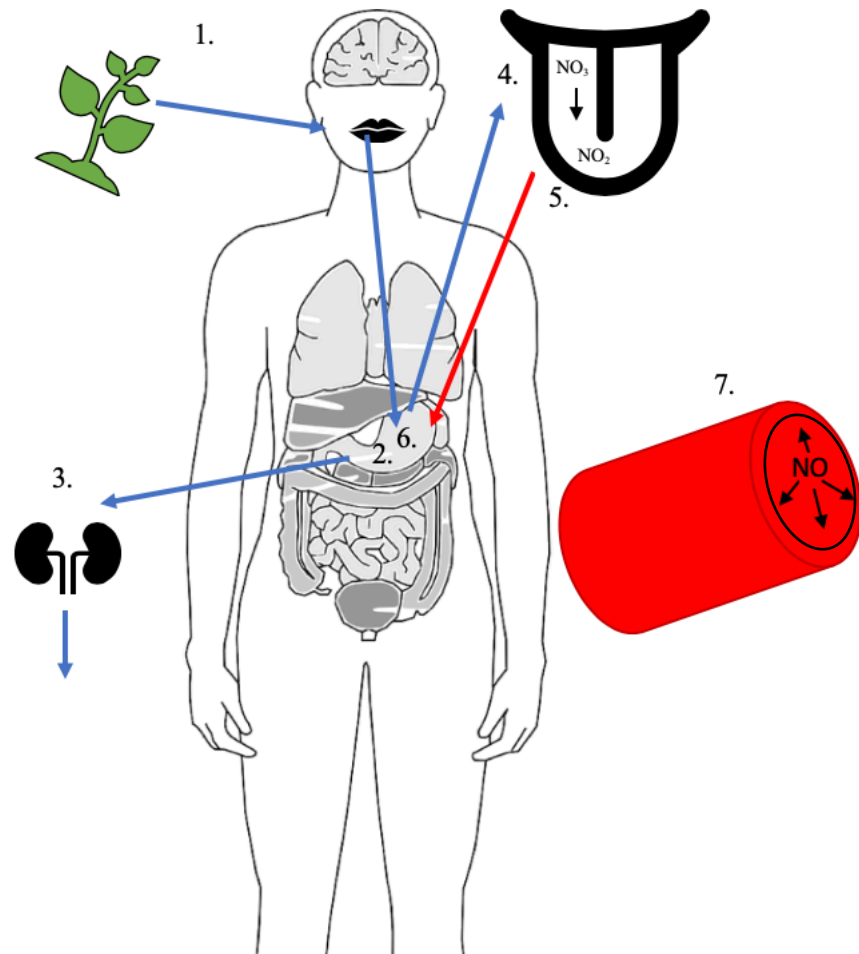


Figure 2.2.1. The entero-salivary circulation of dietary nitrate. Nitrate is represented by the blue arrows and nitrite is represented by the red arrows. NO_3^- ; nitrate, NO_2^- ; nitrite, NO ; nitric oxide. Ingested nitrate (1) is rapidly absorbed across the upper gastrointestinal tract into the systemic circulation (2). Around 70% of the nitrate is excreted by the kidneys (3) but about 25% enters the entero-salivary systems. Nitrate-rich saliva is secreted into the oral cavity (4) where facultative anaerobic bacteria reduce nitrate to nitrite (approx. 20%) (5) via the action of nitrate reductase enzymes. Nitrite-rich saliva is then swallowed and further metabolised to nitric oxide and other nitrogen intermediates nonenzymatically in the acidic stomach (6). Some nitrite enters the intestine and is absorbed by the blood cells and plasma and enters the system circulation (7) where it gets reduced to nitric oxide in the blood and in other tissues. The nitrate that was not reduced in the oral cavity re-enters the systemic circulation, allowing it to be reduced further to nitric oxide. Image by <https://pixabay.com/users/OpenClipart-Vectors-30363>

2.3.1. Dietary nitrate supplementation: Its history and potential benefits

Diet and partaking in exercise are amongst the many factors that can affect an individual's health and quality of life. Dietary nitrate interventions increase nitric oxide production and may have important implications for health and disease. As a result, an extensive

amount of current research is investigating the use of dietary nitrate to enhance human health and exercise capacity. Therefore, the purpose of this review was to critically evaluate the effects of dietary nitrate supplementation on exercise capacity (physiological and competitive) and cardiovascular parameters in healthy young and older adults.

Many studies have observed increased plasma nitrate and nitrite concentrations after ingestion in the form of sodium nitrate, with analogous effects after participants consumed concentrated beetroot (Bailey et al., 2009; Govoni et al., 2008; Lansley et al., 2011; Larsen, Weitzberg, Lundberg, & Ekblom, 2007; Larsen et al., 2011; Vanhatalo et al., 2010; Wylie et al., 2013). The increased nitrite concentration elevates the bioavailability of nitric oxide, this enhanced bioavailability can have a number of physiological effects within the human body (Bailey et al., 2009; Govoni et al., 2008; Lansley et al., 2011; Larsen et al., 2007; Larsen et al., 2011; Vanhatalo et al., 2010; Wylie et al., 2013). This research has led to the assumption that the bio-conversion of nitrate-nitrite- nitric oxide pathway is attributed to the cardioprotective and exercise enhancing qualities (Benjamin, 1994; Kenjale et al., 2011; Lundberg & Govoni, 2004; Vanhatalo et al., 2011). With research signifying the novel nitrate based therapeutic supplement as potential for treatment or reduction in risk of CVD (Lundberg et al., 2008). Recently, there has been growing interest in the root vegetable beetroot (*Beta vulgaris rubra*), predominantly driven by its nitrate content and its significant implications in managing health and disease (Jones, Thompson, Wylie, & Vanhatalo, 2018; Lundberg et al., 2008). It is important to note; beetroot is also rich in other bioactive compounds, to which little attention has been paid (Clifford et al., 2017; Wootton-Beard & Ryan, 2011)

2.3.2. Toxicity

European adults on average consume 1 – 2 mmol/d of nitrate (Alexander et al., 2008). It is estimated in the Western diet roughly 60 – 80% of daily intakes of nitrate is consumed from vegetables with preservatives in meats and water supply contributing to the rest

(Ysart et al., 1999). The European Authority for Food Safety advises an acceptable consumption of nitrate is 3.7 mg nitrate/kg of bw/day, this equates to circa 5 mmol per day for person weighing 80 - 85 kg (Alexander et al., 2008). For many years, it was considered nitrate and nitrite were carcinogens, however, the latest evidence suggests nitrate may be a significant bioactive compound in salad vegetables which may promote human health (Bryan & Ivy, 2015). Despite this, there is no epidemiological verification to suggest that nitrate is carcinogenic in humans, with epidemiological research suggesting vegetables reduce the risk of cancer (Terry, Terry, & Wolk, 2001).

2.3.3. The physiological effects of dietary nitrate intake on exercise

A well-established tenet of human exercise physiology outlines that the oxygen cost of cycling for any individual at a given work rate is highly predictable (for every increase in 1 Watt there is a 10 mL/min increase in oxygen consumption), irrespective of age, health, cardiorespiratory fitness and nutritional/pharmacological interventions (Bailey et al., 2009; Bailey et al., 2010; Cermak, Gibala, & van Loon, 2012; Lansley et al., 2010). In 2007, in a double-blind, crossover study by Larsen et al. (2007), an unexpected reduction in oxygen cost during submaximal cycling after three days of sodium nitrate supplementation (0.1 mmol/kg body mass per day) was reported. During submaximal exercise (45 - 80% of $\text{VO}_{2\text{max}}$) a mean reduction of 5% of oxygen consumption (VO_2) was observed in the sodium nitrate supplementation group in comparison to equimolar amount of sodium chloride. The results indicated that nitrate supplementation enhanced exercise efficiency and improved performance by reducing the energy required for work at the same exercise intensity. Interestingly, the study found there was no difference in heart rate, lactate, ventilation or respiratory exchange ratio. These results indirectly suggested no change in non-oxidative energy supply and instead an efficiency of muscle metabolism. Proposing that the oxygen sparing effect may be due to more efficient aerobic metabolism or increased mechanical efficiency. These findings were replicated

and validated in studies observing a natural, nitrate-rich, beetroot juice after 3 – 15 days of supplementation, reporting a similar approximate 5% decrease in VO_2 during submaximal exercise (Bailey et al., 2009; Hernández et al., 2012; Larsen et al., 2007; Vanhatalo et al., 2010). The results from the studies suggested that dietary nitrate consumption allows for additional muscular work to be executed per unit of time for the same cost of energy (Jones, 2014). This discovery of nitrate offered a new intervention to enhance muscular work during exercise. Larsen et al., (2010) went on to corroborate these findings after sixty minutes following an acute sodium nitrate ingestion dose corresponding to the amount found in 100–300 g of a nitrate-rich vegetable such as spinach or beetroot. Vanhatalo et al., (2010) also observed this reduction in oxygen consumption and muscular efficiency after 2.5 hours, 5 and 15 days of consuming beetroot juice (~ 300 mg) compared to a low-calorie blackcurrant cordial, with negligible nitrate content, acting as a placebo. Interestingly, the decrease was not greater following an extended period of supplementation, showing that over time humans do not develop an intolerance to nitrate supplementation (Vanhatalo et al., 2010).

The observed reduction in oxygen consumption after nitrate supplementation is of great practical significance in terms of exercise tolerance and performance. The maximum rate of oxygen consumption is a gold standard measurement in exercise physiology, used to measure the functional limit of the cardiovascular system and is interpreted as an index of cardiorespiratory fitness. Two factors are considered to equate the maximum rate of oxygen consumption; cardiac output (stroke volume x heart rate) and the capacity to extract oxygen from the blood for mitochondrial respiration. Nitrate supplementation has displayed a surprising capacity to improve exercise efficiency, by permitting more muscular activity to be performed per unit time for the same energy cost.

This revelation generated a lot of interest in nitrate supplementation and since then research has explored other types of exercise, such as kayaking, walking and rowing

(Bailey et al., 2009; Bailey et al., 2010; Lansley et al., 2011; Larsen et al., 2011; Muggeridge et al., 2013; Peeling, Cox, Bullock, & Burke, 2015; Vanhatalo et al., 2010). Results have observed a reduction of oxygen consumption after nitrate administration and reduced time to fatigue and thus, an enhanced exercise performance (Bailey et al., 2009; Bailey et al., 2010; Lansley et al., 2011; F. J. Larsen et al., 2011; Muggeridge et al., 2013; Peeling et al., 2015; Vanhatalo et al., 2010). A systematic review and meta-analysis by Hoon et al., (2013) on the efficacy of nitrate supplementation in young, healthy participants, reported a noteworthy modest benefit in time to exhaustion test (ES = 0.79, 95% CI: 0.23 – 1.35; P = 0.006).

Improvements in time to exhaustion protocols measure exercise capacity and do not necessarily imply an enhanced athletic performance in a practical setting. The early research in nitrate consumption focused on metabolic efficiency and exercise tolerance in healthy participants and found improved exercise tolerance in the range from 16-25% during controlled continuous work rate exercise (Bailey et al., 2009; Bailey et al., 2010; Lansley et al., 2011). The extent of improvement in real world exercise performance would be predicted to be much lower, using the estimates of Hopkins et al., (1999), this would be calculated to resemble an improvement in time trials by 1-3%.

The ergogenic effect of nitrate supplementation has been greatly studied and results have varied (See Figure 2.3 and 2.4). The efficacy of acute nitrate supplementation in TT performance on moderately trained athletes (< 60 mL/kg/min) are equivocal (Cermak, Gibala, & van Loon, 2012; Cermak, Res, et al., 2012; Lansley et al., 2011; Muggeridge et al., 2015; Porcelli et al., 2015). Lansley et al., (2011) was in accordance with Hopkins et al., (1999) estimation following an acute ingestion of beetroot juice in competitive cyclists. Both the 4 km and 16.1 km TTs improved by 2.8% and 2.7%, respectively (Lansley et al., 2011). Participants maintained the same oxygen uptake, however, were able to produce a higher power output after beetroot juice ingestion (Lansley et al., 2011).

Previous research suggests nitrate supplement containing more than ≥ 5 mmol per serving, appears to be the minimal dose required to enhance exercise performance in most participants (Jones et al., 2018). Hoon et al., (2013) meta-analysis found a minor benefit for both time trials and incremental exercise performance tests (ES = 0.11, 95% CI: -0.16 – 0.37; ES – 0.26, 95% CI -0.10 – 0.62, respectively). The analysis by Hoon et al., (2013) indicated that sedentary or recreationally active cohort athletic performances were most likely to benefit from nitrate ingestion (Hoon, Johnson, Chapman, & Burke, 2013).

Previous research observing older adults, has reported both acute and prolonged dietary nitrate supplementation to improve oxygen uptake during exercise and increase time to fatigue, thus promoting exercise performance (Berry et al., 2015; Kelly et al., 2013; Kenjale et al., 2011). A review on performance and health benefits of dietary nitrate supplementation in older adults observed from the four out of the five studies to improve physiological performance and positive health related outcomes (Stanaway, Rutherford-Markwick, Page, & Ali, 2017).

While an enhanced exercise efficiency has been observed in recreationally active participants after dietary nitrate supplementation, there has been an inconsistent reporting in trained athletes (See Figure 2.4). Research suggests persistent training over a long period enhances running economy and cycling proficiency (Coyle, 2005; Jones, 2006). It is also important to note that trained athletes have a higher resting plasma nitrate and nitrite and a greater maximal VO_2 than untrained individuals (Porcelli et al., 2015). It is suggested the restricted efficacy of nitrate supplementation in highly trained athletes (maximal oxygen consumption ≥ 60 mL/kg/min) is due to the already advanced oxidative metabolic system and the superior availability in nitric oxide (Porcelli et al., 2015). Highly trained athletes may require a higher dose for performance benefits (Peeling et al., 2015).

2.3.4. Dose and duration

Nitrate pharmacokinetics suggest that plasma peak nitrate and nitrite activity occurs 2-3 hours post ingestion, with small or no activity after twenty-four hours (Ashor, Lara, & Siervo, 2017; Vanhatalo et al., 2010; Webb et al., 2008). Therefore, it is recommended individuals should consume nitrate 2-3 hours prior to the desired effect (Hoon et al., 2013). The effectiveness of dietary nitrate depends on numerous factors, including training status and the dosage (Jones, 2014).

A study observed a variety of acute doses of nitrate supplementation and their effect on exercise tolerance; 70 mL (containing 260 mg/4.2 mmol of nitrate), 140 mL (containing 521 mg/8.4 mmol of nitrate) and 280 mL (containing 1043 mg/16.8 mmol of nitrate) of beetroot juice in recreationally active males (Wylie et al., 2013). After 2.5 hours of consumption the 140 mL and the 280 mL of concentrated beetroot juice lowered oxygen uptake measured during submaximal exercise by 2% and 3%, respectively, and improved exercise tolerance by 14% and 12%, respectively. No benefit was observed with the 70 mL beetroot juice in terms of exercise economy and tolerance (Wylie et al., 2013). Studies observing oxygen uptake during submaximal cycling have reported 2-5% decrease after acute consumption of 322 mg (5.2 mmol) and 373 mg (6 mmol) of nitrate (Vanhatalo et al., 2010; Wylie et al., 2016). While an acute dose of 186 mg (3 mmol) showed no oxygen uptake lowering effect during submaximal exercise (Wylie et al., 2016). The results from the acute studies suggest that an acute dietary nitrate containing 310-527 mg (5.0 – 8.5 mmol) may improve exercise economy (Wylie et al., 2013). While most studies show an ergogenic effect after 6 – 8 mmol of nitrate is consumed (Domínguez et al., 2017).

Studies observing nitrate supplementation over a longer period; 15 days (5.2 mmol/322 mg of nitrate per day) and 28 days (6 mmol/372 mg of nitrate per day), observed an improvement in exercise economy, however, this improvement was no greater or less than the improvement observed on the first day of supplementation (Vanhatalo et al.,

2010; Wylie et al., 2016). Additionally, a study supplementing ~ 3 mmol of nitrate up to 28 days did not observe an improvement in exercise economy or performance, highlighting the duration of the supplement has little effect on the dose-response relationship (Wylie et al., 2016). Additionally, highly trained athletes may require a higher dose for performance benefits as results are inconsistent (Peeling et al., 2015; Porcelli et al., 2015). Peeling et al., (2015), observed highly trained kayakers completed 500 m 1.7% faster after 9.6 mmol of nitrate in comparison to 4.8 mmol of nitrate which was reported to be ineffective. Research has identified highly trained individuals to respond positively to nitrate supplementation and improve TT performance, however, further research is needed to identify what factors govern sensitivity to nitrate supplementation (Wilkerson et al., 2012). It still remains unclear as to why there is an inconsistency in the efficacy of nitrate supplementation observed in highly trained participants. It may be partly related to the higher synthesis of nitric oxide from the NOS dependent pathway that reduces the potential for added nitric oxide production through nitrate-nitrite- nitric oxide pathway (McConnell et al., 2007). The application of this dose-response relationship to other outcomes such as blood pressure or cohorts such as clinical populations and females are currently unknown.

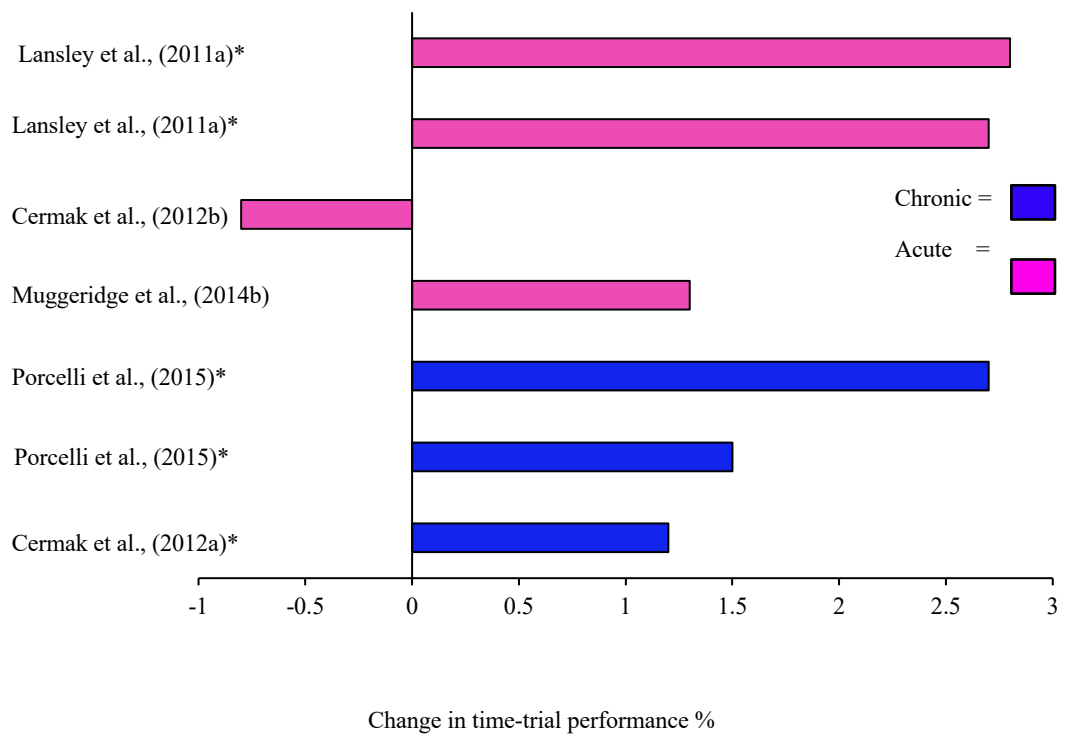


Figure 2.3.1. Percentage change in time-trial performance following dietary nitrate supplementation in recreational and moderately trained athletes (peak $\text{VO}_2 < 60 \text{ ml/kg/min}$). Acute supplementation (pink bars) and chronic supplementation (blue bars). * denotes a significant improvement in performance.

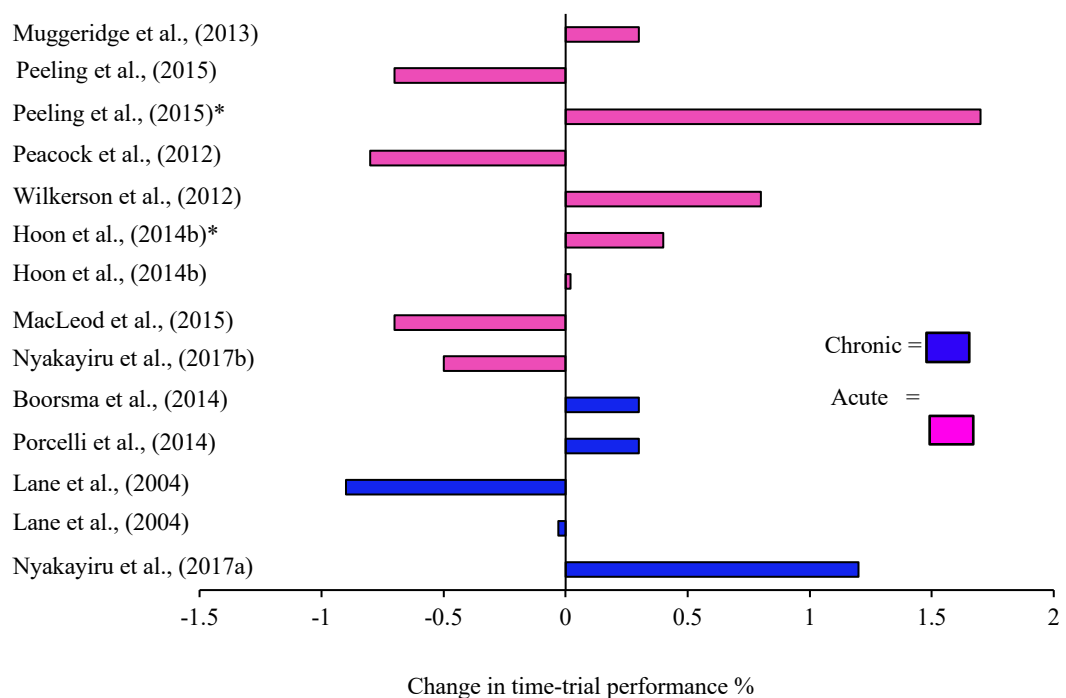


Figure 2.4.1. Percentage change in time-trial performance following dietary nitrate supplementation in highly trained and elite athletes (peak $\text{VO}_2 > 60 \text{ ml/kg/min}$). Acute supplementation (pink bars) and chronic supplementation (blue bars). * denotes a significant improvement in performance.

2.3.5. Physiological mechanisms of dietary nitrate

The aforementioned improvements in time to fatigue and exercise performance following nitrate supplementation are often attributed to enhanced blood flow to the working muscle, improvements in mitochondrial efficiency and/or improved muscle contractile function (Jones, 2014).

Given the diverse capacity of nitric oxide in physiology, there are several proposed mechanisms for this oxygen sparing effect (Clerc, Rigoulet, Lerverve, & Fontaine, 2007). The reduction in oxygen cost may be related to nitric oxide regulating mitochondrial function by direct binding to cytochrome c oxidase (Clementi & Nisoli, 2005). This binding is reversible, it is competing with oxygen and inhibits enzyme activity, indirectly regulating oxygen utilisation and potentially controlling reactive oxygen species by

redistributing oxygen to neighbouring cells, a process that may have beneficial effects in a diseased population (Clementi & Nisoli, 2005; Palmer, Dennis, Noakes, & Hawley, 1996). Research suggests nitric oxide may improve oxidative phosphorylation by reducing mitochondrial proton leak or reducing the expression of uncoupling proteins (Currell & Jeukendrup, 2008; Foster, Green, Snyder, & Thompson, 1993). Larsen et al., (2011) assessed mitochondrial function after three days of consuming inorganic nitrate, in an achievable daily amount of 0.1 mmol/ kg/ day, divided into three doses. The study showed a 19% increase in mitochondrial oxidative phosphorylation efficiency (ATP produced per oxygen consumed) during exercise, suggesting a decrease in proton leakage or slippage across the inner mitochondrial membrane. This result was closely correlated with the reduction of oxygen consumption during exercise after nitrate supplementation ($r = - 0.80$). The study further investigated mitochondrial protein expression involved in proton conductance and found a decrease in the output of ADP/ATP translocase, a protein known to be involved in proton conductance and responsible for protein leakage under physiological conditions (Larsen et al., 2011).

Other research in this area has observed a reduction in total ATP cost of muscle force production after nitrate supplementation (Bailey et al., 2010). Recreationally active men ($n = 7$) consumed 500 ml/day of beetroot juice (5.1 mmol) everyday. On day six of supplementation, participants completed step exercises until task failure during which the right quadriceps muscle metabolism was observed using phosphorus magnetic resonance spectroscopy. The study found nitrate supplementation to reduce whole body oxygen cost due to the reduced ATP cost of muscle force production. This was determined by the sparing of intramuscular phosphocreatine concentration (PCr) and a blunting of the increase in adenosine diphosphate (ADP) concentration during low- and high-intensity knee extensor exercise tests (Bailey et al., 2010). It was proposed this could occur by the possible effects of nitric oxide on the sarcoplasmic reticulum calcium ATPase or the

actin-myosin ATPase (Evangelista et al., 2010; Ishii et al., 1998; Viner, William & Schoneich, 2002). The lower ATP cost of force production would potentially blunt the changes in intramuscular substrates and metabolites that stimulate mitochondrial respiration such as PCr, ADP, inorganic phosphate (Pi), and may account for the lower oxygen cost during exercise. The reduction of muscle PCr and the accretion of Pi and ADP have been associated with the development of muscular fatigue during high-intensity exercise (Allen, Lamb & Westerblad, 2007). Following nitrate supplementation, the reduced alterations in energy substrates and metabolites may consequently explain the augmented tolerance to exercise (Jones, 2014).

2.3.6. The effects of dietary nitrate intake on cardiovascular parameters

The beneficial effects of a diet rich in fruit and vegetables upon cardiovascular health and longevity are well researched (Bazzano et al., 2002; Gilchrist, Winyard, & Benjamin, 2010; Ness & Powles, 1997; Visioli, Bogani, Grande, & Galli, 2005). Substantial evidence suggests dietary nitrate, in the form nitrate rich beetroot juice decrease blood pressure significantly in young healthy adults (Bailey et al., 2010; Kapil, Khambata, Robertson, Caulfield, & Ahluwalia, 2015; Vanhatalo et al., 2010; Webb et al., 2008). The increased production of nitric oxide stimulates smooth muscle cell relaxation via the synthesis of cyclic guanosine monophosphate (Forstermann et al., 1986). A meta-analysis observed systolic blood pressure (-3.55; 95% CI:-4.55 to -2.54 mmHg) and diastolic blood pressure (-1.32; 95% CI: -1.97 to -0.68 mmHg) were significantly lower in the beetroot juice supplemented groups than in the control groups (Bahadoran, Mirmiran, Kabir, Azizi, & Ghasemi, 2017). Suggesting that nitrate supplementation, in the form of beetroot juice, is potentially an economic and safe dietary strategy for managing blood pressure (Bahadoran et al., 2017). Interestingly, the study observed a weak effect size in a meta-analysis of trials that used nitrate depleted beetroot juice as a placebo. The study

attributed other bioactive compounds, such as polyphenols, available in beetroot that could mediate dilatory reactions (Bahadoran et al., 2017; Clifford et al., 2015).

In addition to this, studies have observed nitric oxide to exert anti-inflammatory effects, decrease endothelin production, reduce platelet activation and adhesion, decrease oxidation of LDL and smooth muscle proliferation (Justice et al., 2014; Jädert et al., 2012; Jädert, Phillipson, Holm, Lundberg, & Borniquel, 2014; Webb et al., 2008). It is because of these positive effects researchers suggest that a reduction in nitric oxide bioavailability may have a major deleterious effect on endothelial function (Siervo et al., 2018).

A multitude of factors, such as health/fitness status, medication, gender and lifestyle choices may affect the blood pressure response to increase dietary nitrate. An individual with an inherently low blood pressure, females and highly trained individuals tend to exhibit smaller blood pressure reductions in response to a standardised nitrate dose than their hypertensive, male and untrained participants (Kapil et al., 2010; Wilkerson et al., 2012; Jones, 2018). It has been suggested this may be due to nitrite and blood pressure baseline levels, or due to the difference in commensal bacteria on the dorsal surface of the tongue (Kapil et al., 2010).

One of the most important roles of nitric oxide is to maintain endothelial function (Davignon & Ganz, 2004; Hobbs, Goulding, et al., 2013). The endothelium plays a significant role in the maintenance of vascular homeostasis by regulating vascular tone, platelet function, thrombotic activity, vascular tone and the fragile balance between the release of vasoconstricting and vasodilating agents (Davignon & Ganz, 2004). Endothelial dysfunction is a primary risk factor for several cardiovascular disorders and has been associated in the development of hypertension and atherosclerosis (Joris & Mensink, 2013; Lidder & Webb, 2013). Therefore, understanding the potential benefits of a natural nitric oxide donor from green leafy vegetables or root vegetables is important,

as it may have an easily accessible, cost-effective and important role in preserving or restoring endothelial function.

Although limited, there are several reports that acute consumption of beetroot can evoke positive changes in endothelial function in healthy population (Hobbs, Goulding, et al., 2013; Joris & Mensink, 2013; Webb et al., 2008). The first research group to examine the efficacy of beetroot supplementation on endothelial function in a healthy cohort was by Webb and colleagues (2008). Flow mediated dilation technique was used to measure brachial artery endothelial function, brachial artery dilation was calculated at baseline and after 20 minutes ischemic insult. The ischemic method was efficacious in causing endothelial dysfunction, as demonstrated by the dilation reaction decrease by 60% from pre-to post-brachial artery flow (Webb et al., 2008). Conversely, when participants were given an acute serving of beetroot juice (500 mL; containing 23 mmol of nitrate) two hours prior to the measurement, the brachial artery flow mediated dilation was maintained at pre-ischemic levels, signifying the nitrate supplement attempted to preserve endothelial function (Webb et al., 2008). The study also reported a significant attenuation in *ex vivo* platelet aggregation in response to collagen and ADP after dietary nitrate supplementation (Webb et al., 2008). Similarly, Hobbs et al., (2013) investigated the ingestion of a novel beetroot bread (100 g of bread containing 1.1 mmol of nitrate) on peripheral arterial stiffness and micro-vascular function in a healthy males. Pulse wave velocity and augmentation index (assessed to indicate arterial stiffness) were unchanged by the dietary intervention (Hobbs, George, et al., 2013). However, changes in cutaneous perfusion using laser doppler imaging observed significant microvascular dilation, indicating improved endothelium-independent vasodilation six hours post ingestion in comparison to the control bread (Hobbs, Goulding, et al., 2013). Additionally diastolic blood pressure was also reported to reduced significantly (Hobbs, Goulding, et al., 2013). Notably, this study demonstrated enriching bread with beetroot may provide a novel therapeutic stance

in the management of hypertension and proof that a small amount of nitrate can significantly enhance intravascular function (Hobbs, Goulding, et al., 2013). Moreover, it was reported nitrate supplementation (beetroot juice; 500 mg of nitrate) attenuates postprandial impairment of FMD in a healthy overweight and obese cohort in accordance with increase in nitrite concentrations (Joris & Mensink, 2013). The brachial artery FMD decreased in the control by ~1.6% in comparison to the nitrate condition (500 mg of nitrate; 140 ml of beetroot juice) observed a 0.4% decrease, indicating a mediated protective effect on postprandial endothelial function (Joris & Mensink, 2013). However, whether or not an improvement of the recurrent but temporary postprandial attenuation of the FMD response has a beneficial health affects has yet to be investigated.

Nitrate-rich sources have been researched, spinach (3 mmol of nitrate) and flavonoid-rich apples were combined to observe blood pressure and FMD in a healthy cohort (Bondonno et al., 2014). Interestingly, when consumed on their own the supplements caused improved FMD and systolic blood pressure, however when provided together the two interventions did not result in additive effects but still observed significant improvements (Bondonno et al., 2014). The authors proposed that administering nitrate-rich spinach and flavonoid rich apples concurrently caused increased nitric oxide production in the acidic environment of the stomach with a reduced availability of nitrite to be absorbed in the circulation (Bondonno et al., 2014). The reported interaction and less than added effects of the combination supplementation of both foods on plasma nitrite concentrations are consistent with this suggestion.

2.3.7. Effects of nitrate supplementation in hypoxia

Hypoxia is defined as a decrease in the supply of oxygen to an insufficient level that can no longer maintain cellular function, a frequent issue seen in disease states and aged

populations (Brahimi-Horn, Chiche, & Pouysségur, 2007; Gilany & Vafakhah, 2010). An insufficient oxygen supply can cause down regulation of protein synthesis (Koritzinsky et al., 2006; Shih & Claffey, 1998). The essential requirement of oxygen by all aerobic organisms has elicited the evolution of a complex cellular response to hypoxia, with the hypoxia inducible factor at the centre of this response. Hypoxia inducible factor plays a major role in the response to low partial pressures of oxygen and is recognised to be profoundly modified by nitric oxide (Hagen, Taylor, Lam, & Moncada, 2003; Wang, Jiang, Rue, & Semenza, 1995).

A decreased portion of oxygen in inspired air causes a decrease in arterial oxygen and a reduction in intracellular partial pressure of oxygen (Richardson, Noyszewski, Kendrick, Leigh, & Wagner, 1995). Hypoxia induced vasodilation occurs to help restore sufficient oxygen supply (Heinonen et al., 2010). This increased blood flow response is suggested to be mediated by nitric oxide, adenosine, ATP sensitive potassium channels and prostaglandins (Casey, Madery, Elvebak, et al., 2009; Casey, Madery, Pike, et al., 2009; Heinonen et al., 2010).

Nitric oxide is a key regulator in response to adaptation to hypoxia and is linked in a number of hypoxic vasodilatory pathways, such as, increased energy supply to demand and maintenance of muscle perfusion (Casey, Madery, Elvebak, et al., 2009). The enzymatic specific pathway for nitric oxide generation requires essential co-factors and oxygen for nitric oxide production, it has been suggested in conditions where oxygen may be reduced, such as hypoxia, the nitrate-nitrite-nitric oxide pathway offers a complementary compensatory pathway for nitric oxide generation (Lundberg, Carlstrom, Larsen, & Weitzberg, 2011). In hypoxia, nitric oxide is known to reallocate oxygen by averting the stabilisation of the hypoxia inducible factor and moderate oxygen distribution in the tissue (Hagen et al., 2003; Thomas, Liu, Kantrow, & Lancaster, 2001).

Highlighting an added nitric oxide role in enhancing cellular respiration in hypoxia (Hagen et al., 2003).

Hypoxic conditions reduce nitric oxide synthesis via NOS, but the nitrate-nitrite-nitric oxide pathway is likely accelerated at low oxygen tension levels (Dumais, Nault, Tsertsvadze, & Forbes, 2011; Lundberg et al., 2008; McQuillan, Leung, Marsden, Kostyk, & Kourembanas, 1994). In a hypoxic environment, the decreased haemoglobin oxygenation increases the deoxyhaemoglobin content (Cosby et al., 2003; Lundberg et al., 2008). Research has shown deoxyhaemoglobin and deoxymyoglobin facilitate the reduction of nitrite and therefore nitric oxide availability for smooth muscle vasodilation (Cosby et al., 2003; Lundberg et al., 2008; Shiva et al., 2007). For example, a study reported high levels of circulating bioactive nitric oxide by products and low haemoglobin to be related with augmented forearm blood flow (Erzurum et al., 2007). Increasing dietary nitrate and therefore nitric oxide production may have an ergogenic effect or improve exercise tolerance at a high altitude or may have therapeutic applications in conditions where oxygen delivery is reduced (cardiovascular, pulmonary disorders or as a consequence of ageing). From this, it might be logical to speculate that dietary nitrate can have a superior advantageous physiological effect in hypoxia in comparison to normoxia. Furthermore, when comparing research in hypoxic settings it is important to consider the variation in study designs (duration and type of hypoxia, cohort, passive or active) and measurement location (peripheral, pulmonary and cerebral vasculature) as these may influence nitric oxide metabolism and overall results.

In 2011, Vanhatalo and colleagues observed a significant increase in plasma nitrite concentration and a reduced amount of metabolic muscle perturbations (PCr, P_i and pH) in a hypoxic environment (14.5% oxygen) during high intensity knee extensors and emulated exercise tolerance to that observed in a normoxic (20.9% oxygen) setting after consuming 0.75 L of beetroot juice (9.3 mmol of nitrate) in comparison to nitrate depleted

placebo. Restoration to normoxic levels was also observed in the phosphocreatine recovery kinetics in the hypoxic environment post nitrate supplementation (Vanhatalo et al., 2011). Indicating nitrate supplementation may enhance oxidative phosphorylation and may have a therapeutic application in a hypoxic environment.

Research has consistently reported no change in post-exercise PCr kinetics after nitrate supplementation in a normoxic setting, suggesting an enhanced energy system and therefore enhanced mitochondrial ATP re-synthesis in a hypoxic setting maybe due to the enhanced oxygen delivery rather than a reduced oxygen cost of ATP re-synthesis (Fulford et al., 2013; Lansley et al., 2010; Vanhatalo, Jones, Blackwell, Winyard, & Fulford, 2014). This was shown following high-intensity knee extensor exercise by an increase in magnetic resonance signal which reflects change in the perfusion in oxygenation of the vascular compartment after consuming dietary nitrate compared to placebo in a hypoxic environment (Vanhatalo et al., 2014). Suggesting that nitrate supplementation in a hypoxic environment may enhance the oxygen gradient across the microcirculation and the muscle cells, therefore enabling a speedier rate of mitochondrial ATP re-synthesis inferred by a faster PCr re-synthesis rate (Vanhatalo et al., 2011; Vanhatalo et al., 2014). It is important to note in a hypoxic environment nitrate supplementation may also alter metabolic control and the efficiency of the mitochondria contributing also to mitochondrial ATP re-synthesis (Larsen et al., 2011; Vanhatalo et al., 2014).

Assessment of muscle oxygenation after dietary nitrate using near infrared spectroscopy highlighted the significance of the ergogenic mechanism of increased oxygen delivery in a hypoxic environment (Masschelein et al., 2012; Shannon et al., 2017). Shannon et al., (2017) reported ~ 4% increase in total oxygenation index in *m. gastrocnemius* during a 3 km TT in hypoxic environments (11.7% and 14% oxygen) after an acute ingestion of nitrate (12.5 mmol of nitrate) supplementation. Comparably, following six days of nitrate supplementation a study reported a 4% increase in total oxygenation index in the *m. vastus*

lateralis in a hypoxic environment (11% oxygen) during incremental exercise test till exhaustion (Masschelein et al., 2012). In this study, the control supplement used was an apple-blackcurrant juice containing < 5 mmol nitrate/kg. Although the energy content in the form of carbohydrates, fats and protein were similar between the two beverages, blackcurrants and apples both contain other bioactive compounds, such as quercetin and catechin and other antioxidants and polyphenols which could also affect physiological responses and therefore, may not have acted as control (Boyer & Liu, 2004; Karjalainen et al., 2008; Masschelein et al., 2012). Similarly, an increase by 3.6% in total oxygenation of *m. vastus lateralis* during cycling compared to placebo (nitrate depleted beetroot juice), however, this result was not statistically significant (Kelly et al., 2014). Research has found exercise capacity to be enhanced in a hypoxic setting in healthy individuals after 24 hours – 6 days nitrate supplementation, however, there are variable exercise performance results in a hypoxic setting (11-14.5% oxygen) (Arnold, Oliver, Lewis-Jones, Wylie, & Macdonald, 2015; Aucouturier, Boissiere, Pawlak-Chaouch, Cuvelier, & Gamelin, 2015; Bourdillon et al., 2015; Gasier, Reinhold, Loiselle, Soutiere, & Fothergill, 2017; Horiuchi et al., 2017; Kelly et al., 2014; Muggeridge et al., 2014; Nyback et al., 2017; Rossetti et al., 2017; Shannon et al., 2017; Vanhatalo et al., 2011). With some studies showing an improved TT following nitrate supplementation (6 -15 mmol of nitrate), while others observing no statistically significant effect on exercise performance (Arnold et al., 2015; Muggeridge et al., 2014; Shannon et al., 2016; Shannon et al., 2017). The duration of supplementation may be an important factor contributing to the variables results, as all but one of the studies were acutely supplemented (Bourdillon et al., 2015). The chronically supplemented (3 days) nitrate study observing trained cyclists in a hypoxic setting (11% oxygen) did not improve exercise performance (15 km TT) after consuming sodium nitrate (nitrate: 0.1mmol/kg/day,) or placebo (sodium chloride, 0.1mmol/kg/day) in identical gelatin capsules (Bourdillon et al., 2015).

However, the high training status of the participants may have attenuated the ergogenic effects of nitrate during hypoxic TTs in these studies but not all of these studies (Arnold et al., 2015; Muggeridge et al., 2014; Nyback et al., 2017; Shannon et al., 2016).

Based on the increased muscle oxygenation from previous research and the positive effect of dietary nitrate supplementation on endothelial function at sea level, research observing the efficacy of nitrate supplementation in augmenting endothelial function under hypoxic stress are required and very limited (Bondonno et al., 2014; Kapil et al., 2010; Masschelein et al., 2012; Webb et al., 2008). Bakker et al., (2015) observed an offset of altitude induced reduced endothelial function following the ingestion of beetroot juice (5 mmol of nitrate) during an expedition in Nepal (28 days > 2500 m, including a peak of 3700 m) with ten healthy young participants (mean \pm SD age 25 ± 5 years). Blood pressure did not lower, however, the authors suggested the method used may have been less sensitive in contrast to studies at sea level (Bakker et al., 2015; Kapil et al., 2010; Webb et al., 2008). Whereas flow mediated dilatation is mainly dependent on nitric oxide activity, there are several factors that can influence blood pressure in a hypoxic environment such as raised circulating catecholamine, endothelin concentration and modification of sympathetic activity (Hainsworth & Drinkhill, 2007). Similar blood pressure results were replicated in Hennis et al., (2016) in a group of young healthy male students (16 ± 1 years) during ascent to Everest Base Camp. This research demonstrated that dietary nitrate may represent a cost-effective therapeutic strategy for maintaining endothelial function in low land-living cohorts visiting high altitudes. However, as field-based research is known for heterogeneous degree of noise, future studies would require greater sample size.

Hypoxia plays a major role in the reduced functional capacity in a variety of pathological conditions (Allen, Giordano, & Kevil, 2012; Ellis et al., 2010). The comprehension of the beneficiary effects of dietary nitrate in a hypoxic environment may provide significant

inferences for ageing and/or diseased populations. As improving tolerance in hypoxia in an ostensibly healthy cohort may translate into improved quality of life and functional capacity in aged and/or diseased populations.

2.4. Other major bioactive compounds

Dietary nitrate has emerged advantageous in comparison to pharmacological, inorganic nitrate with regards to oxygen consumption throughout high intensity exercise and blood pressure reduction (Flueck, Bogdanova, Mettler, & Perret, 2015; Siervo et al., 2013). Though nitrate does appear to be accountable for most of the physiological benefits, nitrate consumption in the form of food/juice confers additional benefits. Beetroot and other vegetables high in nitrate are packed with other bioactive compounds (e.g. ascorbic acid, magnesium, folate, flavonoids, antioxidants polyphenols, etc) which may augment nitric oxide production from nitrate and provide added effects (Rodriguez-Mateos et al., 2015).

Studies have reported advantageous effects in cycling, running and rowing, with the majority positive findings reported after beetroot consumption in comparison to sodium nitrate or potassium nitrate intake (Bailey et al., 2009; Bailey et al., 2010; Bescos et al., 2012; Bond, Morton, & Braakhuis, 2012; Cermak, Gibala & van Loon., 2012; Christensen, Nyberg, & Bangsbo, 2013; Lansley et a., 2011; Peacock et al., 2012). It is important to acknowledge beetroot is rich in numerous bioactive compounds that may contribute to the aforementioned health benefits. Bioactive compounds are phytochemicals found in plants that have the ability to modify metabolic processes, resulting in better health (Galanakis, 2016). Epidemiological research on the Mediterranean populace, has reported the lowest occurrence of various degenerative disorders, which have been attributed to oxidative stress (Casanova et al., 2013;

Kapiszewska, Soltys, Visioli, Cierniak, & Zajac, 2005). This positive occurrence appears to be linked with the nutritious plant-based diet containing complex bioactive compounds (Casanova et al., 2013).

Beetroot contains a high number of phytochemicals such as, carotenoids, ascorbic acid, flavonoids and phenolic acids which have antioxidant properties (Georgiev et al., 2010; Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002; Wootton-Beard & Ryan, 2011). It is also one of the only vegetables to contain betalains, a unique group of highly bioactive pigments (Vulić et al., 2014). Several studies have observed betalains to have high antioxidant and anti-inflammatory capabilities *in vivo* animal models and *in vitro* (Pavlov, Georgiev, & Ilieva, 2005; Tesoriere, Allegra, Butera, & Livrea, 2004; Vidal, Lopez-Nicolas, Gandia-Herrero, & Garcia-Carmona, 2014; Vulić et al., 2013; Zielinska-Przyjemska, Olejnik, Dobrowolska-Zachwieja, & Grajek, 2009). This has prompted interest for the potential beneficial role of beetroot in clinical pathologies characterised by oxidative stress and chronic inflammation (Das, Williams, Das, & Kukreja, 2013; Ninfali & Angelino, 2013; Pietrzkowski et al., 2010; Vulić et al., 2014). Two studies (crossover, double blind, randomised design) reported after 7 days of betalain-rich beetroot formulation without nitrate or sugar, significantly enhanced endurance exercise, improved recovery and decreased muscle damage compared to the control (Flueck et al., 2015; Montenegro et al., 2017; Van Hoorebeke, Trias, Davis, Lozada, & Casazza, 2016). Results from these studies suggest other bioactive components may have important contributions to ergogenic/cardiovascular/health outcomes. Additionally, research in the past has compared nitrate-depleted beetroot juice to nitrate rich beetroot juice, however, as nitrate depleted beetroot has numerous bioactive compounds, this might restrict the potential to detect significant differences.

Dietary antioxidants, anthocyanates, flavonoids and polyphenols upregulate NOS expression, reduce oxidative stress, and enhance nitric oxide availability (Rodriguez-

Mateos et al., 2015). These bioactive compounds and nitrate may have separate and synergistic additional effects (Rodriguez-Mateos et al., 2015). Certain plant foods (e.g. tea, vegetables, cocoa) comprise of a variety of bioactive compounds with blood pressure lowering/performance enhancing effects (fibre, folate, potassium) and these have been related with increased nitric oxide and cardiac/exercise benefits (Ashworth, Mitchell, Blackwell, Vanhatalo, & Jones, 2015; Jonvik et al., 2016; Jovanovski et al., 2015). A study observed plasma nitrate to increase to a similar extent after consuming beetroot, green vegetables or sodium nitrate on separate occasion (Jonvik et al., 2016). Interestingly, plasma nitrite and blood pressure lowering response were significantly larger and more elongated after green vegetable ($P = 0.016$) (Jonvik et al., 2016).

2.5. Watercress

Nasturtium officinale, more commonly known as watercress, is a semi-aquatic plant from the family *Brassicaceae* (together with cabbage, broccoli, brussels sprouts and mustard). A hardy perennial plant, native to western Asia, India, Europe and Africa, contains a high concentrate of nutrients (Aires, Carvalho, Rosa, & Saavedra, 2013; Cruz, Vieira, & Silva, 2008). The various phytochemicals and nutrient content of watercress in the diet help protect and promote health (Zeb, 2015). Watercress is a nutritious vegetable, high in nitrate and as mentioned an exceptional source of bioactive compounds (named polyphenol and glucosinolates) which synergistically may contribute towards health benefits and clinical pathologies (Hord et al., 2009; O'Neill et al., 2001; Philosoph-Hadas, Meir, Akiri, & Kanner, 1994; Sadeghi et al., 2014; Yazdanparast, Bahramikia, & Ardestani, 2008; Zeb, 2015). However, there are limited studies in humans observing the efficacy of watercress in the context of a high in nitrate supplement.

Research has highlighted watercress's bioactive compounds and their beneficial role in anti-genotoxic and anti-carcinogenic processes both *in vitro* and *in vivo* (Boyd et al., 2006; Gill et al., 2007; Rose, Faulkner, Williamson, & Mithen, 2000). Up until now, research has observed watercress as an anti-carcinogenic and antioxidant capacity in human participants (Fogarty et al., 2013; Gill et al., 2007). For example, in a single blind, randomised, crossover design study, Gill et al., (2007) reported a watercress intervention (85 g daily for eight weeks) to ameliorate DNA damage and increase antioxidant potential in humans (Gill et al., 2007). It was suggested the augmented antioxidant activity in the blood was attributed to the isothiocyanate concentration in the circulation through inhibition of carcinogenesis (Dyba et al., 2010). The reduction in DNA damage might be related, in part, to isothiocyanates formed from the glucosinolates. This decrease in DNA damage was concomitant with a significant increase in lutein (100%), beta-carotene (33%), alpha-tocopherol (26%) and ascorbic acid (35%), the authors proposed these bioactive compounds may have also contributed to this reduction in DNA damage. Further sub-group analysis found a significant interaction with participants who smoked, signified by a lower baseline total antioxidant status, indicating a toxin burden in this subgroup. With this in mind, the study suggested a greater efficacy of watercress supplementation as shown by a greater reduction in DNA damage. The study reported no effect on endogenous antioxidant enzymes such as glutathione peroxidase and superoxide dismutase. It is worth noting, the cohort age ranged from 19-55 years and the participants were not subjected to a restrictive diet, so the results are applicable to the general population (Gill et al., 2007).

Similarly, Fogarty et al., (2013) reported a decrease in exercise induced DNA damage, lipid peroxidation and decreased hydrogen peroxide accumulation after both acute and chronic (8 weeks) watercress supplementation (85 g) in healthy males. In contrast to Gill et al., (2007), the study reported no change in beta-carotene but a marked increase in lipid

soluble antioxidants such as gamma-tocopherol, alpha-tocopherol and xanthophyll, indicating watercress to be an antioxidant rich source. It was proposed the increase of xanthophyll concentration subsequent to watercress ingestion, contributed to the reduced lymphocyte DNA damage (Fogarty et al., 2013; Haegele et al., 2000; Packer, Witt, & Tritschler, 1995). Xanthophyll is a scavenger of singlet oxygen and known for its protective effect of DNA via antioxidant activity (Haegele et al., 2000; Packer et al., 1995). The decreased hydrogen peroxide accumulation would lend itself to indicate watercress may provide effective *in vivo* protection which may be due to the elevated lipid soluble antioxidants and their capacity to scavenge superoxide (Fogarty et al., 2013). Moreover, this study indicated no additional benefits were gained from 8 weeks of watercress supplementation compared to acute supplementation (Fogarty et al., 2013).

2.6. Conclusion

Evidence suggests due to the bioactive properties of this semi-aquatic plant, watercress supplementation may function as a useful strategy to boost endogenous antioxidant defences and assist to defend cellular components from oxidative stress (Casanova et al., 2013; Fogarty et al., 2013; Gill et al., 2007). To the best of the author's knowledge, watercress as a nitrate supplement has yet to be investigated.

Given the reported ability of pharmacological sodium nitrate and beetroot to enhance exercise performance and cardiovascular parameters, we sought to determine whether similar effects are observed when the nitrate dose is administered in the form of nitrate rich watercress juice. This is important as watercress is a natural food product rich in other bioactive compounds, that can be readily ingested as part of the normal diet and has anti-genotoxic and anti-carcinogenic processes both *in vitro* and *in vivo* (Boyd et al., 2006; Gill et al., 2007; Rose et al., 2000).

In summary, this thesis involved three experimental trials with the following hypotheses:

Study 1 hypothesis; Acute nitrate supplementation will increase plasma nitrate and nitrite concentration, reduces blood pressure, increases forearm blood flow, alters oxidative/nitrosylative stress markers and enhances 16.1 km time trial performance in a physically active, healthy, young, male cohort.

Study 2 hypothesis; Acute watercress supplementation will increase plasma nitrate and nitrite concentration, enhances vascular parameters and alters oxidative/nitrosylative stress markers in both the normoxic and hypoxic conditions in comparison to the control conditions in a physically active, healthy, aged population.

Study 3 hypothesis; We hypothesised the healthy, physically active cohort would demonstrate a repeatable performance over the 16.1 km time trial performance one week apart. We hypothesised the venous occlusion plethysmography occlusion results and, the oxidative and nitrosylative stress markers measures one week apart would not be repeatable and to have a high variation.

The following Chapter 3 describes the general methods used in the three experimental trials (Chapter's 4, 5 and 6).

Chapter 3: General Methods

3.1. Introduction

The methods described below are implemented in the experimental chapters in this thesis (Chapter 4, 5 and 6); therefore, descriptions of measures unique to individual experimental chapters are not included but can be located in the methods section of the specific chapter.

3.1.1. Ethical approval and informed consent

The research in this thesis was approved by the Sports, Health and Exercise Science Department Ethics Committee at the University of Hull (approval number 1516013 and 1617162, see appendices A and B) before commencing data collection complied with the Declaration of Helsinki. All exercise tests were conducted in an exercise physiology laboratory at sea level with an ambient temperature of 20 - 23 °C. Healthy active participants between the age of 18 and 73 years were recruited through emails and posters. Participants received an information form outlining the experimental protocol and what was required of them. Written consent for the study and a medical history questionnaire was filled out on the participants first visit the lab, any questions were answered before testing. Participants were told they could withdraw from the study at any point. The potential risks and benefits were clearly stated, participants were assigned a unique identification number preserving anonymity. The participants were informed the data collected may be published in an academic journal or presented at a conference.

3.1.2. Health and safety

All testing procedures followed the health and safety guidelines of the Sports, Health and Exercise Science School, School of Life Sciences, Faculty of Health Sciences, University of Hull. The Physical Activity Readiness Questionnaire was reviewed and signed at the start of each visit. The laboratory was clean, safe and suitable for the exercise testing of participants. All respiratory equipment and heart rate monitors were submerged in Milton

solution for a minimum of 15 minutes, rinsed in water for 15 minutes and dried prior to use. All venous blood samples collected during testing were performed by a trained member of the research team who had attended training at Castle Hill Hospital and received Hepatitis B inoculation. During blood collection a laboratory coat, nitrile gloves and goggles were worn at all times. In accordance with institutional risk assessments, all sharps and all materials that came into contact with the blood were disposed of in clinical sharps waste bins for incineration.

3.1.3. Participants

Participants were recruited from the University and the local community. Participants were non-smokers, not taking any dietary supplements at the time of data collection and believed to be free from disease. All participants were physically active (as defined by the World Health Organisation someone who completes 150 minutes of moderate intensity aerobic physical activity a week or do at least 75 minutes of vigorous-intensity physical activity throughout the week, or an equivalent combination of moderate- and vigorous-intensity activity).

3.2. Procedures

3.2.1. Anthropometric measurements

Participants were asked to remove any heavy clothing and footwear before the measure was recorded. On arrival participant's body mass was measured to the nearest 0.05 kg using a SECA balance scale (Vogel & Halke, Hamburg, Germany). Height was measured to the nearest 0.1 cm with a stadiometer (Holtain Ltd, Crymych, Dyfed, Wales).

3.2.2. Blood pressure

Blood pressure of the brachial artery was measured using an automated sphygmomanometer (Omron M6, Omron Healthcare UK LTD, Milton Keynes, UK). Participants were instructed to remain seated in an upright position using the back support from the chair, and the participant's legs uncrossed, feet flat on the ground. All measurements were taken from the left arm, the arm was supported on a table, and the cuff was placed above the elbow. Measurements were recorded to the nearest systolic and diastolic (millimetres of mercury, mmHg). Triplicate measurements were taken after the participant remained seated for 10 minutes. The mean of the final two measurements was recorded.

3.2.3. Heart rate

Heart rate was collected during exercise using a telemetry device (Polar A1HRM, Polar Electro Oy, Finland). An adjustable strap connected to a belt containing two electrodes with a transmitting device was carefully placed approximately over the xiphoid process with electrode gel to augment readings. The heart rate was transmitted via waveforms to the receiver on SRM power meter software (Schoberer Rad Mebtechnik, Konigskamp, Germany) and to the wristwatch allowing for continuous recording of heart rate expressed in beats per minute (beats/min).

3.2.4. Cardiac output and stroke volume

Cardiac output (\dot{Q}) and stroke volume (V_s) were collected using the Innocor™ (Innocor, Odense, Denmark) inert gas rebreathing unit with breath-by-breath ergospirometry and arterial oxygen saturation sensor (Innovision, Odense, Denmark).

In accordance with the Innocor manual prior to measurement, the ambient information was input from the laboratory's weather station (Oregon Scientific, UK), and the flowmetric and oxygen calibrations were completed. Each participant was assigned a new mouthpiece connected to a bacterial filter (Innovision, Odense, Denmark). Throughout the test, a nose clip (Innocor, Odense, Denmark) was worn to prevent any expired gas escaping. Participants breathed through an airtight closed-circuit system (Innocor, Odense, Denmark) containing a gas mixture of 0.5% nitrous oxide (blood soluble gas), 0.1% sulphur hexafluoride (blood soluble gas), 28% oxygen in nitrogen in a 4-litre rubber bag. Rebreathing was performed over 30 seconds with a gas volume of 300% of the predicted tidal volume and a breathing rate of 18 min⁻¹. Gas was sampled continuously from the mouthpiece for analysis by the infrared gas analyser. Participants were instructed to breathe synchronously to a graphical tachometer on the computer screen to control for constant ventilation rate. Participants were also requested with every breath to empty the rebreathing bag to ensure consistent ventilation volume. The computer software calculated the cardiac output from the rate of uptake of nitrogen into the blood (slope of the regression line through logarithmically transformed expiratory nitrogen concentration plotted against time), while the system's gas volume was corrected using the end-tidal sulphur hexafluoride concentration. Participants performed 5 - 8 breaths, after correction for system volume changes using sulphur hexafluoride concentration the first two or three breaths were excluded from the analysis due to initial incomplete gas mixing.

3.2.5. Venous occlusion plethysmography

Venous Occlusion Plethysmography (VOP) was used as it provides an alternate of endothelial function in the small, resistance arteries and whole limb blood flow. Forearm blood flow was measured using VOP (EC-6, D.E. Hokanson, Inc., Bellevue, WA, USA) and was set up in conformity with Hokanson guidelines using the participant's dominant arm. A strain gauge was applied to the broadest part of the participant's forearm; circumference was measured to allow for correct strain gauge size selection (maximum circumference minus 2 cm). The output signal was transmitted to the non-invasive Vascular Program (NIVP) 3 calibrated with arterial inflow studies software (Hokanson, Bellevue, USA). A cuff was placed around the participant's bicep (SC10D, Hokanson, Bellevue, USA) connected to a rapid cuff inflator (E20, Hokanson, Bellevue, USA) to occlude venous outflow. During the testing, the cuff was set above venous arterial blood pressure (50 mmHg) but below arterial pressure. A wrist cuff (Standard Adult Cuff, Accoson, Essex, United Kingdom) was inflated to 200 mmHg (supra-arterial pressure) for constant pressure to exclude circulation to the hand due to the complex arterio-venous shunts; this was inflated for 2 minutes before each test begun and remained occluded for the forearm blood flow measurement.

Test protocol: The participant was seated for 20 minutes preceding each test, the procedure was explained during this time. Once set up was complete the participant laid in a supine position with arm above the level of the heart, supported at the elbow and at the wrist with foam pillows to ensure the strain gauge did not touch the surface. Once the wrist cuff had occluded the blood flow (~200 mmHg for 2 minutes), the recording began on the software, and the bicep cuff was inflated to 50 mmHg for 10 seconds at a time, three times. During the 10 seconds intervals, the changes in the forearm were detected by the silastic strain gauge through changes in the electrical resistance. Three readings were recorded at rest, 30 minutes post supplementation and 120 minutes post-supplementation.

Data were extracted using proprietary software (Lab Chart Reader, ADInstruments, UK) and blood flow was expressed millilitres (mL) per minute per decilitre (dL) of forearm tissue.

3.2.6. Peak oxygen uptake

Before commencing study 2 and study 3 participants completed an incremental exercise test to volitional exhaustion to calculate the peak oxygen uptake ($\dot{V}O_{2\text{peak}}$) and peak power output. Breath by breath data was measured using a Cortex Metalyzer 3B (Cortex Biophysic, Leipzig, Germany) gas analysis system. Following the manufacturer's instruction, the Cortex Metalyzer 3B (Cortex Biophysic, Leipzig, Germany) was calibrated before each test. The turbine flow meter was calibrated with a 3-litre syringe (model 5530, Hans Rudolph, Kansas, USA) to determine minute ventilation. Certified standard calibration gases of 17.35% O₂ and 5.7% CO₂ (Cranlea & Company, Bournville, United Kingdom) were used for gas analysis calibration. Ambient conditions including barometric pressure (mmHg), room temperature (°C) and relative humidity (%) were recorded from a weather station (Oregon Scientific, UK) in the laboratory.

All tests were performed on an SRM (Schoberer Rad Mebtechnik, Konigskamp, Germany) cycle ergometer in a temperature-controlled laboratory (20 - 23 °C). Before the test, the cycle ergometer was adjusted appropriately, and saddle and handlebar settings were recorded for any subsequent tests. Participants warmed up for 5 minutes with 50 W resistance, once warm-up was complete the test began straight away with watts increasing by 25 W/min. The test ended when the participant reached volitional exhaustion and was unable to continue or when the pedal rate dropped below 60 RPM. During the incremental test heart rate was recorded every minute and rate of perceived exertion (RPE) using the Borg scale (1973) were recorded every second minute throughout the test. Gas analysis was continuously collected throughout the entirety of the test.

3.2.7. Rate of perceived exertion

The Borg's scale (1973) was used to quantify the rate of perceived exertion (RPE). The Borg scale displays numeric values ranging from 6 indicating "very, very light" or 20 indicating "maximum", the scale was used by participants throughout the peak oxygen uptake test.

3.2.8. Collection of venous blood

Participants assumed a supine position before tourniquet was tightly secured around the distal region of the bicep. A protuberant ante-cubital forearm vein was selected and wiped using a sterilised swab saturated with 70% v/v isopropyl alcohol (Bunzl Retail and Healthcare Supplies Limited, UK). The Vacutainer (Greiner-Bio One Ltd, Gloucestershire, United Kingdom) method was used to collect all blood samples. Blood was collected using di-potassium ethylene diamine tetra-acetic acid (EDTA) or serum separation tubes (SST).

After blood collection, EDTA vacutainers were placed on ice whilst SST's could clot in the dark at room temperature (20 - 25 °C) for ten minutes before centrifugation at 1300 x g (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) for ten minutes. Plasma and serum were removed using a 1ml pipette and transferred to two 2 ml Eppendorf and frozen (- 80 °C) for later analysis.

3.2.9. Supplementation

Nitrate supplementation was administered in the form of watercress juice, beetroot juice and sodium nitrate. The dosage was selected in line with the commercially available beetroot juice (~ 400 mg of nitrate) (James White, Suffolk, United Kingdom), which is estimated to be around 4 - 12 times greater than the average daily nitrate intake in the USA and Europe (Bailey et al., 2009; Hernández et al., 2012; Jones et al., 2018; Larsen et al., 2007; Vanhatalo et al., 2010). The watercress juice (lot number 406062016) was

supplied by Vitacress, Southampton, UK. Beetroot juice was purchased from the company Beet it, James White Drinks, Ipswich, UK. Sodium nitrate was purchased from Sigma-Aldrich Company, Dorset, UK. Participants were instructed to ingest the supplements or water (<0.1 mg of nitrate) (Buxton Water, UK) within 60 seconds.

A washout period of a minimum of 7 days separated each supplementation period. Participants were instructed to follow their regular dietary habits throughout the testing period. Participants were warned the supplementation may cause temporary urine discoloration (beeturia) and red stools. In addition to this, participants were asked to abstain from using antibacterial mouthwash and chewing gum for the duration of the study since this has been shown to blunt the conversion of nitrate to nitrite- in the oral cavity (Govoni et al., 2008). All supplementation was well tolerated with no adverse problems reported.

3.3. Biochemical analysis

3.3.1. Nitrate/Nitrite

Nitrate/Nitrite was determined according to the manufacturer's guidelines, using a commercially available fluorometric assay kit (Cayman Chemical, Ann Arbor, USA). Both plasma and serum samples were thawed on ice and filtered using a Microcon-10kDa Centrifugal Filter Unit with an Ultracel-10 membrane (Millipore, Cork, Ireland). The Microcon® was inserted into the tube, and 100 µl of sample was pipetted into the device ensuring not to touch the membrane with the pipette tip. The tube was then sealed with a cap, and all samples are placed in a centrifuge for 20 minutes at 13100 g (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK). The tube was then separated from the filter device and placed on ice till further assay instructions.

A standard curve in a total volume of 1 ml was prepared using the nitrate standard and assay buffer with a range of 0.156 - 10 μ M. Assay buffer (80 μ l) was added to the two blank wells in the 96-well solid white plate, followed by 30 μ l of assay buffer and 50 μ l of the standard were pipetted into the remaining 12 wells. Subsequent to this 10 μ l of the sample were aliquot in duplicates, and 70 μ l of assay buffer was added to adjust the volume to 80 μ l in the sample wells. Enzyme cofactor mixture was aliquotted (10 μ l) followed by nitrate reductase (10 μ l) to each well, the plate was then covered and left to incubate at room temperature (20 - 25 $^{\circ}$ C) for 2 hours to ensure >95% conversion. After required incubation time 10 μ l of DAN reagent was added into each well followed by a 10-minute incubation. Finally, 20 μ l of sodium hydroxide (2.8 M) was added to each well. The plate reader (Tecan, Infinite M200 pro, software, Magellan) quantified the nitrate in the wells using an excitation wavelength of 360 - 365 nm and a slit width for emission wavelength of 430 nm.

For the measurement of nitrite, a standard curve in a total volume of 1 ml was prepared using the nitrite standard and assay buffer with a range of 0.156 - 10 μ M. Assay buffer (100 μ l) was added to the blank wells in the 96-well solid white plate, followed by 50 μ l of assay buffer and 50 μ l of the standard were pipetted into the remaining 12 wells. After this ten μ l of the sample were aliquot in duplicates, ensuring no bubbles entered the well. Ninety μ l of assay buffer was added to adjust the volume to 100 μ l in the plate. DAN reagent (10 μ l) was added into each well followed by a 10 - minute incubation. Finally, 20 μ l of sodium hydroxide (2.8 M) was added to each well, followed by the plate being read using an excitation wavelength 360 - 365 nm and a slit width for emission wavelength of 430 nm in a plate reader (Tecan, Infinite M200 pro, software, Magellan) quantifying the nitrite in the wells.

3.3.2. Nitrotyrosine

Nitrotyrosine was measured using a commercially available Enzyme-Linked Immunosorbent Assay kit (ab210603, Abcam, Cambridge UK). The Nitrotyrosine standard and assay buffer were prepared in a total volume of 500 μl to create the standard curve with a range of 62.5 – 800 μM . Fifty μl of standards were added to the wells, followed by 50 μl of samples, all in duplicate. Nitrotyrosine Antibody Preparation was then added to each well except the blank. The blank contained 50 μl of the Standard and Sample Diluent and 50 μl of Antibody Diluent; the plate was then covered and left to incubate at room temperature (20 - 25 $^{\circ}\text{C}$) for 1 hour. After the incubation, each well was washed with the assay wash buffer (300 μl) four times before adding 100 μl of TMB substrate into each well. The plate was carefully covered and placed in the dark at room temperature (20 - 25 $^{\circ}\text{C}$) for 30 minutes, allowing the enzymatic reaction to take place yielding a blue solution. This reaction was terminated by adding 100 μl of the stop solution to each well. Absorbance was read immediately on a plate reader (Tecan, Infinite M200 pro, software, Magellan) at a wavelength of 450 nm.

Microsoft Excel (version 15.26) was used for analysis; the standard curve was calculated using the blank corrected values against the known concentrations of the Nitrotyrosine. With the standard curve the Pearson coefficient of determination (R^2) value was examined to indicate the correlation coefficient. The assay was deemed successful when the R^2 was > 0.98 . The values for each sample were calculated from each duplicate well, and the concentration was determined in μM by dividing the average value of each well against the slope of the line of best fit for the Nitrotyrosine standard curve. The concentration found in μM was then multiplied by the dilution factor of the sample to determine the final concentration of each sample.

3.3.3. Lipid hydroperoxides

Lipid peroxidation was measured using the ferrous oxidation in xylenol orange (FOX-1) assay in line with established methods (Jiang et al., 1992). This method determines the estimation of oxidation of ferrous to ferric ions by hydroperoxides at a low pH with xylenol orange (Jiang, Hunt, & Wolff, 1992). The xylenol orange binds to the ferric ions yields a purple/blue-coloured reaction.

Samples were defrosted on ice and centrifuged (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) at 1750 g for 5 minutes at 4°C. Standards concentration ranged from 0 – 5 µM and were prepared using hydrogen peroxide (Alfa Aesar, L1400, Lot: 10194511) mixed with distilled water (18.2 MΩ). The reagent was prepared in a total volume of 100 ml of 25 mM sulphuric acid (Amresco, 0499, Lot: 1125C446), containing 100 µM of xylenol orange (21120010, Sigma-Aldrich, Dorset, UK), 100 µM sorbitol (Alfa Aesar, 36404, Lot: T30A064) and 250 µM ammonium iron (II) sulphate (Acros Organics, 423721000; Lot: A03655885). In a clear bottom 96-well plate format (Corning, Costar, CLS3599, Sigma) 20 µl standards or samples in duplicates were pipetted into each well, followed by 180 µl of FOX-1 with each well consisting of a final volume of 200 µl per well. The plate was placed in the dark at room temperatures (20 - 25 °C) for 30 minutes to incubate. Absorbance was then measured at 560 nm, using a multimodal plate reader (Tecan, Infinite M200 pro series, software: Magellan).

Microsoft Excel (version 15.26) was used for analysis; the standard curve was calculated using the blank corrected values against the known concentrations of the hydrogen peroxide. With the standard curve; the y-intercept was set to 0 and Pearson coefficient of determination (R^2) value was examined to indicate the correlation coefficient. The assay was deemed successful when the R^2 was > 0.98 . The values for each sample was calculated from each duplicate well, and the concentration was determined in µM by dividing the average value of each well against the slope of the line of best fit for the

hydrogen peroxide standard curve. The concentration found in μM was then multiplied by the dilution factor of the sample to determine the final concentration of each sample.

A number of samples were found to be above the standard curve concentration. Consequently, these samples were diluted and re-tested to find optimum dilution; this was indicated when the samples were found in the mid-range of the standard curve.

3.3.4. Protein Carbonyls

The method described by Levine (2002) was used to determine carbonylation of protein. Both plasma and serum samples were thawed on ice and centrifuged (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) at 1750 g for 5 minutes at 4°C. The 2,4-Dinitrophenylhydrazine (DNPH) (Lot: BCBS2288V, Sigma-Aldrich, Dorset, UK) was prepared by adding 500 μl of 0.2 M stock to 9.5 ml of phosphoric acid 0.5 M (Lot: MKBV7289V, Sigma-Aldrich, Dorset, UK) to create a 10 mM solution. From the Bradford protein assay, the equivalent of 1 mg of protein of sample was added per well, each sample was in duplicate, and PBS acting as a standard was also in duplicate. Followed by 100 μl of distilled water (18.2 M Ω) and 100 μl of the DNPH solution were added. The plate was placed in the dark at room temperature (20 - 25 °C) for 10 minutes to incubate. Following incubation 50 μl of 6 M sodium hydroxide (Batch: 15E260035, VWR Chemicals, Leicestershire, UK) was added to each well and incubated in the dark at room temperature (20 - 25 °C) for 10 minutes. Absorbance was then measured at 450 nm using a multimodal plate reader (Tecan, Infinite M200 pro series, software: Magellan). Protein carbonyl content was expressed as $\mu\text{M}/\text{mg}$ cellular protein.

Microsoft Excel (version 15.26) was used for analysis of the protein carbonyl assay. Samples were blank corrected and then, using the Lambert-Beer law to calculate the concentration of carbonyls using equation 1.

Equation 1.

$$C=A/\epsilon L$$

Where: **C** = concentration

A = Absorbance of the sample (unitless)

ϵ = molar extinction coefficient or molar absorptivity of the protein ($M^{-1} \text{ cm}^{-1}$)

L = light path length (cm)

From this the carbonyl content ($\mu\text{M}/\text{mg}$) was calculated from protein carbonyl concentration per amount of corrected protein.

3.3.5. Bradford protein assay

The Bradford protein assay for the measurement of protein concentration for both plasma and serum samples used. Bovine serum albumin (422361V, VWR Leicestershire, UK) standards were prepared in a total volume of 100 μl of phosphate-buffered saline (PBS), with concentrations ranging from 0 – 1.4 mg/ml. Using a clear bottom 96-well plate (Corning, Costar, CLS3599, Sigma-Aldrich, Dorset, UK) 10 μl of sample was added with 190 μl of PBS to each well. Before the next step, each well was methodically mixed with a multichannel pipette. Samples were then diluted one more time, 50 μl of each well in the original plate was carefully transferred to a new clear bottom 96-well plate, and 150 μl of PBS was added to each well. Once carefully mixed, 5 μl of the samples were transferred into the final plate along with five μl of standards, with 195 μl of Bradford reagent (SLBV5669, Sigma-Aldrich, Dorset, UK) was added on top. The plate was placed in the dark, at room temperature (20 - 25 $^{\circ}\text{C}$), left to incubate for 20 minutes. Absorbance was measured at wavelength 595 nm using a multimodal plate reader (Tecan, Infinite M200 pro series, software: Magellan).

Microsoft Excel (version 15.26) was used for analysis of the Bradford protein assay; the standard curve was calculated using the blank corrected values against the known concentration of the bovine serum. With the standard curve; the y-intercept was set to 0 and Pearson coefficient of determination (R^2) value was examined to indicate the amount of variance in the samples after they were corrected against the blank. The assay was deemed successful when the R^2 was > 0.98 . The values for each sample was calculated from duplicate wells, and the concentration was determined in $\mu\text{g}/\mu\text{l}$ by dividing the average value of each well against the slope of the line best fit for the bovine serum standard curve. The concentration found in μg was then multiplied by the dilution factor of the sample to determine the final concentration of each sample.

3.4. Temperature and humidity regulation

Laboratory temperature and humidity were controlled and recorded using a weather station (Oregon Scientific, UK).

3.5. Statistical analysis

Computerised statistical analysis was performed using the Statistics Package for Social Sciences (SPSS) (version 24, IBM Corp., NY. USA). All variables were analysed for normal distribution by descriptive statistics and the Shapiro-Wilk test ($P < 0.05$). Normally distributed continuous variables are presented as mean \pm standard deviation and non-normally distributed continuous variables as median (inter-quartile range; 25% and 75%), and finally, categorical variables as frequencies (percentages). Normal distribution is an arrangement of a data set in which the highest frequency is in the middle of the range and the smaller frequencies toward either extreme, in the shape of a symmetrical bell-shaped curve. Parametric statistical tests were performed for normally distributed data and non-parametric tests were performed for non-normally distributed data.

Chapter 4:
**Effect of nitrate supplementation on
vasodilatory responses, oxidative stress
biomarkers and a 16.1 km cycling time
trial**

4.1. Introduction

In recreational and athletic populations, ergogenic aids are frequently consumed with the assumption that the supplement will enhance exercise performance (Burke, Castell, & Stear, 2009). Specifically, in sports events that require high levels of effort for a prolonged period, such as track and cycling, supplements may be most beneficial (Burke et al., 2009; Jeukendrup & Gleeson, 2018).

Nitrate supplementation has become increasingly popular in recent years, and has become a commercially available and a well-established product either by ingesting sodium nitrate or beetroot juice, (Hoon et al., 2013; Jones et al., 2018; McMahon, Leveritt, & Pavey, 2017). Evidence has shown dietary nitrate to reduce oxygen uptake during submaximal exercise and to improve exercise tolerance when observing exercise to exhaustion protocols (Bailey et al., 2009; Bailey et al., 2010; Cermak et al., 2012; Lansley, Winyard, Fulford, et al., 2011; Larsen, Weitzberg, Lundberg, & Ekblom, 2007; Vanhatalo et al., 2010). Previously Lansley et al., (2011) reported a 2.8% and 2.7% improvement over 4 km and 16.1km time trial performance (TT) following a single dose of beetroot juice (500 ml of ~ 6.2 mmol of nitrate). The consumption of this nitrate-rich supplement is thought to enhance vasodilation, improve blood flow dynamics, augment mitochondrial respiration by possibly reducing electron leakage, reduce the oxygen cost of mitochondrial ATP re-synthesis, improve skeletal muscle contractility and reduce fatigue development and thus has become a popular ergogenic aid (Bailey et al., 2009; Bailey et al., 2010; Stamler & Meissner, 2001; Vanhatalo et al., 2010; Webb et al., 2008).

Beetroot juice is currently one of the more popular nitrate ergogenic aids. Studies observing beetroot juice have reported enhanced muscle efficiency by reduced oxygen cost during submaximal exercise, thus increasing endurance exercise and augmented muscle contractile function, resulting in improved muscle power and sprint-type exercise

(Bailey et al., 2009; Thompson et al., 2016; Vanhatalo et al., 2010; Wylie et al., 2016). Research suggests the effectiveness of beetroot juice is mostly attributed to the high inorganic nitrate content (typically ranging from 300 – 520 mg of nitrate), although this does not facilitate any specific physiological function (Alexander et al., 2008). It is the reduction to nitric oxide, a diverse messenger molecule that these aforementioned advantageous physiological effects are attributed to (Hobbs, et al., 2013). However, nitrate is not the only constituent of beetroot proposed to have beneficial effects in health and disease.

The majority of nitrate research has utilised beetroot juice and has often directed its mechanism of action towards the nitrate content, there are of course a whole range of active chemicals within any food source that could aid in enhancing exercise performance that may not necessarily be nitrate dependent. Furthermore, it is likely that the biochemical compounds within whole foods will act in a synergistic manner across a whole range of physiological reactions. Beetroot is also found to be a rich source of bioactive compounds, such as ascorbic acid, carotenoids, phenolics and betalains (Kujala et al., 2002; Wootton-Beard & Ryan, 2011). The dark rich purple colour and yellow pigments are known as betalains, presenting potential for antioxidant and other bioactive capabilities (Kanner, Harel, & Granit, 2001). Studies have observed a wide array of physiological actions such as effects on blood pressure, endothelial function, platelet reactivity and antioxidant defence systems, to name a few (Duffy et al., 2001; Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Holt et al., 2002; Sies et al., 2005; Taubert, Berkels, Roesen, & Klaus, 2003; Yang, Hong, Hou, & Sang, 2004). Although the mechanism by which flavanols mediate vascular effects are not fully known, research suggests flavanol-rich foods are able to improve nitric oxide bioavailability and, thereby, increase vasodilation, highlighting the importance of the different effects of phytochemical compounds in food (Auger, Chaabi, Anselm, Lobstein, & Schini-Kerth,

2010; Duffy et al., 2001; Schroeter et al., 2006). Due to the complex nature of beetroot, it is unclear which bioactive components underlie these effects.

Thus, comparison with a different plant-derived drink rich in nitrate could offer insights into the function constituents of plant derived ergogenic aids. Watercress (*Rorippa nasturtium-aquaticum*) is high in nitrate and is an exceptional source of bioactive compounds; including, several phytochemicals; glucosinolates, caftaric acid, carotenoids such as lutein and beta-carotene and flavonoids, such as kaempferol, and quercetin (Hord et al., 2009; O'Neill et al., 2001; Zeb, 2015). It has previously been shown that watercress attenuates DNA damage in healthy adults and reduces the risk of cancer (Gill et al., 2007). Fogarty et al., (2013) corroborated this after watercress supplementation exhibited a prophylactic effect after exhaustive exercise, observing a reduction DNA damage, and a decrease in lipid peroxidation and hydrogen peroxide concentration compared to the control. Furthermore, watercress in animal and cell models have been shown to exert a range of cardio-protective effects and a favourable role in anti-genotoxic and anti-cancer processes (Boyd et al., 2006; Gill et al., 2007; Rose, Faulkner, Williamson, & Mithen, 2000). These studies have shown an enhanced antioxidant status, a reduction in total cholesterol concentration, glucose levels, and with a reduction in oxidative damage and immune enhancing effects (Asadi, Mirvaghefi, Nematollahi, Banaee, & Ahmadi, 2012; Bahramikia & Yazdanparast, 2010; Mousa-Al-Reza Hadjzadeh, Moradi, & Ghorbani, 2015; Shahani, Behzadfar, Jahani, Ghasemi, & Shaki, 2017). The efficacy of watercress supplementation from human trials are limited, it is postulated the health-related benefits associated with watercress ingestion might at least partly result from the downstream metabolites of the glucosinolates, beta-carotene, lutein and alpha-tocopherol (Fogarty et al., 2013; Gill et al., 2007).

Therefore, watercress juice and beetroot juice both rich in nitrate and other bioactive compounds were compared to isolated sodium nitrate to attempt to understand the

aforementioned mechanisms and offer insights into the function constituents of plant derived ergogenic aids.

The purpose of this study was to determine the effect of an alternative nitrate supplement, watercress juice (Vitacress, Southampton, UK), to a well-established, commercially available, concentrated beetroot juice (Beet It; James Whites Drinks, Suffolk, United Kingdom), to sodium nitrate, and water acting as the controls in a healthy active population. The study aimed to assess the acute effect of the nitrate matched supplements on 16.1 km time trial, cardiovascular parameters and oxidative stress biomarkers in a healthy active population.

4.1.1. Hypotheses

We hypothesised that the acute dietary nitrate supplements will increase plasma nitrate and nitrite concentration, reduce resting blood pressure, increase blood flow volume, alter oxidative/nitrosylative stress markers and enhance exercise performance in an active healthy male population.

4.2. Methods

4.2.1. Participants

Ten healthy active males ($n = 10$) volunteered to participate in the present investigation and were recruited using email and poster advertisement in the University of Hull staff and student population. All participants completed a physical activity readiness questionnaire and as a minimum requirement volunteers were required to be physically active as defined by the World Health Organisation (engage with 150 minutes of moderate-intensity aerobic physical activity throughout the week or, at least 75 minutes of vigorous-intensity aerobic physical activity throughout the week, or an equivalent combination of moderate- and vigorous-intensity activity). Further inclusion/exclusion criteria are outlined in Table 4.1. Only one participant did not comply with the study protocol and was excluded. Prior to participation the protocol and the potential risks were outlined with verbal and written informed consent granted. The experiment was approved by the Research Ethics Committee of the Sports, Health and Exercise Science School at the University of Hull and conformed to the standards set by the Declaration of Helsinki.

Table 4.1. Inclusion/exclusion criteria

Inclusion criteria	Exclusion criteria
Between 18 – 50 years old	Smoking
Physically active	History of cardiovascular/metabolic disease
Male	Medication
Healthy	Supplements

4.2.2. Study design

Using a randomised (counter-balanced), single blind (to the participant) cross-over design, each participant reported to the laboratory on five occasions over a five - week period. During the first visit, participants performed a maximal incremental test to exhaustion for determination of VO_{2peak} and peak power output (PPO) on a cycle ergometer (Schoberer Rad Messtechnik (SRM), Germany), anthropometric data was also recorded (Table 4.2.). Participants were requested to refrain from caffeine and alcohol, six and twenty-four hours before the first experimental session, respectively. Participants were instructed to avoid any exercise forty-eight hours prior to exercise.

Participants were randomly (Research Randomizer Version 4.0, Urbaniak & Plous 2013) assigned a single dose nitrate matched supplement (400 mg of nitrate) or a control supplement, Buxton water containing < 0.1 mg/l, (Buxton, UK). All tests were completed at 9 am of day (± 1 h) to minimise diurnal variation. Before the first supplementation session, participants were requested to fill out a qualitative activity and food diary for forty-eight hours, participants were instructed to repeat this diary each time before visiting the lab.

4.2.3. Visit 1: Baseline measures

4.2.3.1. Anthropometric

Anthropometric measurements were performed as described in General methods Chapter 3, section 3.2.1.

4.2.3.2. Blood pressure

Participants were asked to relax for ten minutes in a temperature (20 - 23 °C) controlled, quiet room. Blood pressure readings were recorded using an Omron (Omron M6, Omron Healthcare UK LTD, Milton Keynes, UK) automated sphygmomanometer, measurements were taken in triplicate. For detailed protocol please see General Methods Chapter 3, section 3.2.2.

4.2.3.3. *Peak oxygen uptake*

Prior to the $\dot{V}O_{2\text{peak}}$ test the participant's saddle and handlebar height and handlebar position were recorded and reproduced for subsequent visits. All tests took place on an SRM (Schoberer Rad Mebtechnik, Konigskamp, Germany) cycle ergometer in a temperature-controlled laboratory (20 - 23 °C). Online gas was quantified using a Cortex Metalyzer 3B (Cortex Biophysic, Leipzig, Germany) for breath by breath analysis. Participants warmed up for five minutes with 50 W resistance, once warm up was complete the test began straight away with watts increasing by 25 W/min. The test ended when the participant reached volitional exhaustion and was unable to continue or when the revolutions per minute dropped below sixty. During the incremental test, heart rate was noted every minute and every second minute rate of perceived of exertion (RPE) using the Borg scale (1973) was noted. Gas analysis was continuously collected.

Table 4.2. Anthropometric measures

Characteristic	Mean \pm SD
Body mass (kg)	74.9 \pm 7.3
Age (yrs)	31 \pm 7
Height (cm)	178.7 \pm 6.0
BMI (kg/m²)	23.4 \pm 2.0
VO_{2peak} (ml/kg/min)	52.4 \pm 6.1
PPO (W)	303.3 \pm 43.6

All data are presented as mean \pm standard deviation. Kg = kilograms, yrs = years, cm = centimetres, BMI = body mass index, kg/m² = kilograms per metres squared, VO_{2peak} = peak oxygen consumption, ml/kg/min = millilitres per kilogram per minute, PPO = peak power output, W = watts, (n = 9).

4.2.4. Visits 2 – 5: Supplementation visits

See Figure 4.1.1 for an overview of the experimental protocol. Approximately one week after the maximal exercise test, each participant completed the first of the four separate supplementation sessions. Participants fasted for a minimum twelve hours (water only permitted) prior to attending the laboratory. During the first supplementation visit participants were given low nitrate water (Buxton water Ltd, Buxton, UK) to consume post supplementation and prior to starting the 16.1 km time trial (TT). The amount of water was calculated using National Institute for health and care excellence guidelines: 34 ml/kg/day (NICE guidelines, 2017) for over the course of two hours. Participants consumed the same amount of water for all four supplementation sessions. Participants were forbidden from consuming food during all sessions. Once baseline measures were

recorded participants were randomly assigned in a single-blind crossover design to consume a single dose of either: 184 ml of watercress (Vitacress, Southampton, United Kingdom), 70 ml of beetroot juice (Beet It, Suffolk, UK), 400 mg (~ 6.45 mmol) of sodium nitrate (VWR, Leicestershire, UK) dissolved in water (Buxton water, Buxton, UK) or 184 ml of water (Buxton water Ltd, Buxton, UK) acting as a control. Participants were instructed to consume supplement within 20 seconds. There was a minimum of seven days wash out period between each supplement. Participants were requested to avoid mouthwash and chewing gum for the duration of the study period as it has been reported to attenuate the conversion of nitrate to nitrite (Govoni et al., 2008).

4.2.4.1. Anthropometric

On arrival participant's mass (Vogel & Halke, Hamburg Germany) was recorded to ensure no significant changes over the four-week period.

4.2.4.2. Blood pressure

Blood pressure measurements were recorded at baseline, 120 minutes post supplementation (See General Methods Chapter 3, section 3.2.2.).

4.2.4.3. Venous occlusion plethysmography

Blood flow volume measurements were recorded at baseline and 120 minutes post supplementation. See the General Methods Chapter 3, section 3.2.5.

4.2.4.4. Cardiac output

Cardiac output was recorded at baseline and two hours post supplementation. Please see the General Methods Chapter 3, section 3.2.4. for protocol.

4.2.4.5. Blood collection

Participants fasted for twelve hours overnight prior to provision of a venous blood sample. This protocol restricts the dietary effect of blood borne metabolites such as antioxidants and lipids (Pronk, 1993). Samples were obtained for serum and plasma, as described in

general methods (See section 3.2.8.), at baseline, two hours post supplementation and post exercise.

After blood collection, EDTA vacutainers were placed on ice whilst SST's clotted in the dark at room temperature (10 minutes) before centrifugation at 1300 x g (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) for ten minutes. Plasma and serum were removed using a 1 ml pipette and transferred to a 2 ml cryovial and immediately frozen (- 80 °C) prior to analysis.

4.2.4.6. Time Trial

Participants completed 16.1 km time trial (TT) on the SRMTM ergometer with integrated heart rate monitoring (Polar Electro, Finland), see Chapter 3, section 3.2.3. in General Methods. Participants completed four supplementation visits, seven days apart. Time trials were repeated at the same time of day, to avoid diurnal variation over the course of the four weeks. Participants were asked to cover the 16.1 km distance as quick as possible. Before each trial participants performed five minutes warm up. The ergometer (SRM) was programmed in the mode “open end test”. In this mode, power output varies with pedal rate and/or a gear change. Participants started the test in gear nine and could change gear once the test had started. Each TT began after a numerical countdown from a stationary start. For each TT power, heart rate, distance and time was recorded by the SRM software. To simulate a time trial, no blood samples or cardiorespiratory measurements were taken during the time trial. To avoid experimental bias, the only feedback available to cyclists during the trial was the distance covered. No verbal encouragement was given so to ensure every participant had the same setting. Exercise tests were performed at sea level (4 m), this is important as the reduction of nitrite to nitric oxide is potentiated in hypoxia (Vanhatalo et al., 2011). Once the 16.1 km TT was completed a final venous blood sample was collected, followed by blood flow

measurement and cardiac output. A schematic overview of experimental protocol is outlined in Figure 4.1.1.

4.2.5. Biochemical analysis

4.2.5.1. Nitrate and nitrite

Nitrate and nitrite were determined using a commercially available fluorometric kit (Cayman Chemical, Ann Arbor, USA) (General Methods Chapter 3, section 3.3.4). The intra-assay coefficient of variation (COV) was < 6% for nitrite and < 5% for nitrate.

4.2.5.2. Nitrotyrosine

Nitrotyrosine was measured using a commercially available Enzyme-Linked Immunosorbent Assay kit (ab210603, Abcam, Cambridge UK) (General Methods Chapter 3, section 3.3.5). The intra-assay COV was < 6%.

4.2.5.3. Lipid hydroperoxides

Lipid hydroperoxides were measured spectrophotometrically using the method of Wolff (1994) (General Methods Chapter 3, section 3.3.1). The intra-assay COV was < 6%.

4.2.5.4. Protein Carbonyls

Protein Carbonyls derivatives were measured according to the method of Levine (2002) (General Methods Chapter 3, section 3.3.3). The intra-assay COV was < 7%.

4.2.5.5. Bradford assay

Protein concentration was measured spectrophotometrically using the Bradford assay (General Methods Chapter 3, section 3.3.2). The intra-assay COV was < 5%.

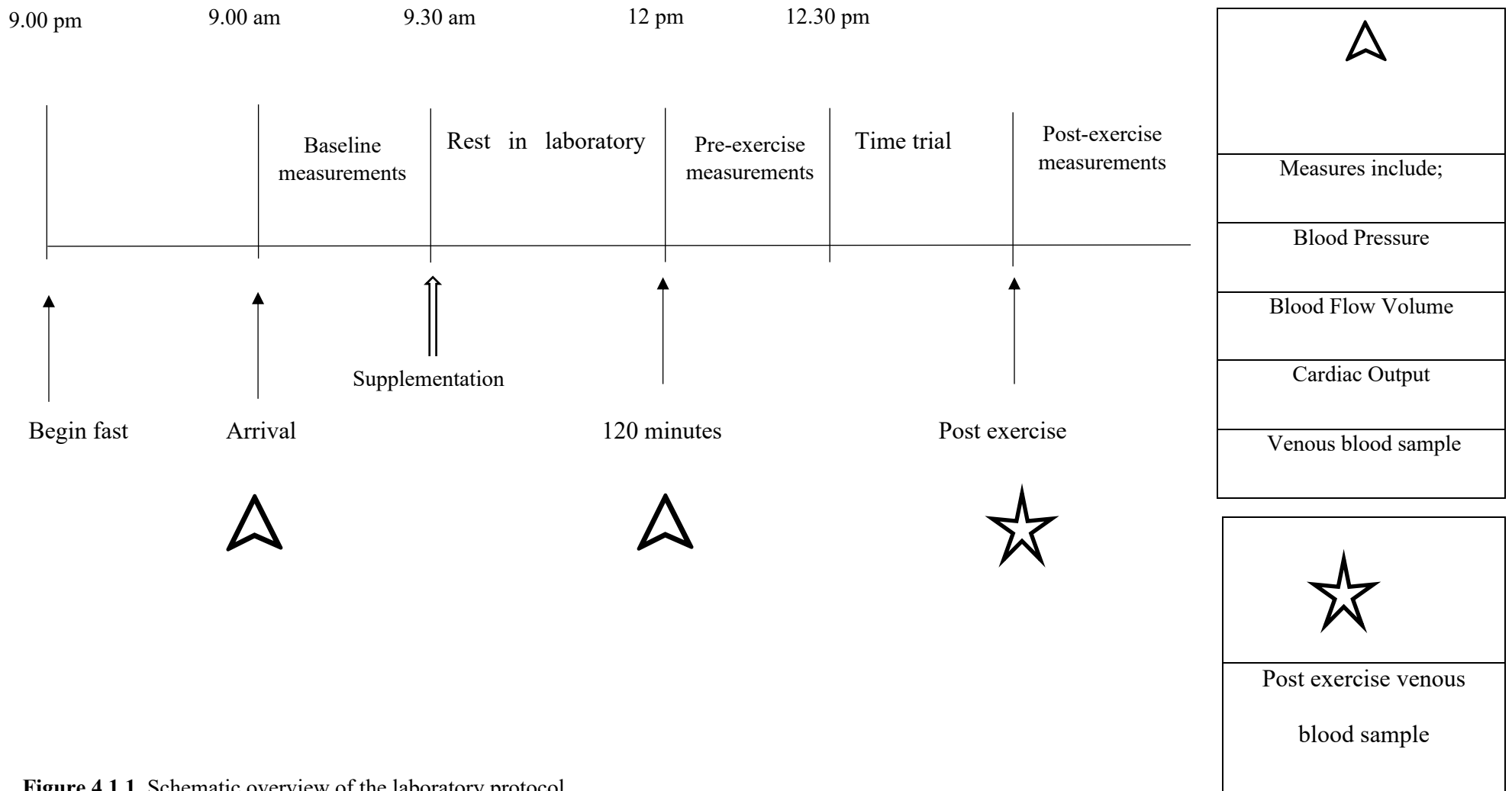


Figure 4.1.1. Schematic overview of the laboratory protocol

4.2.6. Statistical analysis

Data was analysed using IBM SPSS (version 24, IBM Corp., NY, USA). Prior to statistical analysis a Grubb's test was run and examination of studentized residuals for values greater than ± 3 standard deviations to detect outliers. The adherence rate for the experiment was 90% with one participant's data being removed prior to analysis due to non-compliance with the study protocol. Therefore, the data was analysed and presented as $n = 9$. All variables were analysed for normal distribution by descriptive statistics and the Shapiro-Wilk test ($P < 0.05$). Normally distributed continuous variables are presented as mean \pm standard deviation and non-normally distributed continuous variables as median (inter-quartile range; 25% and 75%), and finally, variables as frequencies (percentages). A one-way repeated measure analysis of variance (ANOVA) was used to determine whether there was a statistically significant difference in supplementation on time trial performance times. A two-way repeated measures ANOVA was used to examine differences between supplements over time. Bonferroni post-hoc analysis was run to identify where the significant difference occurred. Cohen's d effect sizes (ES) were calculated to determine the practical significance, with the magnitude of effects considered trivial (< 0.19) small ($0.2 - 0.59$), medium ($0.6 - 1.19$) and large ($1.2 - 1.99$) and very large (> 2.00) (Hopkins et al., 2009). Statistical significance was set at $P < 0.05$ prior to analyses.

4.3. Results

Markers of vasodilation

4.3.1. Nitrate and nitrite

Data for plasma nitrate and nitrite concentration at baseline, two hours post-ingestion and post-exercise are presented in Figure 4.2.1 and Figure 4.3.1. Baseline values for plasma nitrate concentration ($P = 0.48$) and nitrite ($P = 0.18$) were not significantly different between the four interventions. At two hours post-ingestion and post-exercise plasma nitrate concentrations were significantly elevated ($P < 0.001$; $ES > 0.8$) in all nitrate supplements (see Figure 4.2.2), in comparison, the water supplementation did not change significantly from baseline ($P = 0.855$). Individual nitrate and nitrite values over time for each supplement is shown in Figure 4.2.2 and Figure 4.3.2, respectively. Plasma nitrite concentrations were not significantly elevated for all four interventions at the two hours post-ingestion time point or post exercise ($P = 0.36$; Figure 4.3.3.).

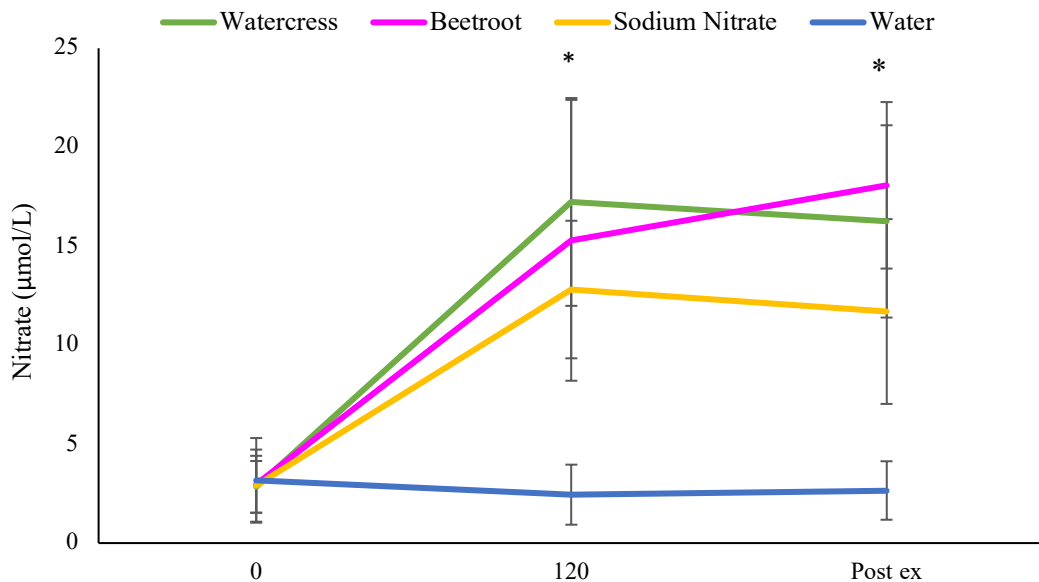


Figure 4.2.1. Plasma nitrate concentrations in all four supplements at 0, 120 minutes and post exercise ($n = 9$). Values are mean \pm SD. * Denotes significant difference for all three nitrate supplements vs water supplementation, ($P < 0.05$).

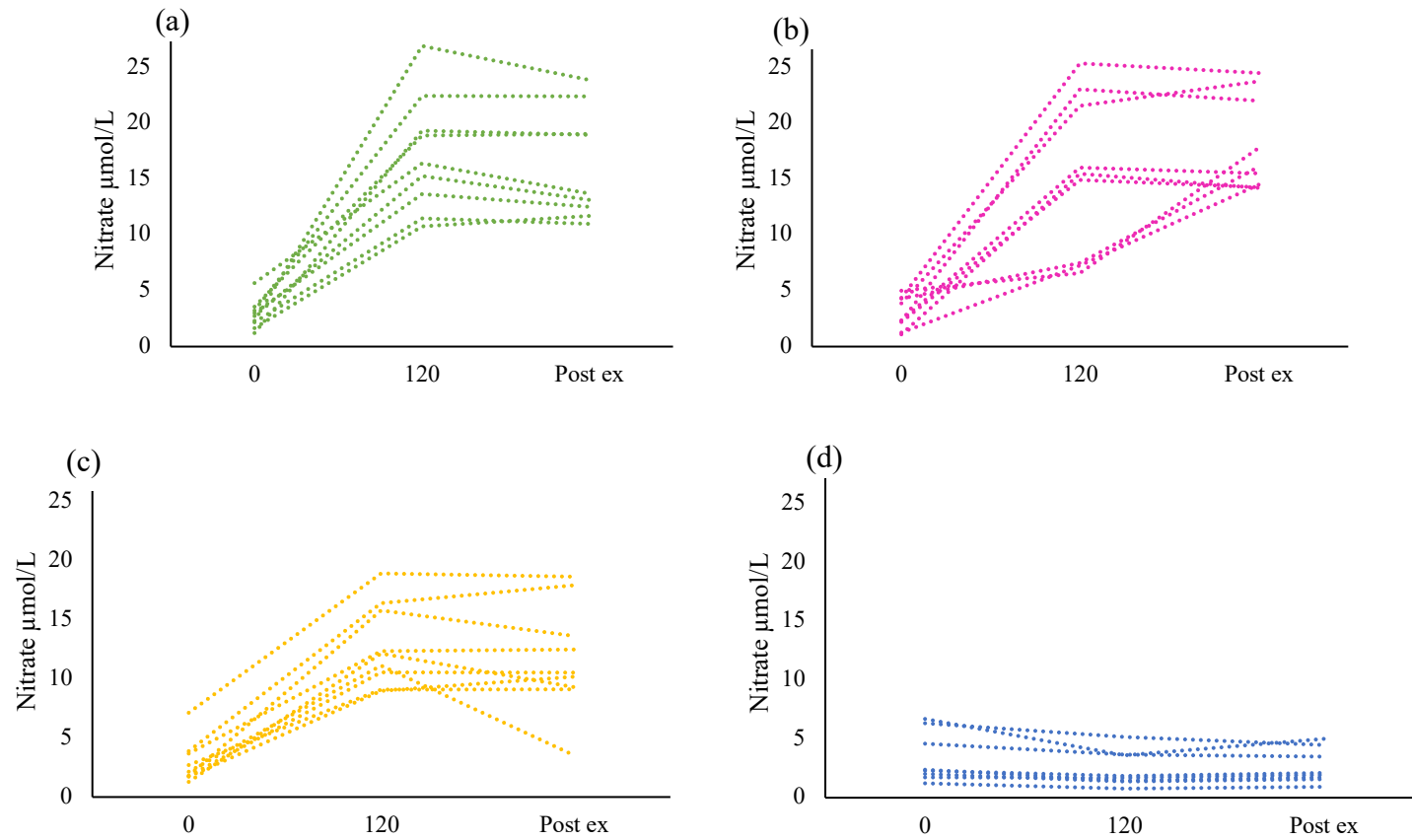


Figure 4.2.2. Individual nitrate concentration in plasma at 0, 120 minutes and post exercise, (a) watercress, (b) beetroot, (c) sodium nitrate, (d) water (n = 9).

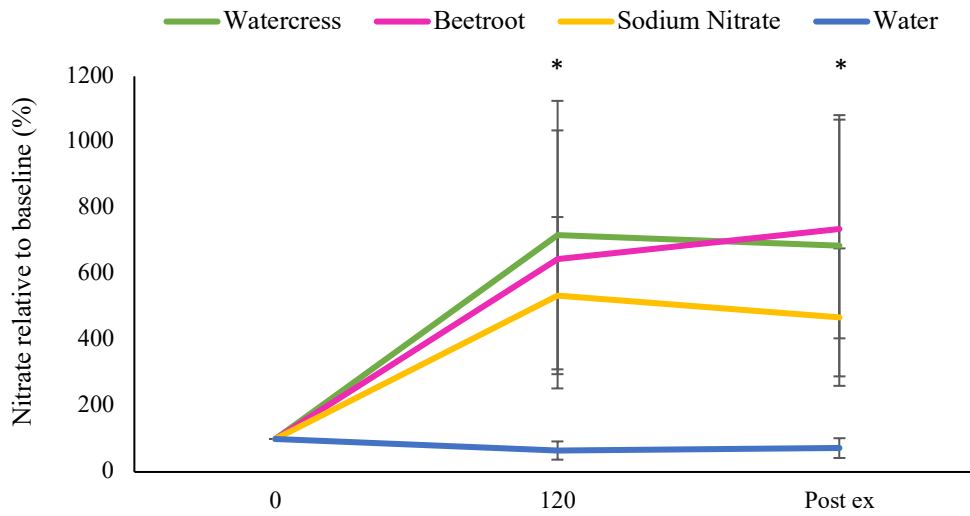


Figure 4.2.3. Change of nitrate concentration relative to baseline for all supplements at 0 (baseline = 0), 120 minutes and post exercise (n = 9). Values are mean \pm SD.

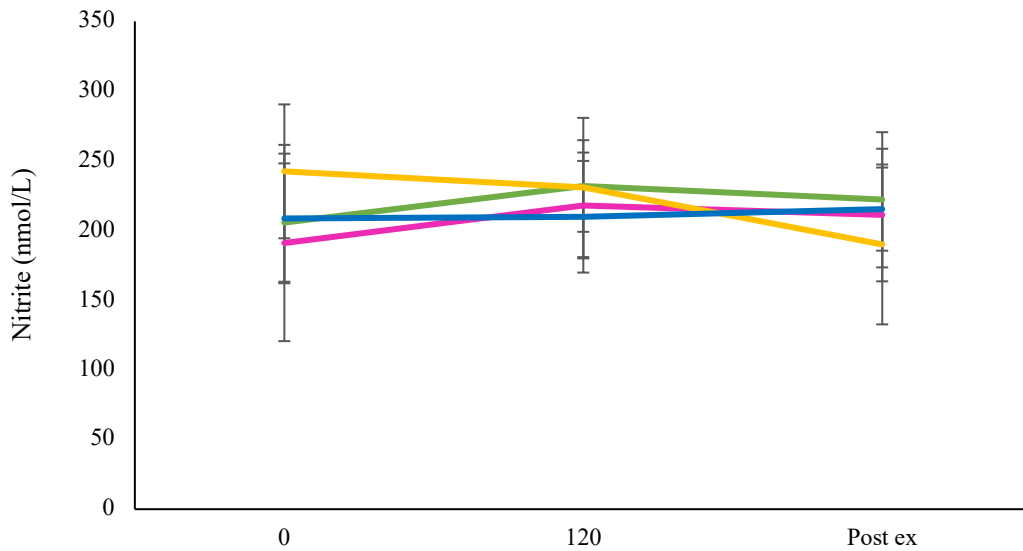


Figure 4.3.1. Plasma nitrite concentrations in all four supplements at 0, 120 minutes and post exercise, (n = 9). Values are mean \pm SD.

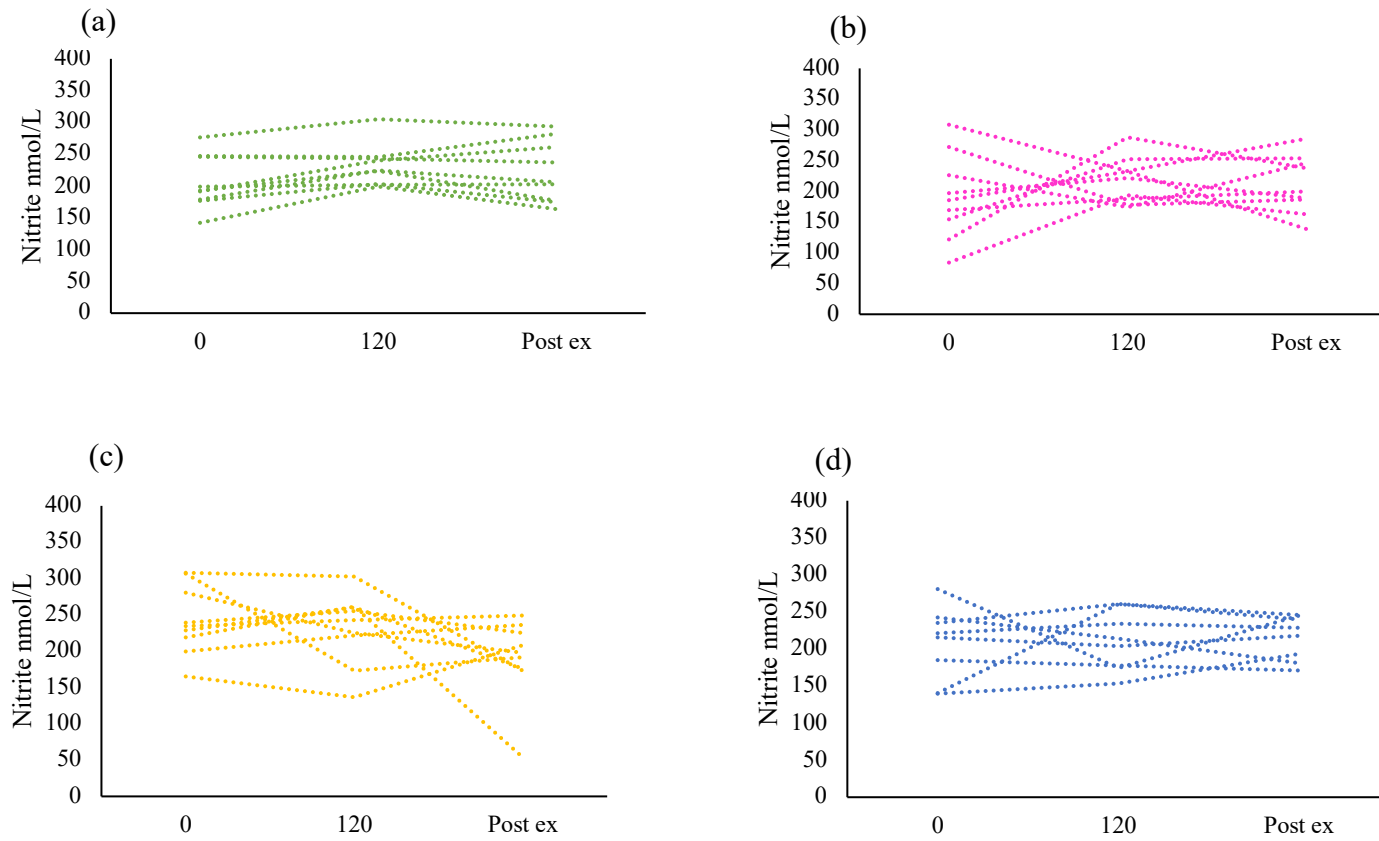


Figure 4.3.2. Individual nitrite concentration in plasma at 0, 120 minutes and post exercise, (a) watercress, (b) beetroot, (c) sodium nitrate, (d) water (n = 9).

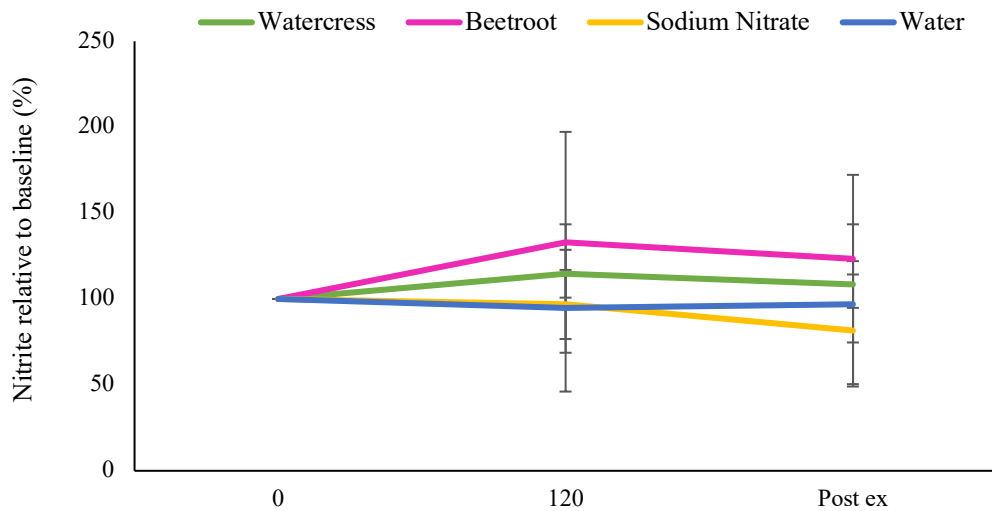


Figure 4.3.3. Change of nitrite relative to baseline for all supplements at 0 (baseline = 0), 120 mins post and post exercise (n = 9). Values are mean \pm SD.

4.3.2. Blood flow volume

A two-way repeated measure ANOVA observed a significant change over time ($F(1,8) = 8.087$, $P = 0.022$). Post-hoc analysis with a Bonferroni test revealed water supplementation blood flow volume significantly decreased from baseline $0.042 (\pm 0.034) \text{ %/min}$ to 120 minutes post supplementation $0.035 (\pm 0.028) \text{ %/min}$ ($P = 0.032$), a small ES (0.32) was evident (see Figure 4.4.2.). The watercress supplementation increased slightly (7%) from 0.041 %/min to 0.045 %/min , however, this was not significant. All other supplements decreased two hours post-ingestion. The water supplementation over the two hours consistently decreased for all participants, in comparison to the three nitrate supplements which observed mixed effects, see Figure 4.4.1. To control for inter individual variability Figure 4.4.3 displays percentage for all four supplements relative to baseline.

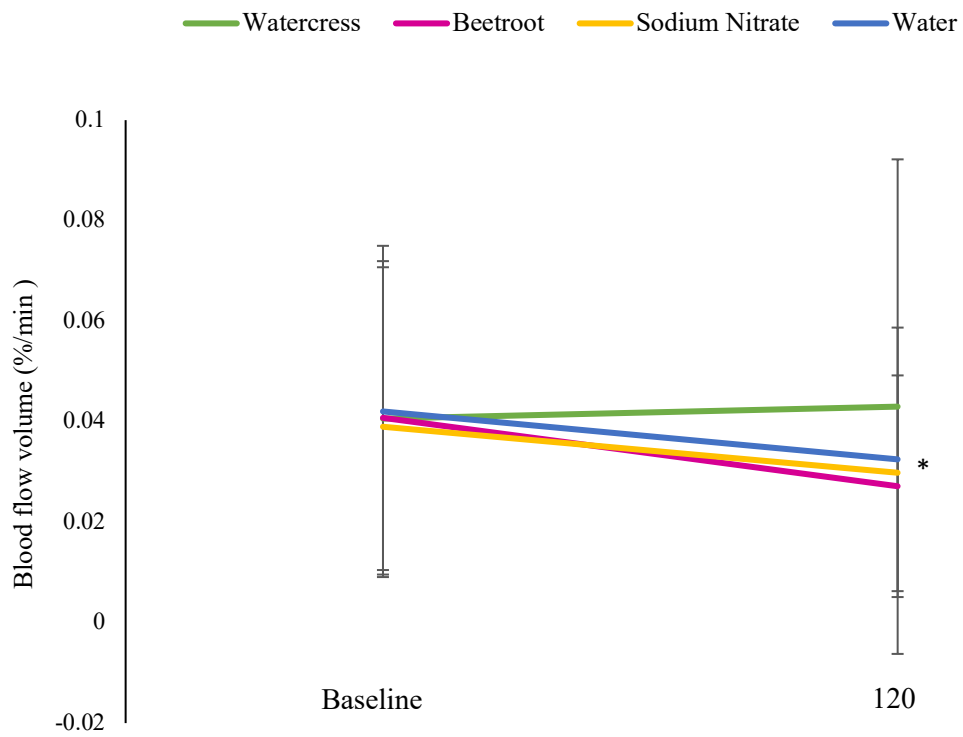


Figure 4.4.1. Comparison of blood flow volume (%/min) at baseline to 120 minutes post-ingestion (n= 8). *denotes difference compared to rest (< 0.05). Values are mean \pm SD.

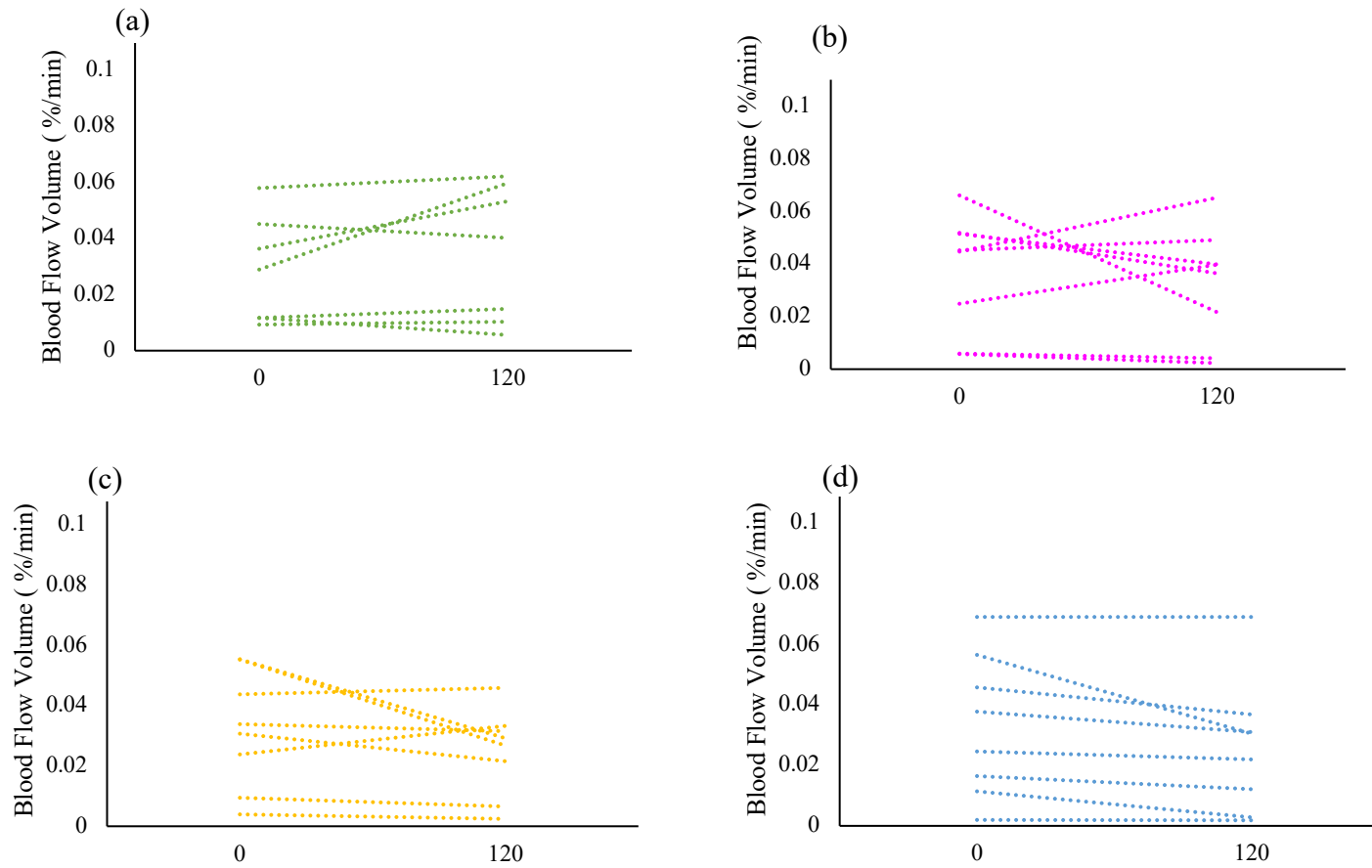


Figure 4.4.2. Individual blood flow volume (%/min) at 0 and 120 minutes, (a) watercress, (b) beetroot, (c) sodium nitrate, (d) water (n= 8).

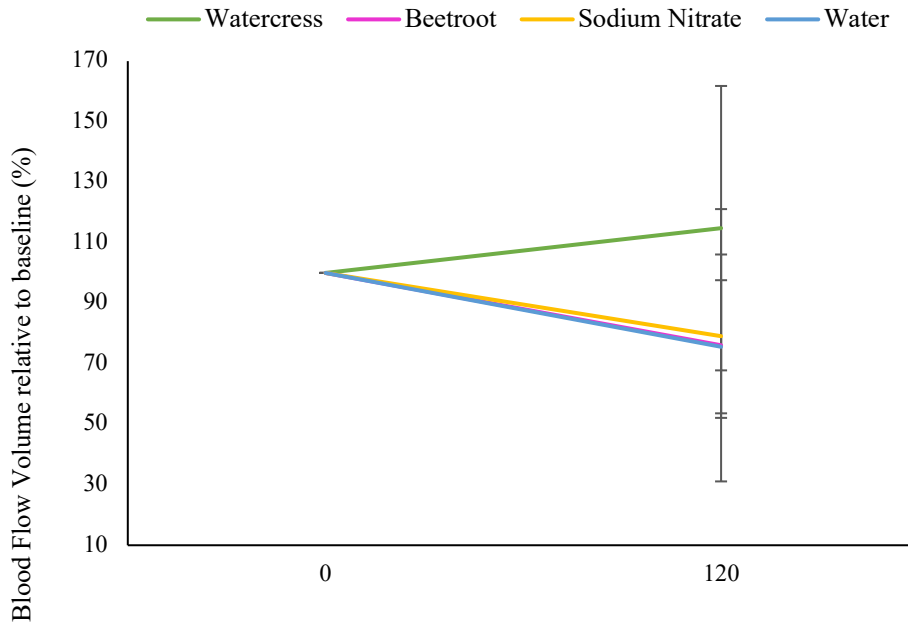


Figure 4.4.3. Change of blood flow volume relative to baseline (baseline = 0) for all four supplements at 0 and 120 minutes (n = 8). Values are mean \pm SD.

4.3.3. Blood pressure

Resting systolic and diastolic blood pressure at baseline were not significantly different between the four interventions (systolic: $P = 0.86$; diastolic: $P = 0.70$). No significant differences were found from baseline to two hours post-ingestion for blood pressure measures in all four supplements ($P > 0.05$), see Table 4.3.

Table 4.3. Systolic and diastolic blood pressure for each condition.

	0 mins	120 mins
SYS BP (mm Hg)		
Watercress	125 ± 11	124 ± 12
Beetroot	124 ± 11	126 ± 13
Sodium Nitrate	125 ± 11	123 ± 9
Water	126 ± 11	128 ± 14
DIA BP (mm Hg)		
Watercress	72 ± 9	70 ± 12
Beetroot	71 ± 9	71 ± 8
Sodium Nitrate	71 ± 7	70 ± 8
Water	73 ± 9	75 ± 10

Values are mean ± SD (n = 9). SYS BP; systolic blood pressure, DIA BP; diastolic blood pressure

4.3.4. Cardiac output

Resting cardiac output did not significantly differ at baseline ($P = 0.21$). A two repeated measure ANOVA observed no significant differences between treatment and time for cardiac output measures in all four supplements ($F(3, 15) = .752, P = 0.538$). In the watercress condition the baseline measure increased from 3.7 ± 0.8 to 4.4 ± 1.3 L/min, ES 0.40 (as seen in Figure 4.5.1. and Figure 4.5.2). Whereas, the rest of the conditions decreased with the beetroot and water condition observing ES 0.25, 0.26, respectively.

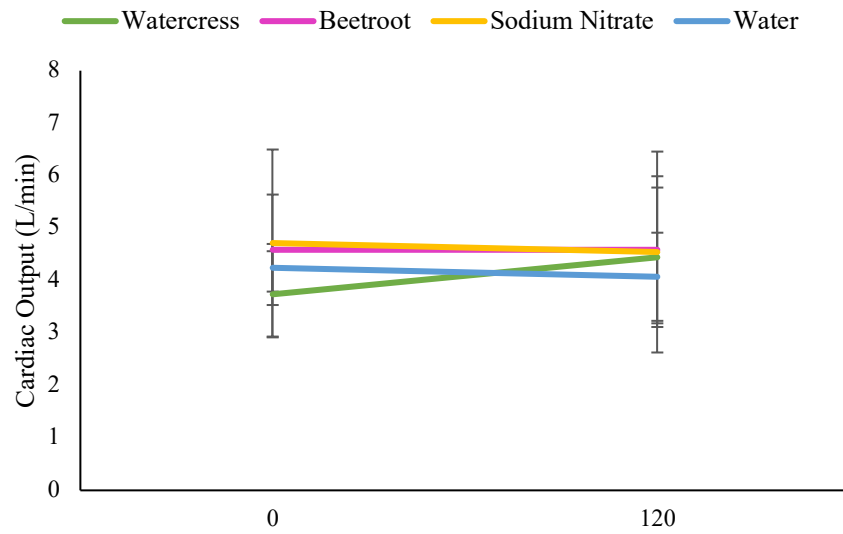


Figure 4.5.1. Comparison of cardiac output at baseline to 120 minutes post-ingestion (n= 6). Values are mean \pm SD.

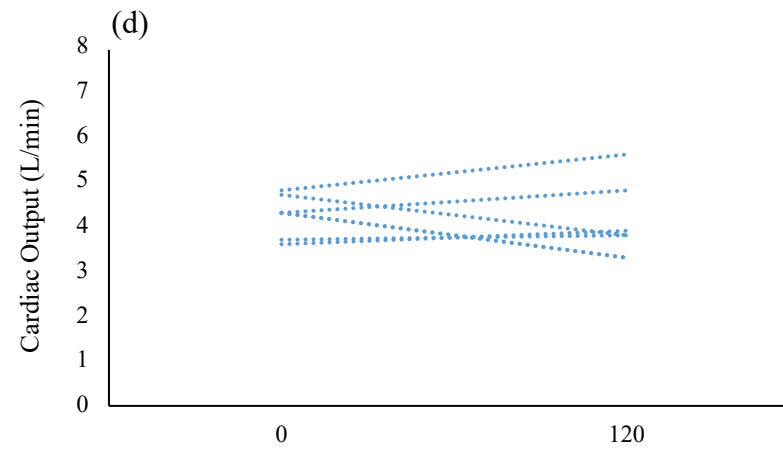
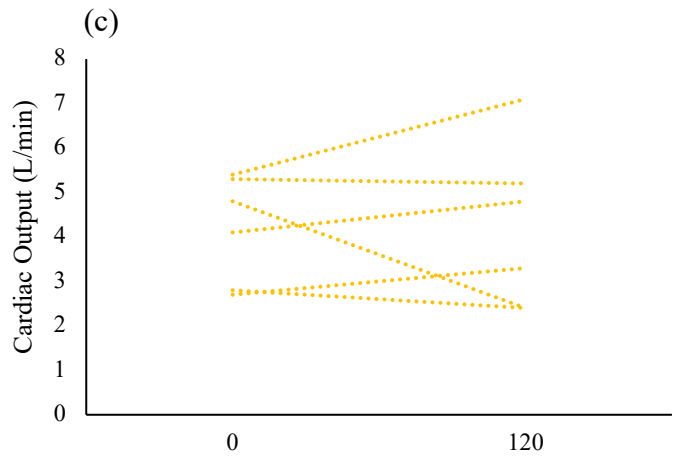
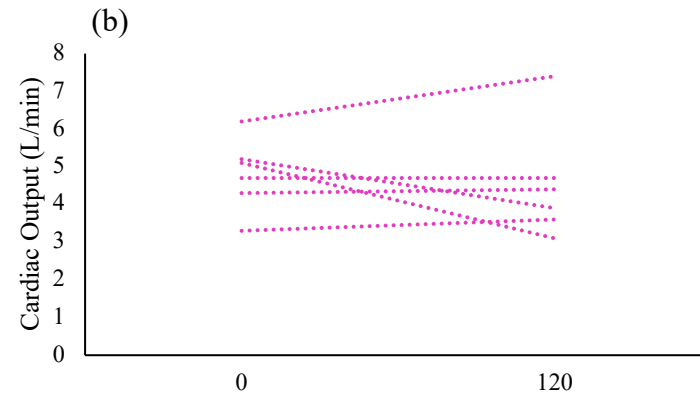
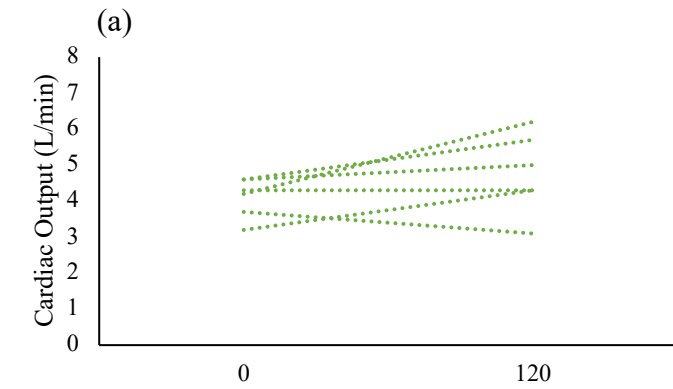


Figure 4.5.2. Individual cardiac output (L/min) at 0 and 120 minutes, (a) watercress, (b) beetroot, (c) sodium nitrate, (d) water (n = 6).

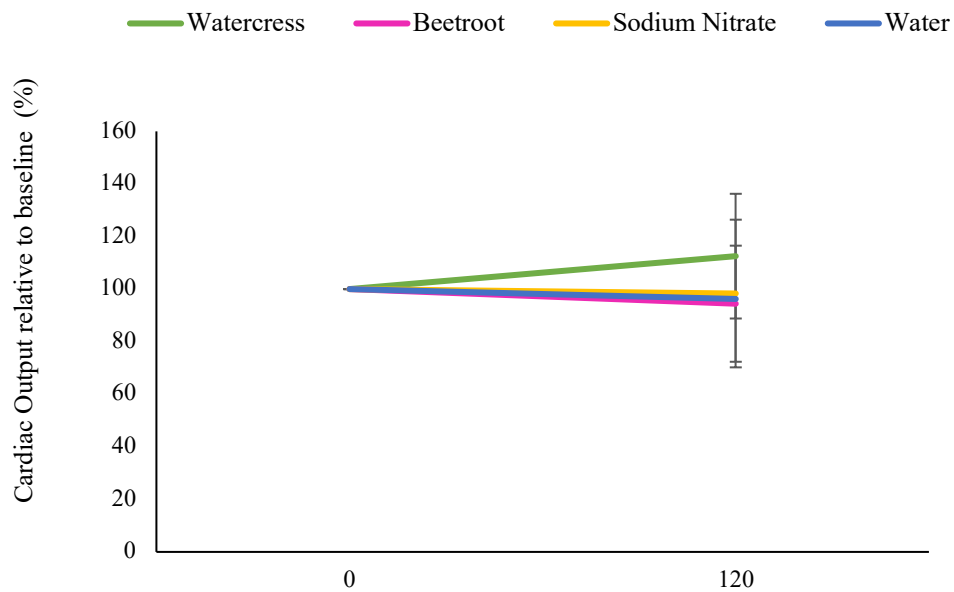


Figure 4.5.3. Change of cardiac output relative to baseline for all four supplements at 0 (baseline = 0) and 120 minutes (n = 6). Values are mean \pm SD.

4.3.5. Time trial performances

A one-way repeated measures ANOVA observed no statistically significant effect between supplements on time trial performance, ($F(3, 24) = 1.271$, $P = 0.307$). Watercress supplementation had the quickest time (30 seconds faster, 1.4%) than the slowest supplementation water, percentage change relative water supplementation is presented in Figure 4.6.1. Further, Cohen's effect size value ($ES = 0.28$) for watercress supplementation suggested a small practical significance, while beetroot juice and sodium nitrate effect size suggested trivial significance ($ES = .06$, $ES = 0.12$, respectively) in comparison to the control, water. Individual responses to supplementation are shown in Figure 4.6.2. Five of the nine participants recorded their fastest time trial time after watercress consumption, while two participants observed their faster time trial time after consuming water.

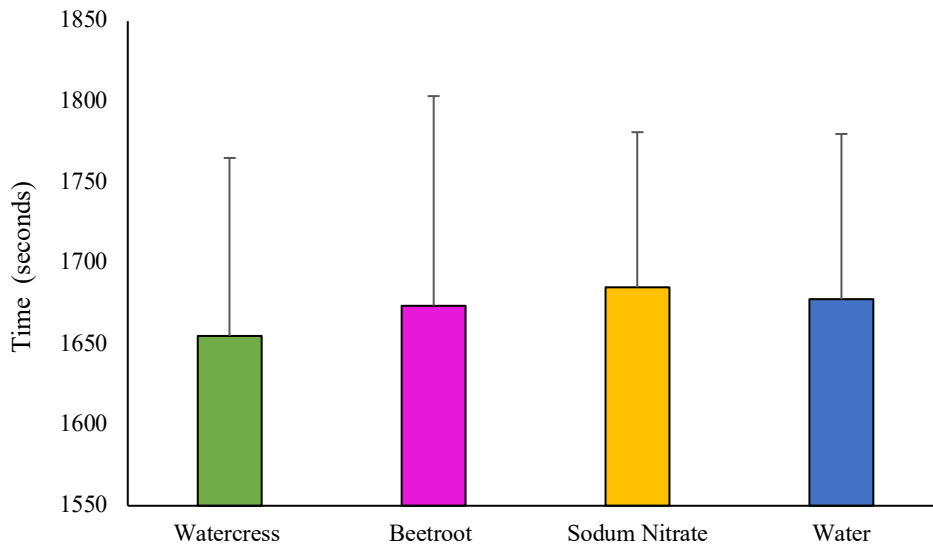


Figure 4.6.1. Time to complete 16.1 km TT for all four conditions (n = 9). Data are presented as mean \pm standard deviation.

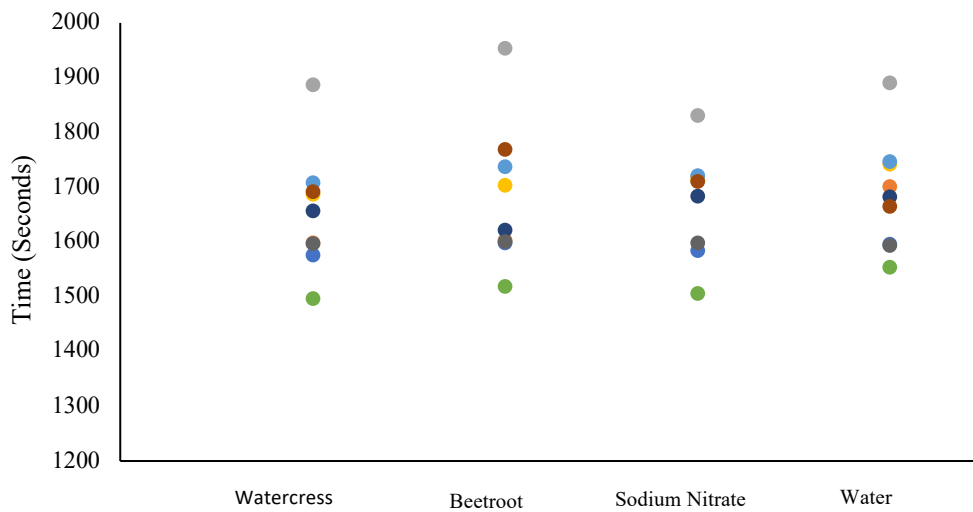


Figure 4.6.2. Individual time trial times in all four supplements (n = 9).

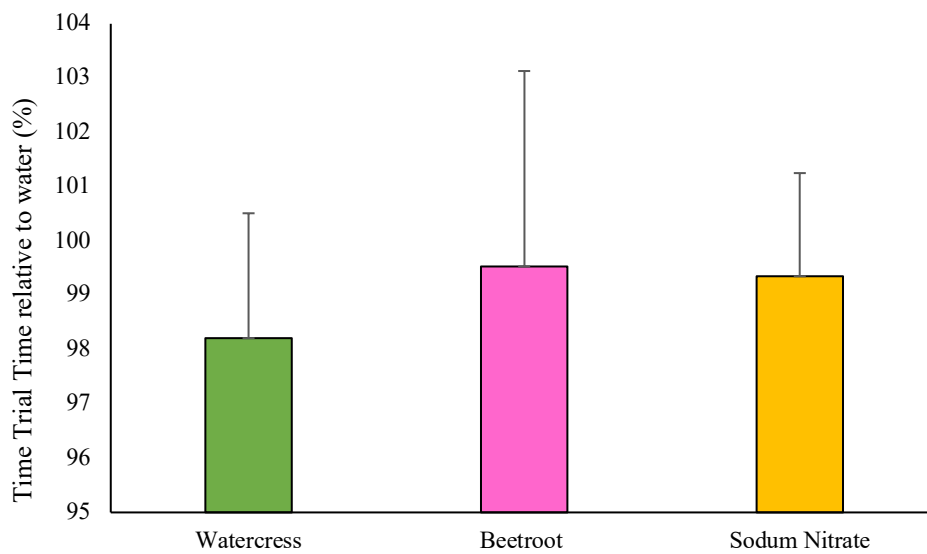


Figure 4.6.3. Data are presented as time to complete 16.1 km as percentage change relative to the water condition (n = 9). Values are mean + SD.

4.3.6. Power output and cadence

A one-way repeated measures ANOVA was conducted to determine whether there was a statistically significant difference for mean power and cadence across all four conditions. There was no significant differences between all four conditions in mean power (Watercress: 170 ± 30 W; Beetroot: 165 ± 30 W; Sodium Nitrate: 167 ± 26 W; Water: 164 ± 24 W; $P = 0.34$) and mean cadence (Watercress: 91 ± 8 rpm; Beetroot juice: 93 ± 8 rpm; Sodium Nitrate; 92 ± 9 rpm; Water; 94 ± 7 rpm; $P = 0.50$).

Markers of oxidative and nitrosylative stress markers

4.3.7. Nitrotyrosine

Nitrotyrosine concentration was not normally distributed ($P < 0.05$), as assessed by Shapiro-Wilk's test of normality on the studentized residuals. A Friedman test was run to determine if there were differences in nitrotyrosine concentration in each supplementation group. The nitrotyrosine concentration in the sodium nitrate group was significantly different at different time points, $\chi^2(2) = 10.750$, $P = 0.005$. Post hoc Wilcoxon signed rank test determined that there was a statistically significant median decrease in nitrotyrosine concentration from baseline (0 minutes) (Median = 198.5 ng/mL) to 120 minutes post ingestion (Median = 82.1 ng/mL) time point, $z = -2.51$, $P = 0.012$. see Table 4.4. Considerable variation was observed in the range of 6.57 – 878.25 ng/mL.

Table 4.4. Nitrotyrosine concentration at 0, 120 minutes and post exercise.

	0 mins	120 mins	Post exercise
Nitrotyrosine (ng/ml)			
Watercress	143 (115 – 173)	130 (97 – 300)	299 (163 – 337)
Relative to baseline (%)	x	↓ 9.1	↑ 109.1
Beetroot	87 (60 – 174)	85 (60 – 188)	152 (80 – 385)
Relative to baseline (%)	x	↓ 2.3	↑ 74.7
Sodium Nitrate	201 (76 – 326)	82 (25 – 221)	131 (36 – 226) *
Relative to baseline (%)	x	↓ 59.2	↓ 34.8
Water	105 (29 – 152)	52 (28 – 107)	120 (27 – 197)
Relative to baseline (%)	x	↓ 50.5	↑ 14.3

Values are median and interquartile ranges (n = 9). ↑=increase, ↓=decrease. * Significant difference from baseline (0 mins), P < 0.05.

4.3.8. Lipid hydroperoxides

Baseline values for lipid peroxidation concentration did not significantly differ between the four interventions ($F(3,21) = 1.59$, $P = 0.221$). Two way repeated measure ANOVA revealed there was no statistically significant interaction between treatment and time, ($F(6, 48) = 1.211$, $P = 0.317$), see Table 4.5.

4.3.9. Protein carbonyls

Baseline values for protein carbonyls concentration did not significantly differ between the four interventions ($F(3,21) = 1.532$, $P = 0.236$). Two way repeated measure ANOVA revealed there was no statistically significant interaction between treatment and time, ($F(6, 42) = 1.260$, $P = 0.297$), see Table 4.6.

Table 4.5. LOOH values at baseline, 120 minutes post ingestion and post exercise for all four supplements.

	0 mins	120 mins	Post exercise
Lipid hydroperoxide (μmol/L)			
Watercress	1.6 ± 1.0	1.9 ± 1.3	3.8 ± 1.6
Relative to baseline (%)	x	↑ 18.7	↑ 137.5
Beetroot	2.3 ± 1.2	2.4 ± 1.4	2.3 ± 1.5
Relative to baseline (%)	x	↑ 4.3	0
Sodium Nitrate	1.2 ± 0.9	3.4 ± 2.9	1.6 ± 1.1
Relative to baseline (%)	x	↑ 183.3	↑ 33.3
Water	2.2 ± 1.4	3.8 ± 3.9	3.1 ± 2.5
Relative to baseline (%)	x	↑ 72.7	↑ 40.9

Values are mean ± SD. LOOH, lipid hydroperoxide; (n = 9). ↑ = increase

Table 4.6. PC values at baseline, 120 minutes post ingestion and post exercise for all four supplements.

	0 mins	120 mins	Post exercise
Protein Carbonyls ($\mu\text{M}/\text{mg}$ protein)			
Watercress	32.2 \pm 42.0	68.1 \pm 99.2	77.4 \pm 44.3
Relative to baseline (%)	x	↑ 111.5	↑ 140.3
Beetroot	38.5 \pm 34.1	82.2 \pm 77.1	72.4 \pm 89.5
Relative to baseline (%)	x	↑ 113.5	↑ 88.1
Sodium Nitrate	42.4 \pm 89.5	52.3 \pm 64.5	37.7 \pm 58.3
Relative to baseline (%)	x	↑ 23.3	↓ 11.1
Water	35.3 \pm 60.9	57.8 \pm 77.7	55.3 \pm 59.7
Relative to baseline (%)	x	↑ 63.2	↑ 56.6

Values are mean \pm SD. PC, protein carbonyls (n = 9). **↑**= increase, **↓**=decrease

4.4. Discussion

The main findings of this study are that acute dietary nitrate supplementation did not significantly enhance cardiovascular parameters, 16.1 km TT performance or alter oxidative stress markers in healthy, physically active males. This was surprising due to reports that nitrate supplementation improved 4.0, 10.0 and 16.1 km TT performance in moderately trained athletes (Cermak et al., 2012; Lansley et al., 2011). In the current study the watercress supplementation reported the fastest 16.1 km TT with a 1.4 % improvement compared to the water condition. While the beetroot juice was faster by 0.2 %, however, both did not attain statistical significance.

4.4.1. Effects of dietary nitrate on markers of vasodilation

In line with previous studies, participant's plasma nitrate increased significantly ($P < 0.05$) after consuming dietary nitrate (Bailey et al., 2009; Bailey et al., 2010; Larsen, Weitzberg, Lundberg, & Ekblom, 2010; Webb et al., 2008). In addition, we found nitrate levels to remain stable after exercise, this is also in line with previous research and is likely due to the pharmacokinetics of nitrate after dietary ingestion (Bailey et al., 2010; Lansley et al., 2011; Wylie et al., 2013). With plasma nitrate reported to peak within an hour of consumption and to have a half-life in humans for approximately five hours (Larsen et al., 2010; Webb et al., 2008).

Nitrite plasma levels peak 2 – 3 hours post nitrate consumption (Jones, 2014). In the current investigation, plasma nitrite concentration observed the largest increase in the beetroot condition by 32 % relative to baseline and the watercress condition observed a 14 % increase relative to baseline, although neither significant. Surprisingly, nitrite

did not increase in the sodium nitrate condition at two hours post ingestion. Research suggests nitrite is a more sensitive biomarker of nitric oxide synthesis/availability, as it is the main precursor of nitric oxide and other bioactive nitrogen oxides (Lundberg et al., 2008). Interestingly, for an individual to be classified as a responder to nitrate supplementation, plasma nitrate and nitrite concentrations should show a significant increase after consuming nitrate (Jonvik et al., 2015). This was not observed in the current study, as plasma nitrite from the nitrate supplementation groups were not significantly higher than the water supplementation group at the 120 minutes time point. A greater number of participant's nitrite levels were observed to increase at the 120 minutes time point in comparison to the baseline in the watercress condition (7 of the 9 participants) and the beetroot condition (6 of the 9). Interestingly, the same five participants increased in both conditions. In comparison the sodium nitrate condition observed 5 of the 9 participant's nitrite concentration levels to increase above baseline. Only three participants increased relative to baseline in all three conditions.

The observed increases in plasma nitrite concentration were to a smaller extent in comparison to previous research (Betteridge et al., 2016; Larsen et al., 2010; Vanhatalo et al., 2010; Vanhatalo et al., 2011). For example, Lansley et al., (2011) found after a similar dose of beetroot supplementation (~ 6.2 mmol of nitrate) plasma nitrite values increased by 65% relative to baseline. Studies have showed a significant increase in plasma nitrite concentrations with increasing nitrate dosages, as shown by Wylie et al, (2013) where they analysed nitrite and nitrate concentrations after the ingestion of 4.2 mmol, 8.4 mmol and 16.8 mmol of beetroot juice compared to a placebo. Wylie et al., (2013) and a similar study by Flueck et al., (2016) found inter-individual variability in the response of nitrite concentration after nitrate ingestion,

with the number of non-responders decreasing when the dose was increased (Wylie et al., 2013).

Oral bacteria and gut bacteria are integral for the reduction of nitrate to nitrite and nitric oxide (Tiso and Schechter, 2015). Although participants were restricted of antibacterial mouthwash to control the existence of oral bacteria, it was not possible to control the gut bacteria in the current investigation (Tiso and Schechter, 2015). This may partly explain the lack of increase in plasma nitrite concentration or perhaps the nitrate concentration in the supplements were too low to significantly increase plasma nitrite concentrations. Additional analyses of salivary nitrate/nitrite in addition to gut bacteria may potentially further understanding in the pharmacokinetics after dietary nitrate ingestion and explain why plasma responses may vary between individuals.

Blood pressure did not significantly reduce in all nitrate conditions in comparison to the water condition. In contrast to this, Lansley et al., (2011) reported a 65% increase in plasma nitrite concentration to be associated with a 5% decrease in systolic blood pressure in young healthy participants after they consumed 6.2 mmol of beetroot juice. Plasma nitrite concentration has been shown to be associated with flow mediated dilation via the bioactivation to nitric oxide which can significantly influence arterial stiffness, vascular tone and blood pressure (Casey, Beck, & Braith, 2007; Jin & Loscalzo, 2010). From this, it is postulated that due to the insignificant increase in nitrite levels after nitrate supplementation, blood pressure did not significantly decrease (Casey et al., 2007; Jin & Loscalzo, 2010). The absence in reduction of blood pressure may be due to the health and activity status of the cohort (Kapil et al., 2010). In normotensive participants and highly trained individuals, the efficacy of nitrate may be less evident/absent due to optimum expression and activity of NOS, reduced inflammation/oxidative stress, decreased acidosis/systemic hypoxia (Jones et al.,

2018). It is hypothesised that crosstalk between endothelial NOS and nitrate-nitrite-nitric oxide pathway occurs, limiting nitric oxide generation when its availability is already optimal, thus reducing the risk of hypotension (Bondonno et al., 2015; Kerley, Dolan, & Cormican, 2017). However, this finding is in contrast with earlier work, with numerous studies demonstrating dietary nitrate to significantly modulate blood pressure in healthy humans (Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010; Webb et al., 2008).

The acute doses of the nitrate supplements did not augment blood flow in the forearm, while the water condition significantly decreased blood flow over time in comparison to the nitrate supplements. Interestingly, the watercress condition was the only supplement to slightly increase over time prior to exercise while the rest of the conditions observed a reduction in blood flow. As previously mentioned, the plasma nitrite levels in the watercress condition had increased at a lesser magnitude in comparison to the beetroot condition. This suggests additional bioactive compounds within the watercress juice may have had a protective cardiovascular role, although not significant (Egert et al., 2009).

The reduced bioconversion of nitrate to nitrite may have caused the lack of physiological effects (Webb et al., 2008; Wylie et al., 2013). As the key pathway for dietary nitrate uptake and conversion to nitrite occurs in the oral cavity via the action of commensal bacteria, the inter participant variances in oral microbiome may have attributed to varied nitrite uptake after oral consumption of the nitrate supplements (Hezel & Weitzberg, 2015). A study reported a decrease by 10 mmHg and 8 mm Hg, in systolic and diastolic blood pressure, respectively, after consuming beetroot juice (500ml; 22 mmol), in comparison to this, the same study reported no significant physiological effects after 8 mmol of beetroot juice in healthy men (Wylie et al.,

2016). The present investigation nitrate dose may have been too low (~400 mg). Interestingly, a decrease of 5 mmHg in systolic blood pressure could reduce the risk of death by cardiovascular causes, such as heart disease, by 14% and stroke by 9%. Therefore, a longer period of supplementation or/a greater dose of nitrate may have a significant effect on the variables investigated in this study. Thus, the acute period and the dose administered is a feasible limitation of this study.

4.4.2. Effects of dietary nitrate supplementation on 16.1 km TT performance

Contrary to our working hypothesis, acute nitrate supplementation was not observed to significantly improve 16.1 km TT performance. The result is surprising as previous studies have found nitrate supplements or beetroot juice to enhance TT performance (Cermak, Gibala, & van Loon, 2012; Lansley et al., 2011; Wilkerson et al., 2012). In contrast to this, other research has reported no acute effect of nitrate supplementation in TT performance (Cermak., et al., 2012; Hoon et al., 2014; Muggeridge et al., 2013; Peeling et al., 2015). To explain and comprehend the discrepancies between this study and other nitrate research we must consider the dose and duration of nitrate supplementation, the type of participants (specifically their training status) and the duration/ nature/intensity of the exercise.

In the present study, the acute effects of nitrate supplementation were investigated rather than chronic ingestion, this was chosen with consideration to financial cost and time commitment, allowing for a more user-friendly protocol to be adopted by athletes. We compared a beetroot juice shot (James White, Suffolk, United Kingdom) to watercress juice (Vitacress, Southampton, United Kingdom) and sodium nitrate (VWR, Leicestershire, UK), the dosage was selected in line with the commercially

available beetroot juice (~400 mg of nitrate) (James White, Suffolk, United Kingdom), which is estimated to be around 4 - 12 times greater than the average daily nitrate intake in the USA and Europe (Bailey et al., 2009; Hernández et al., 2012; Jones et al., 2018; Larsen et al., 2007; Vanhatalo et al., 2010).

There is extensive research to suggest that nitrate supplementation can significantly improve moderately trained ($\text{VO}_{2\text{peak}} < 60 \text{ mL/kg/min}$) endurance athlete's performance, the participants in this study all had a $\text{VO}_{2\text{peak}}$ under 60 mL/kg/min (Cermak et al., 2012a; Porcelli et al., 2015; Muggeridge et al., 2014; Lansley et al., 2011). Research proposes this is due to the enhanced bioavailability of nitric oxide provided by the non-enzymatic pathway, and the low muscle oxygenation and acidosis experienced during exercise in the moderately trained muscles favourable for nitrate reduction (Hoon et al., 2013). However, the participants recruited for this study were recreationally active with a mean $\text{VO}_{2\text{peak}} 52.4 \pm 6.1 \text{ ml/kg/min}$.

Although the mean time trial performance was not significantly improved by acute ingestion of nitrate. Interestingly, the five participants who observed an increase in plasma nitrite concentration above the group mean, were the same five participants that recorded their fastest TT performance following watercress consumption in comparison to the rest of the supplementation groups. The explanation for the variable nitrite concentration responses to nitrate supplementation is not clear but might relate to inter-individual differences in muscle oxygenation and acidosis during exercise which might, in turn, be linked to differences in anaerobic capacity, effort, or muscle fibre-type distribution. Other mechanistic bases behind differences in the efficacy of nitrate supplementation, or responsiveness of the participant are not fully understood and require further research.

A recent review suggested the minimum dose to observe an enhanced exercise economy is 5.0 – 8.5 mmol but to elicit an enhanced economy and performance the required nitrate dose of > 8.5 mmol might be necessary (Jones et al., 2018). This may explain as to why a significant increase was not observed, as participants consumed the commercially available beetroot shot (~6.45 mmol of nitrate) which may not have contained enough nitrate for a substantial elevation in plasma nitrite and for an ergogenic effect (Gallarado & Coogan, 2018).

A critical improvement in performance in competitive sport is classed as small as 0.5 - 1.5 %, however, Paton and Hopkins (2006) suggested a smallest worthwhile change of ~ 0.6 % for road TT cyclists (Paton & Hopkins, 2006). The beetroot juice condition observed a 0.2 % improvement in time in comparison to the water condition, while the watercress condition observed an on average 1.4 % improved TT performance. Using these criteria, it is possible that our findings may be of real-world relevance to some athletes. For example, in 2012, at the London Olympics the difference between 12th and first place in the men's 10,000 metres running final was 0.66%, therefore, it is may be wise to advise nitrate supplementation to aid endurance performance, when minor enhancements can be particularly effective.

A similar study observed moderately trained individuals (n = 9) achieve statistically significantly faster 4 km and 16.1 km TT performances (2.8 % and 2.7 %, respectively) on a cycle ergometer after an acute consumption of an analogous concentration of nitrate in the form of beetroot juice (Lansley et al., 2011). Although impractical for athletes, Lansley et al., (2011) gave 0.5 l of beetroot juice 2.5 hours prior to commencing the TTs whereas, in the present study, participants consumed a 70 ml shot (Beet It, James White, UK), with the consideration of avoiding gastrointestinal distress during exercise. The greater TT performance after beetroot consumption

observed by Lansley et al., (2011), may be possible due to the larger volume of the beetroot juice (500 ml versus 70 ml) and with this, an increased availability of other bioactive compounds. Several studies have reported betalain-rich supplements to enhance exercise performance and attributed this ergogenic effect to a number of mechanisms including decreased inflammation, oxidative stress and muscle damage (Montenegro et al., 2017; Mumford et al., 2018; Van Hoorebeke et al., 2016). Plasma betalains were not measured in the present study, a limitation in the design.

In the present investigation oxidative stress biomarkers were not significantly altered in any condition, at any time point. This may be that antioxidant supplementation only offers additional protection in cohorts at an increased risk of oxidative damage due to disease or old age (Fisher-Wellman & Bloomer, 2009). Furthermore, a decrease in exercise-induced oxidative stress by antioxidant supplementation may not be advantageous, as it has been suggested that augmented RONS during and after exercise may be essential for adaptations in antioxidant defences, as well as other physiological parameters (Fisher-Wellman & Bloomer, 2009).

An important advance in the present study was the use of sodium nitrate supplementation and water supplementation, acting as a positive control and a control, respectively, with the sodium nitrate offering insight on the nitrate mechanism. Out of the nine participants one participant guessed the correct control supplement sequence.

Most studies postulate the physiological effects observed from beetroot juice supplementation are mediated through the nitrate – nitrite – nitric oxide pathway (Bailey et al., 2009; Jones et al., 2018; Lansley et al., 2010). In the current investigation, the use of isolated sodium nitrate as a positive control in this study enabled us to highlight the effects of other potentially ergogenic bioactive

components. The unchanged plasma nitrite and TT performance after sodium nitrate compared with the beetroot juice supplement and watercress supplement results may suggest the acute physiological effects are not exclusively attributed to nitrate content or another bioactive component may elicit an ergogenic effect. This does not exclude the possibility, however, that nitrate in beetroot and watercress might act synergistically with these other components to positively affect the physiological responses.

4.4.3. Limitations

A limitation to the current study was the small sample size; however, the experimental design involved a crossover design helping to control variance. To avoid a carrying over effect, the washout period was a minimum of seven days between each intervention. The participants were requested to record food, fluid and activity diaries forty-eight hours prior to attending the first TT session and were requested to reproduce this before all subsequent tests. The participants were instructed to abstain from caffeine, alcohol and restricted physical activity (self-reported). However, we do not know how closely these instructions were adhered to. Plasma nitrite levels have been reported to peak between 2 – 3 hours after nitrate consumption, possibly a blood sample one hour later would have indicated a significant increase in nitrite levels in the nitrate conditions and potential physiological responses (Jones et al., 2018; Webb et al., 2008).

4.4.4. Conclusion

In conclusion, we have shown that acute dietary nitrate supplementation does not significantly alter 16.1 km TT performance in moderately trained individuals. However, the group mean improvement in completion time for the watercress supplementation of 1.4 % may still be meaningful in competition. After watercress supplementation, five out of the nine participants observed an elevation of plasma nitrite above the group mean and the fastest TT performance. Future research should consider exploring the optimal watercress loading regimen required to elevate plasma nitrite and enhance 16.1 km TT performance in young, physically active males.

Assessing nitrate supplementation in a cardiovascular compromised population, rather than young, physically active volunteers may provide new insights. In the Chapter 5, we endeavoured to establish whether a normoxic and hypoxic setting would alter the efficacy of watercress supplementation in a healthy, aged population.

Chapter 5:
**The effect of acute watercress
supplementation on cardiovascular and
oxidative stress parameters in normoxic
and hypoxic environments at rest with an
aged population.**

5.1. Introduction

Irrespective of the significant advances over the past two decades in pharmaceutical therapies for cardiovascular disease (CVD), CVDs remain the most common cause of death worldwide (WHO, 2017). An estimated 17.9 million people died from CVDs in 2016, representing 31% of deaths globally (WHO, 2017). Most types of CVD are linked with the impairment of arteries, while interestingly; the primary risk factor for cardiovascular disease is ageing, suggesting, most forms of CVD are diseases of ageing (Go et al., 2013; Lakatta, 2002; Lonn et al., 2010; Najjar et al., 2005). It is forecasted that over the next five years, for the first time in history, people over 65 years will outnumber children (< 5 years old), increasing the prevalence of CVD and other age-related diseases, and an exacerbating demand on medical care systems (Lutz et al., 2008).

The ageing process results in dysfunctional arteries, of which there are two significant alterations; the stiffening of the large elastic arteries (the carotid and aorta) and the progression of endothelial dysfunction, leading to an increased risk of CVD (Lakatta, 2003; Lakatta & Levy, 2003; Oliver & Webb, 2003; Seals et al., 2011; Widlansky et al., 2003). The degradation and reduction in bioavailability of nitric oxide is a critical feature, linking both alterations during the ageing process (Soucy et al., 2006; Wilkinson et al., 2004). There is a large body of evidence supporting the proposition that reactive oxygen species cause the accelerated degradation and bioavailability of nitric oxide, as seen in ageing and various disease states (Eskurza et al., 2004; Kojda & Harrison, 1999; Ohara, Peterson, & Harrison, 1993; Seals et al., 2011; Taddei et al., 2001). The aetiology of these CVD processes is varied, combining both environmental and genetic factors, however, hypoxia is implemented in the pathology of most of these diseases (Michiels, 2004).

Epidemiological research has suggested fruit and vegetables that are rich in polyphenols have cardiovascular health benefits on blood pressure, platelet activity, cholesterol levels,

and insulin resistance and augment endothelial nitric oxide bioavailability (Bazzano et al., 2002; Davies et al., 2002; Grassi et al., 2005; Joshipura et al., 1999; Liu, 2003; Lundberg & Govoni, 2004; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005). While in recent times, there has been a considerable amount of interest in nitrate supplementation, as it has been shown to modulate vascular tone (Kapil et al., 2010; Lidder & Webb, 2013; Park, Pikhova, Huang, Noguchi, & Schechter, 2013; Richardson et al., 2002; Siervo et al., 2013; Webb et al., 2008). The discovery of the contribution of dietary nitrate to nitric oxide bioavailability offers great potential to improve vascular function in the elderly (Rammos et al., 2016).

Watercress (*Rorippa nasturtium-aquaticum*) is high in nitrate and is an exceptional source of bioactive compounds; including, several phytochemicals; glucosinolates, caftaric acid, carotenoids such as lutein and beta-carotene and flavonoids, such as kaempferol, and quercetin (Hord et al., 2009; O'Neill et al., 2001; Zeb, 2015). It has previously been shown that watercress attenuates DNA damage, reduces the risk of cancer and exert a range of cardioprotective effects and a favourable role in anti-genotoxic and anti-cancer processes in healthy adults, animal and cell models, (Boyd et al., 2006; Fogarty et al., 2013; Gill et al., 2007; Rose et al., 2000). However, the efficacy of watercress supplementation from human trials are limited (Fogarty et al., 2013; Gill et al., 2007).

Given the potential ability of watercress to improve time trial performance observed in the previous chapter (Chapter 4), we sought to determine whether beneficial effects on cardiovascular and oxidative stress parameters are observed after acute ingestion of watercress juice in a healthy aged population, in a hypoxic and normoxic environment. This is of importance as watercress can be readily ingested as part of a healthy diet.

Considering the nitrate-nitrite-nitric oxide pathway is accelerated in hypoxic conditions, we deduced the watercress supplement may modulate the changes in plasma nitrate and nitrite levels and ameliorate the negative effects of hypoxia on vascular system and

oxidative stress markers. The present study aimed to investigate if watercress supplementation modulates vascular function, by using venous occlusion plethysmography and non-invasive direct measurement of vasodilatory capacity using the EndoPAT. oxidative and nitrosylative stress markers, blood pressure, platelet aggregation and nitrate and nitrite measurements were also observed.

5.1.1. Hypotheses

We hypothesised acute watercress supplementation in both hypoxic and normoxic conditions will increase plasma nitrate and nitrite concentration, improve cardiovascular parameters, and reduce blood pressure and oxidative stress markers in comparison to the control conditions in a healthy, aged, physically active population.

5.2. Methods

5.2.1. Participants

Nine active participants ($n = 9$, male = 7, female = 2; see Table 5.2) volunteered to participate in the present investigation and were recruited using email and poster advertisement. Participants reported no overt disease or medications that affect NO metabolism. All participants completed a physical activity readiness questionnaire and as a minimum requirement volunteers were required to be physically active as defined by the World Health Organisation (engage with 150 minutes of moderate-intensity aerobic physical activity throughout the week or, at least 75 minutes of vigorous-intensity aerobic physical activity throughout the week, or an equivalent combination of moderate- and vigorous-intensity activity). Further inclusion/exclusion criteria are outlined in Table 5.1. Prior to participation the protocol and the potential risks were outlined with verbal and written informed consent granted. The study was approved by the Research Ethics Committee at the Sports, Health and Exercise Science School at the University of Hull and conformed to the Declaration of Helsinki.

Table 5.1. Inclusion/exclusion criteria

Inclusion criteria	Exclusion criteria
Between 58 – 75 years old	Smoking
Physically active	History of cardiovascular/metabolic disease
Healthy	Medication
	Supplements

5.2.2. Study design

Using a randomised (Research Randomizer Version 4.0, Urbaniak & Plous 2013), crossover design, each participant reported to the laboratory on five occasions, undergoing a different experimental approach with each visit. Participants completed a baseline visit for $VO_{2\text{peak}}$, and anthropometric measurements followed by;

- 1) Normoxia control
- 2) Hypoxia control
- 3) Normoxia with watercress supplement
- 4) Hypoxia with watercress supplement

In a random order using a randomised (Research Randomizer Version 4.0, Urbaniak & Plous 2013) crossover design. Each visit had a minimum of a seven-day washout period and participants were requested to refrain from caffeine and alcohol, six and twenty-four hours before the first experimental session, respectively.

5.2.3. Visit 1: Baseline measures

During the first visit, participants performed a maximal incremental test to exhaustion for determination of peak oxygen uptake ($VO_{2\text{peak}}$) and peak power output (PPO), on a cycle ergometer (Schoberer Rad Messtechnik (SRM), Germany), anthropometric data was also recorded (Table 5.2). Online gas analysis was collected using a Cortex Metalyzer 3B (Cortex Biophysic, Leipzig, Germany) for breath by breath analysis. Participants warmed up for five minutes with 50 W resistance, once warm up was complete the test began straight away with watts increasing by $25 \text{ W}\cdot\text{min}^{-1}$. The test ended when the participant reached volitional exhaustion and was unable to continue or when the revolutions per minute dropped below sixty. During the incremental test, heart rate was noted every

minute and every second minute rate of perceived of exertion (RPE) using the Borg scale (1973) was noted. Gas analysis was continuously collected. Participants were instructed to avoid any exercise forty-eight hours prior to exercise.

Table 5.2. Anthropometric measures

Characteristic	Mean \pm SD
Body mass (kg)	72.5 \pm 9.0
Age (yrs)	62.6 \pm 5.4
Height (cm)	171.3 \pm 5.4
BMI (kg/m ²)	24.6 \pm 2.4
VO _{2peak} (ml/kg/min)	40.4 \pm 6.2
PPO (W)	264.4 \pm 34.3

All data are presented as mean \pm standard deviation. Kg = kilograms, yrs = years, cm = centimetres, BMI = body mass index, kg/m² = kilograms per metres squared, VO_{2peak} = peak oxygen consumption, ml/kg/min = millilitres per kilogram per minute, PPO = peak power output, W = watts, (n = 9).

5.2.3.1. Anthropometric measurements

Anthropometric measurements were performed as described in chapter (General Methods Chapter 3, section3.2.1).

5.2.3.2. Blood pressure

Participants were asked to relax for ten minutes in a temperature (22 - 24 °C) controlled, quiet room. Blood pressure readings were recorded using an Omron (Omron M6, Omron Healthcare UK LTD, Milton Keynes, UK) automated sphygmomanometer, measurements were taken in triplicate. For detailed protocol please see General Methods Chapter 3, section 3.2.2.

5.2.3.3. Peak oxygen uptake

Prior to the $\text{VO}_{2\text{peak}}$ test the participant's saddle and handlebar height and handlebar position were recorded and reproduced for subsequent visits. All tests took place on an SRM (Schoberer Rad Mebtechnik, Konigskamp, Germany) cycle ergometer in a temperature-controlled laboratory (20 - 23 °C). Online gas analysis was collected using a Cortex Metalyzer 3B (Cortex Biophysic, Leipzig, Germany) for breath by breath analysis. Participants warmed up for five minutes with 50 W resistance, once warm up was complete the test began straight away with watts increasing by 25 W/min. The test ended when the participant reached volitional exhaustion and was unable to continue or when the revolutions per minute dropped below sixty. During the incremental test, heart rate was noted every minute and every second minute rate of perceived of exertion (RPE) using the Borg scale (1973) was noted. Gas analysis was continuously collected.

5.2.4. Visits 2-5

5.2.4.1 Environmental conditions

The environmental chamber (Weiss Technik Ltd, Wales UK) was set at 20.93% oxygen, 23 °C, 50% humidity for the normoxic conditions and 14.5% oxygen, 23 °C and 21% relative humidity for the hypoxic simulation (Vanhatalo et al., 2011). All experimental visits were completed at the same time of day ($\pm 1\text{h}$) to attempt minimise diurnal variation.

5.2.4.2 Supplementation protocol

Before the first supplementation session, participants were requested to fill out a qualitative activity and food diary for forty-eight hours, which participants were asked to repeat this diary each time before each subsequent lab visit.

Participants fasted for a minimum twelve hours (water only permitted) prior to attending the laboratory and completing the standardised baseline measurements (as described below). Once completed participants consumed either a single dose of 184 ml of

watercress juice (containing 400 mg of nitrate) (Vitacress Ltd, Southampton, United Kingdom) or 184 ml of water (Buxton water Ltd, Buxton, UK) acting as control followed by two hours of rest in a normoxic or hypoxic environment. Participants were instructed to consume each supplement within 60 seconds. A minimum of seven day occurred between each laboratory visit to act as a wash out for the experimental supplement. Participants were requested to avoid mouthwash and chewing gum for the duration of the study period (Govoni, et al., 2008).

On arrival to the laboratory baseline measures were collected prior to supplementation. Once all measurements were collected the supplement was consumed prior to entering the environmental chamber. All measurements below were repeated at 30 minutes and 120 minutes post consumption, unless otherwise stated (see Figure 5.1.1.).

5.2.4.3. Blood pressure

Blood pressure measurements were recorded at baseline, 30 minutes and 120 minutes post supplementation. Participants were asked to relax for ten minutes in a temperature (22 - 24 °C) controlled room. Blood pressure readings were recorded using an Omron (Omron M6, Omron Healthcare UK LTD, Milton Keynes, UK) automated sphygmomanometer, measurements were taken in triplicate. For detailed protocol please see General Methods Chapter 3, section 3.2.2.

5.2.4.4. Heart rate and oxygen saturation

In the chamber, participants were seated for two hours during which oxygen saturation and heart rate was measured every 30 minutes. Heart rate and arterial oxygen saturation (SaO₂) were measured during the test protocol using a pulse oximeter device (Nonin PalmSat 2500, Nonin Medical inc, Plymouth, Minnesota, USA) which was attached to the participants index finger. In line with the ethics application, if a participant's oxygen saturation dropped below 85 % the participant would be removed from the chamber.

5.2.4.5. Venous occlusion plethysmography

Blood flow volume measurements were recorded at baseline, 30 minutes and 120 minutes post supplementation. Please see the General Methods Chapter 3, section 3.2.5. for protocol.

5.2.4.6. Endo-PAT

A plethysmography device called the Endo-PAT 2000 (Itamar Medical Ltd, Caesarea, Israel) was used for measuring the reactive hyperaemic index (RHI) assessing pulse volume changes at the fingertips. Fingertips were inspected for any injuries, long nails or abnormalities that may affect the results prior to the test. Single use Endo-PAT sensors were placed on each index finger, a blood pressure cuff (E20, Hokanson, Bellevue, USA) was secured on the non-dominant arm while the other arm acted as control. Participants remained reclined in an armchair for the duration of this test, participants hands were supported at approximately heart level. All tests took place in a temperature controlled (20 - 23 °C), quiet and darkened laboratory room. Participants were requested to remain as still as possible and not to talk throughout the test. The PAT probes were inflated, and the signals were recorded on the Endo-PAT software (Itamar Medical Ltd. Caesarea, Israel) in accordance to the manufacturer's instructions. The protocol involved a 5 minutes baseline recording, followed by the cuff inflated to 200 mmHg for 5 minutes and followed by another 5 minutes of recording after cuff release. The data was then analysed by the software and gave a value for RHI and augmentation index (AI), an indicator of vessel stiffness.

The PAT score is conveyed as a ratio of the post to pre-occlusion average signal level in the occluded arm, relative to the same ration in the control arm, corrected for systemic changes and baseline vascular tone. The reactive hyperaemia index ratio was defined as the ratio between the post- to pre- occlusion average signal size, corrected for systemic

changes and baseline level. This measurement was recorded at baseline and 2 hours post supplementation.

5.2.4.7. Cardiac output

Cardiac output was recorded at baseline and two hours post supplementation. Please see the General Methods Chapter 3, section 3.2.4. for protocol.

5.2.4.8. Blood collection

Participants fasted for twelve hours overnight prior to provision of a venous blood sample. This protocol restricts the dietary effect of blood borne metabolites such as antioxidants and lipids (Pronk, 1993). Samples were obtained for serum and plasma, as described in general methods, section 3.2.8.). At baseline, thirty minutes post supplementation and one hundred twenty minutes post supplementation.

After blood collection, EDTA vacutainers were placed on ice whilst SST's clotted in the dark at room temperature (ten minutes) before centrifugation at 1300 x g (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) for ten minutes. Plasma and serum were removed using a 1 ml pipette and transferred to a 2 ml cryovial and immediately frozen (-80°C) prior to analysis. Whole blood was drawn by venepuncture into evacuated tubes containing 3.2% sodium citrate (Greiner-Bio One Ltd, Gloucestershire, United Kingdom) and inverted 4 times at baseline (prior to entering the chamber) and one hundred twenty minutes post supplementation for measurement of platelet aggregation.

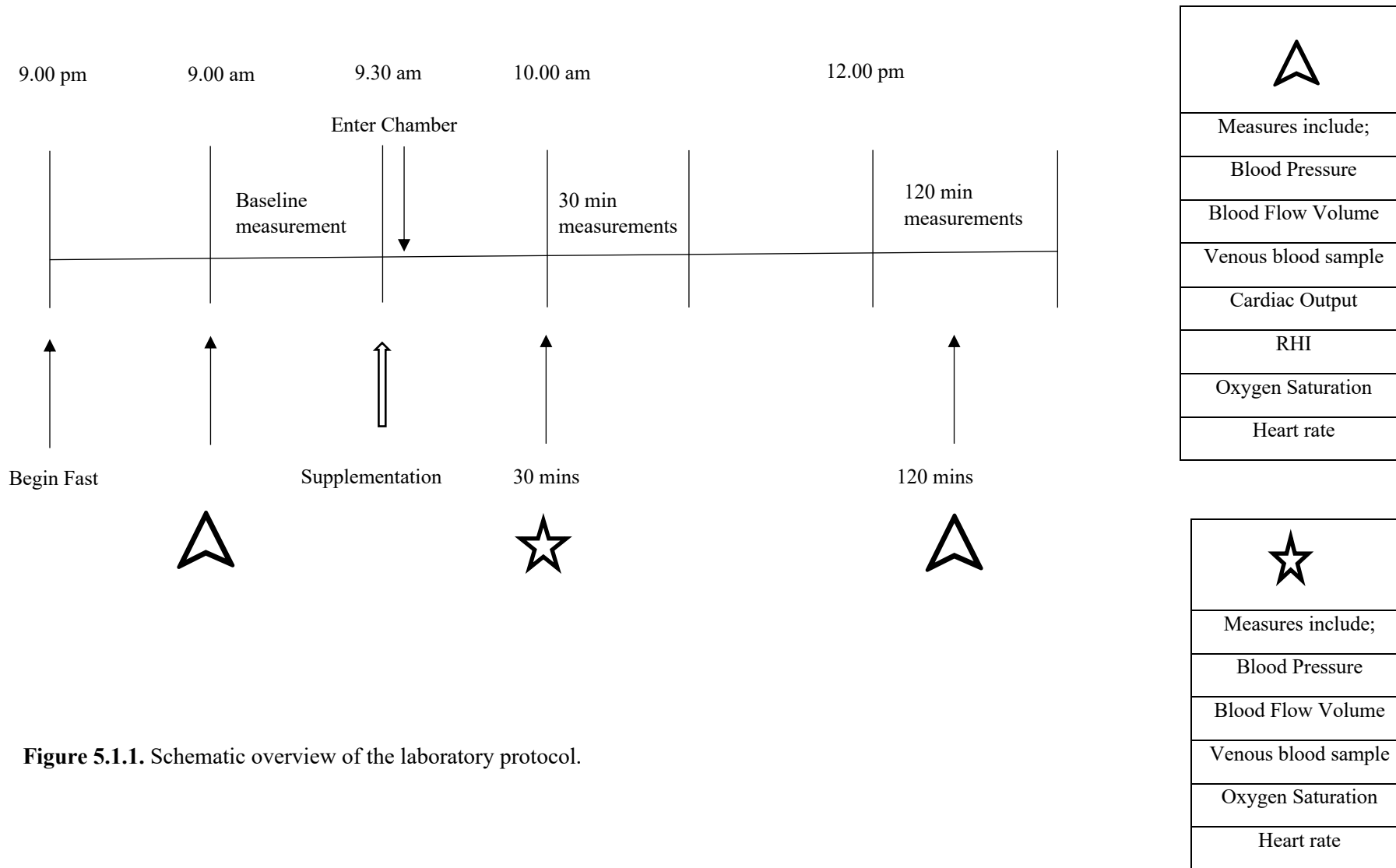


Figure 5.1.1. Schematic overview of the laboratory protocol.

5.2.5. Biochemical analysis

5.2.5.1. Nitrate and nitrite

Nitrate and nitrite were determined using a commercially available fluorometric kit (Cayman Chemical, Ann Arbor, USA) (General Methods Chapter 3, section 3.3.4). The intra-assay coefficient of variation (COV) for nitrate was < 4% and for nitrite < 6%.

5.2.5.2. Nitrotyrosine

Nitrotyrosine was measured using a commercially available Enzyme-Linked Immunosorbent Assay kit (ab210603, Abcam, Cambridge UK) (General Methods Chapter 3, section 3.3.5). The intra-assay COV was < 5%.

5.2.5.3. Lipid hydroperoxides

Lipid hydroperoxides were measured spectrophotometrically using the method of Wolff (1994) (General Methods Chapter 3, section 3.3.1). The intra-assay COV was < 4%.

5.2.5.4. Protein Carbonyls

Protein Carbonyls derivatives were measured according to the method of Levine (2002) (General Methods Chapter 3, section 3.3.3). The intra-assay COV was < 5%.

5.2.5.5. Bradford assay

Protein concentration was measured spectrophotometrically using the Bradford assay (General Methods Chapter 3, section 3.3.2). The intra-assay COV was < 4%.

5.2.5.6. Platelet aggregation

Whole blood was drawn by venepuncture into evacuated tubes containing 3.2% sodium citrate (Greiner-Bio One Ltd, Gloucestershire, United Kingdom) and inverted 4 times. To obtain platelet rich plasma (PRP) whole blood was spun at 200 g for 20 minutes at 4°C. Platelet poor plasma (PPP) was prepared by centrifugation of the remaining blood at 1000

g for 10 minutes at 4°C. To obtain platelet rich plasma (PRP) whole blood was spun at 200 g for 20 minutes at 4°C (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK). Platelet poor plasma (PPP) was prepared by centrifugation of the remaining blood at 1000 g for 10 minutes at 4°C. The effects on platelet aggregation were assessed using a 96-well plate adaptation of the Born method. Using a 96 well flat bottom plate (Thermo Fischer Scientific, Leicestershire, UK) each reading had comprised of one row with 90 µl of PRP in each well in triplicate, 90 µl of PPP in each well in triplicate, and finally another row with 90 µl of PRP in to each well in triplicate. The study used adenosine diphosphate (ADP) (Sigma-Aldrich, Missouri, USA) as an agonist to quantify platelet aggregation. Absorbance was measured at 595 nm every 15 seconds for 4 min at 37°C in a 96-well plate reader, with vigorous shaking for 10 s between readings. Prior to the agonist being added to the first row of PRP, a baseline reading obtained every 20 seconds for a minute using a plate reader (Tecan, Infinite M200 pro, software, Magellan). After the first minute, the plate was taken out and a multichannel pipette was used to add 10 µl of the agonist in to each well in the first row of PRP, the plate was then re-inserted into the plate reader (Tecan, Infinite M200 pro, software, Magellan). PRP alone was taken to be 0% light transmission (representing 0% aggregation); PPP alone representing 100% light transmission (represents 100% aggregation). The percentage change in aggregation was calculated from the formula:

$$(\text{Sample PRP value with agonist} - \text{Control PRP}) / (\text{Control PRP} - \text{Control PPP}) * 100$$

5.2.6. Statistical analysis

Data was analysed using IBM SPSS (version 24, IBM Corp., NY, USA). Prior to statistical analysis a Grubb's test was run and examination of studentized residuals for values greater than ± 3 standard deviations to detect outliers. The adherence rate for the experiment was 100%, therefore, the data was analysed and presented as $n = 9$. All variables were analysed for normal distribution by descriptive statistics and the Shapiro-

Wilk test ($P < 0.05$). Normally distributed continuous variables are presented as mean \pm standard deviation and non-normally distributed continuous variables as median (interquartile ranges; 25% and 75%), and finally, categorical variables as frequencies (percentages). A two-way repeated measures ANOVA was used to examine differences between conditions over time. Bonferroni post-hoc analysis was run to detect where the significant differences occurred. Cohen's d effect sizes (ES) were calculated with the magnitude of effects considered small (0.2), medium (0.6) and large (>1.2). Statistical significance was set at $P < 0.05$ prior to analyses.

5.3. Results

5.3.1. Oxygen saturation

In order to assess the effect of the chamber on inducing hypoxia, we measured oxygen saturation. The hypoxic environment caused a reduction in oxygen saturation that was statistically significant after 30 minutes and 120 minutes; the watercress supplementation had no effect on ameliorating this, as oxygen saturation was the same regardless of treatment. There was no statistically significant difference between oxygen saturation percentage at baseline between all four conditions (Normoxia Watercress $99 \pm 0.70\%$, Hypoxia Watercress $99 \pm 0.53\%$, Normoxia $98 \pm 0.70\%$, Hypoxia $98 \pm 0.46\%$), ($F(3, 24) = 0.808$, $P = 0.502$). Oxygen saturation showed treatment and time interaction effect ($F(6, 48) = 20.91$, $P < 0.005$), with post hoc analysis revealing oxygen saturation significantly decreasing in both hypoxic environments, for both the watercress and the control condition ($P < 0.05$; Figure 5.2.1.). Both hypoxic trials compared to the normoxic trials observed a large ES (> 0.8) at 30 minutes and at 120 minutes oxygen saturation. Oxygen saturation did not differ in the normoxic environment for both, watercress and the control condition ($P > 0.05$). The mean decrease in oxygen saturation percentage for the watercress hypoxic condition relative to baseline was 5% at 30 minutes and 7% at 120 minutes with ES (1.52, 2.15, respectively). The mean decrease in oxygen saturation percentage for the control hypoxic condition relative to baseline was 6% at 30 minutes with and 6% at 120 minutes with ES (1.81, 1.89, respectively). Individual responses to each condition are shown in Figure 5.2.2. Thus, the hypoxic environment seemed to induce a hypoxic phenotype in the healthy volunteers.

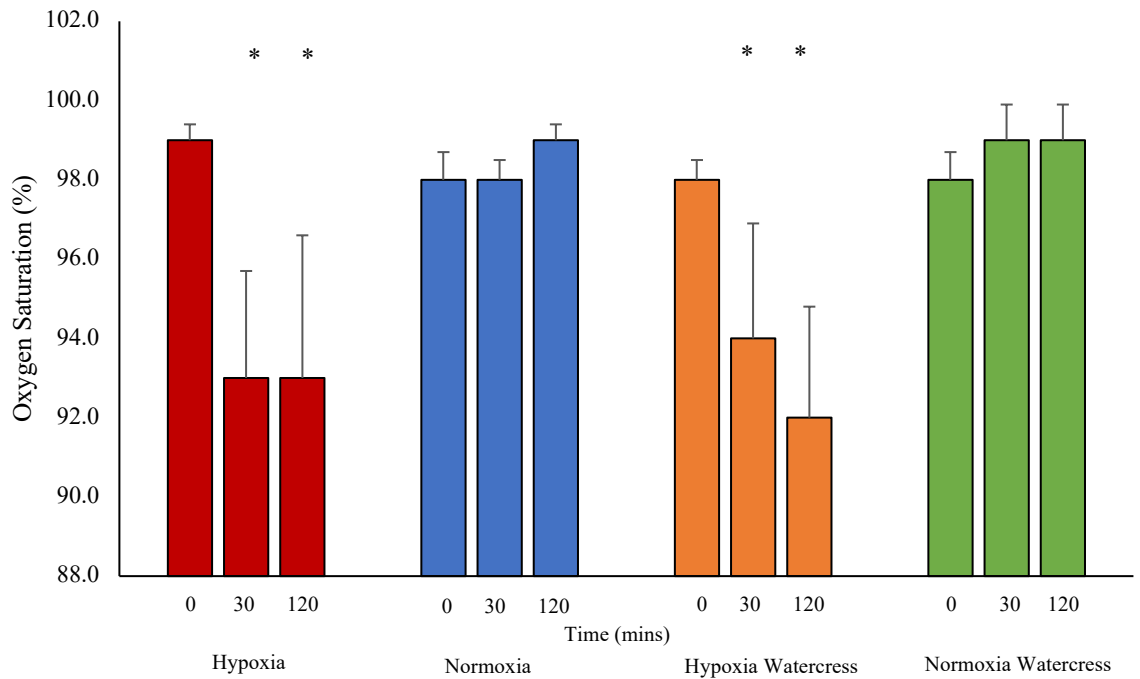


Figure 5.2.1. Change in oxygen saturation (%) in all four conditions at 0 (baseline), 30 and 120 minutes (n=9). Values are mean \pm SD. * significant difference from baseline (0) ($P < 0.05$).

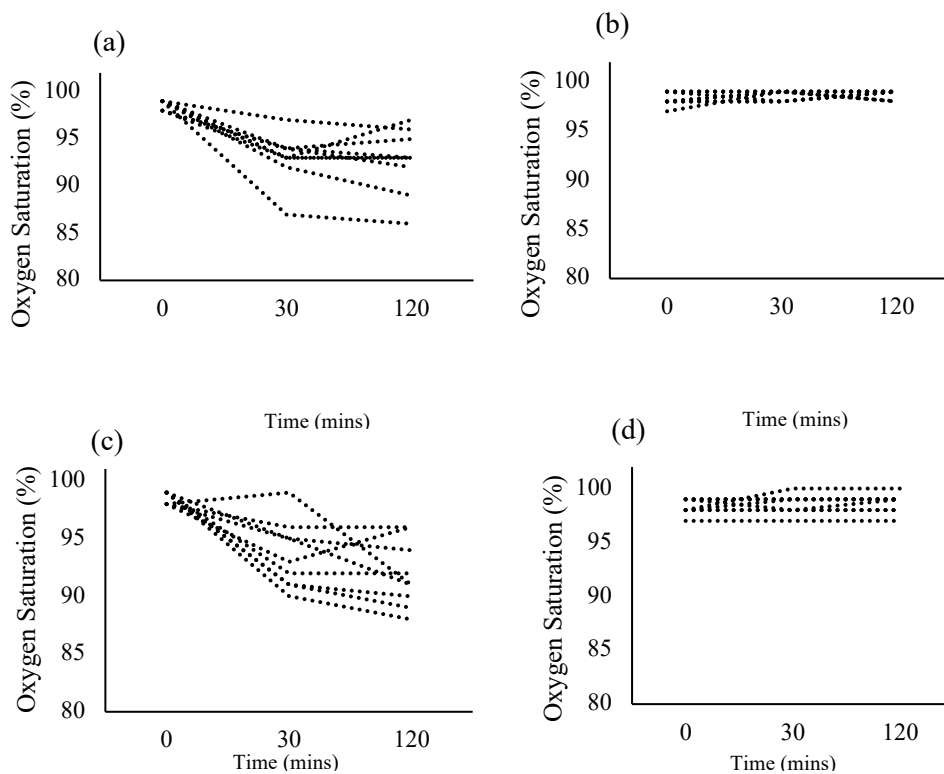


Figure 5.2.2. Individual oxygen saturation (%) at 0 (baseline), 30 and 120 minutes, (a) hypoxia control, (b) normoxia control, (c) hypoxia watercress, (d) normoxia watercress (n = 9).

Markers of vasodilation

5.3.2. Blood flow volume

Blood flow volume was measured for assessing changes in blood flow, an increase in this measure would be indicative of vasodilation. Baseline blood flow volume measures were not significantly different ($F(3, 24) = 0.558$, $P = 0.648$). Statistical analysis using a two-way repeated measures ANOVA showed no significant two-way interaction between condition and time ($F(6, 48) = 1.42$, $P = 0.226$), (Figure 5.3.1). Both normoxic conditions blood flow volume decreased at 120 minutes (Figure. 3.2.3), with water observing a moderate ES (0.62) in comparison to watercress normoxia ES (0.52), however, no condition or time effects were present ($P > 0.05$). In comparison at 120 minutes both hypoxic conditions, watercress increased by 4.17% (0.45 ± 0.01 to $0.048 \pm 0.01\%/min$, ES: 0.27) and the control by 11.84% (0.038 ± 0.01 to $0.042 \pm 0.02\%/min$, ES: 0.30) relative to baseline however, neither were significant ($P > 0.05$; Figure 5.3.1.).

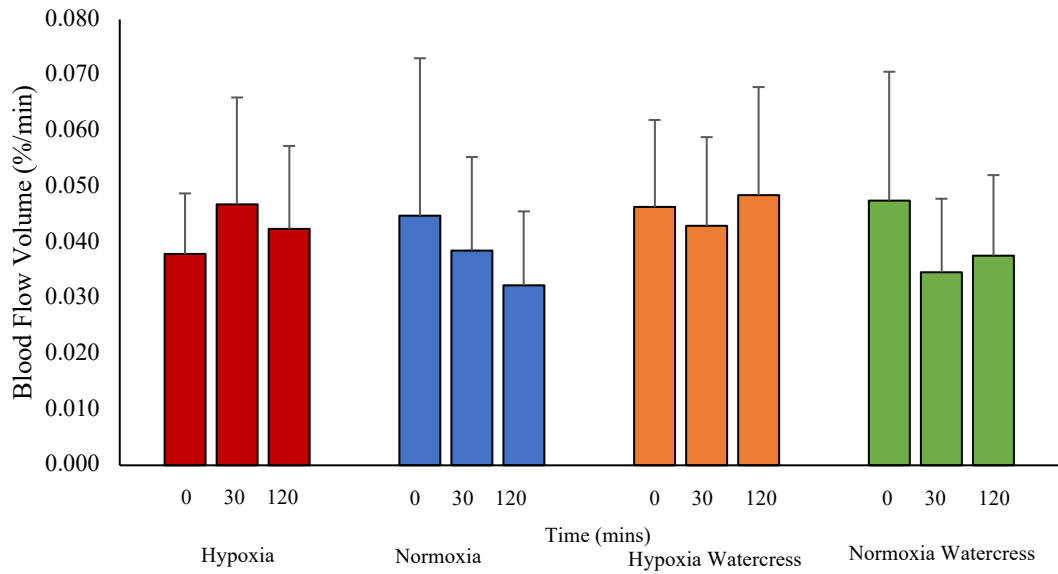


Figure 5.3.1. Comparison of blood flow volume (%/min) at 0 (baseline), 30 and 120 minutes in all four conditions (n = 9). Values are mean \pm SD.

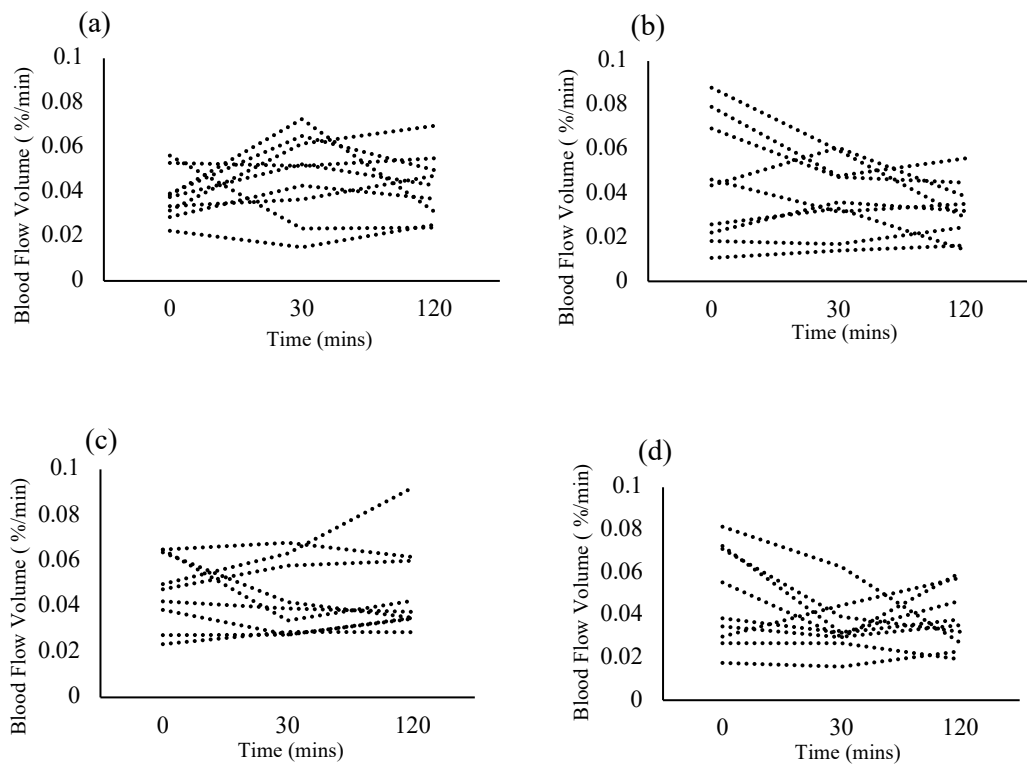


Figure 5.3.2. Individual responses in blood flow volume (%/min) at 0 (baseline), 30 and 120 minutes, (a) hypoxia control, (b) normoxia control, (c) hypoxia watercress, (d) normoxia watercress (n = 9).

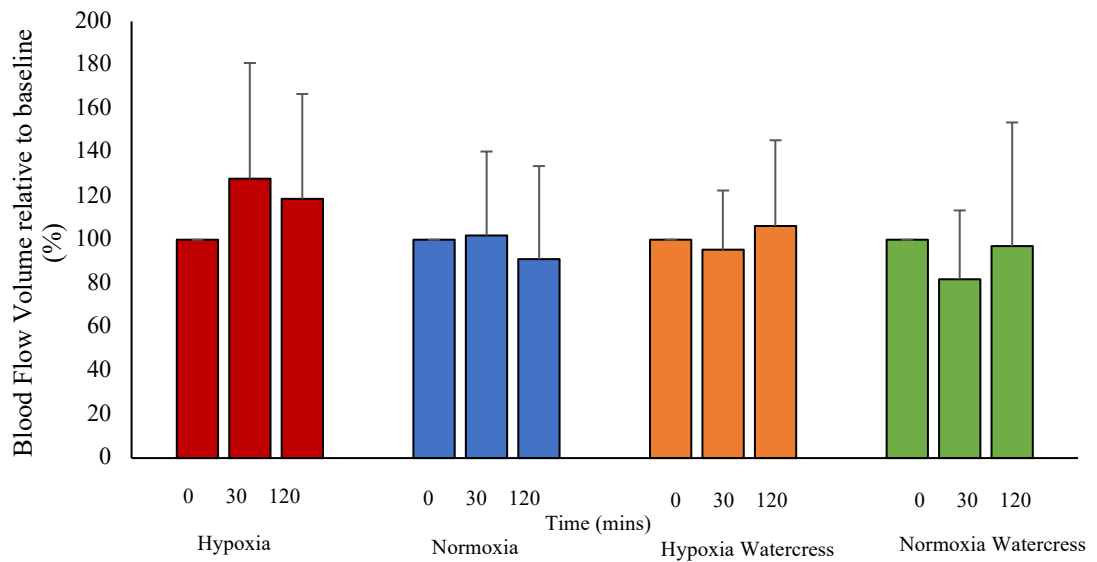


Figure 5.3.3. Change of blood flow volume (%/min) relative to baseline (0) in all four conditions at 0, 30 and 120 minutes (n = 9). Values are mean \pm SD.

5.3.3. EndoPat

Reactive hyperaemia peripheral arterial tonometry was measured as a way of assessing endothelial mediated changes (Yeboah, Crouse, Hsu, Burke, & Herrington, 2007). Baseline RHI measures were not significantly different ($F(3, 12) = 0.77$, $P = 0.529$). Reactive hyperaemia index showed no condition, time or two-way interaction effect ($F(3, .257) = 1.374$, $P = 0.40$), see Table 5.3. All conditions observed a trivial ES (<0.2), except the control normoxic condition observed a large ES (1.50) over time.

Table 5.3. RHI (n = 5), a marker for endothelial function at 0 (baseline) and 120 mins for each condition.

RHI	0 mins	120 mins
Hypoxia Control	1.76 ± 0.39	2.04 ± 0.68
Normoxia Control	2.01 ± 0.19	1.91 ± 0.22
Hypoxia Watercress	1.90 ± 0.24	1.72 ± 0.45
Normoxia Watercress	1.92 ± 0.23	1.97 ± 0.22

Values are mean ± SD. RHI, reactive hyperaemia index.

5.3.4. Blood pressure and cardiac output

Blood pressure and cardiac output were measured as increasing dietary nitrate increases nitric oxide bioavailability, stimulating smooth muscle relaxation via the synthesis of guanosine monophosphate (Murad, 1986). Systolic and diastolic blood pressure and cardiac output did not change significantly after nitrate supplementation in both conditions in comparison to the control environments ($P > 0.05$). Heart rate showed no condition, time, or a two-way interaction effect, ($P > 0.05$). Similarly, there was no condition, time or interaction effects for systolic and diastolic blood pressure and cardiac output, ($P > 0.05$). See Table 5.4. for results.

Table 5.4. Cardiovascular parameters for each condition at 0 (baseline), 30 and 120 mins.

	0 mins	30 mins	120 mins
SYS BP (mm Hg)			
Hypoxia Control	145 ± 25	145 ± 17	148 ± 24
Normoxia Control	148 ± 25	148 ± 22	146 ± 29
Hypoxia Watercress	143 ± 15	143 ± 16	143 ± 12
Normoxia Watercress	147 ± 18	147 ± 21	142 ± 19
DIA BP (mm Hg)			
Hypoxia Control	87 ± 9	85 ± 5	86 ± 9
Normoxia Control	88 ± 7	84 ± 5	86 ± 13
Hypoxia Watercress	86 ± 6	84 ± 5	85 ± 7
Normoxia Watercress	85 ± 7	82 ± 7	83 ± 8
Cardiac Output (L/min)			
Hypoxia Control	4.5 ± 0.7	-	4.8 ± 1.2
Normoxia Control	4.4 ± 0.8	-	4.2 ± 0.9
Hypoxia Watercress	4.2 ± 0.9	-	3.9 ± 1.8
Normoxia Watercress	4.7 ± 0.5	-	3.5 ± 1.0
Heart Rate (bpm)			
Hypoxia Control	54 ± 6	53 ± 5	51 ± 7
Normoxia Control	56 ± 7	53 ± 7	55 ± 7
Hypoxia Watercress	53 ± 8	50 ± 6	51 ± 7
Normoxia Watercress	53 ± 5	52 ± 5	53 ± 5

Values are mean ± standard deviation. SYS = systolic and DIA = diastolic blood pressure (n = 9), cardiac output (n = 5), Heart rate (n = 9), for each condition.

5.3.5. Nitrate

Nitrate concentration was measured to indicate the efficacy of nitrate supplementation and to assess the nitrate- nitrite- nitric oxide pathway (Lundberg & Govoni, 2004). In the previous Chapter 4, using young healthy individuals we saw an increase in nitrate concentration with watercress supplementation. This study has shown that effect occurs in older participants. Nitrate concentration were not statistically significantly different at baseline in all four conditions (Watercress Normoxia 2.8 ± 0.9 $\mu\text{mol/L}$, Watercress Hypoxia 3.0 ± 1.3 $\mu\text{mol/L}$, Normoxia 3.6 ± 1.7 $\mu\text{mol/L}$, Hypoxia 3.3 ± 1.1 $\mu\text{mol/L}$) ($F(3, 21) = 0.652$, $P = 0.591$). A two-way repeated measure ANOVA observed a statistically significant two-way interaction between treatment and time, ($F(6, 36) = 10.79$, $P = .001$). Therefore, simple main effects were run. Nitrate concentration was statistically significantly different at the 30 minutes time point, ($F(3,24) = 15.67$, $P = 0.001$). With Bonferroni post hoc analysis revealing normoxia watercress condition increased significantly in comparison to the control normoxia and control hypoxia conditions, ($P = 0.006$; $ES = 2.51$; $P = 0.028$; $ES = 2.49$, respectively). Similarly, hypoxia watercress condition significantly increased in comparison to the control normoxia and control hypoxia conditions at 30 minutes, ($P = 0.025$; $ES = 2.03$; $P = 0.026$; $ES = 2.0$, respectively). Nitrate concentration was statistically significantly different at the 120 minutes time point, $F(3, 24) = 12.66$, $P = 0.001$. With post hoc analysis observing normoxia watercress to have significantly increased in comparison to normoxia and hypoxia control conditions, ($P = 0.001$; $ES = 1.93$; $P = 0.003$; $ES = 1.14$, respectively) (Figure 5.4.1). Nitrate concentration for both control conditions did not significantly change for the duration of the test, (Figure 5.4.2 and Figure 5.4.3).

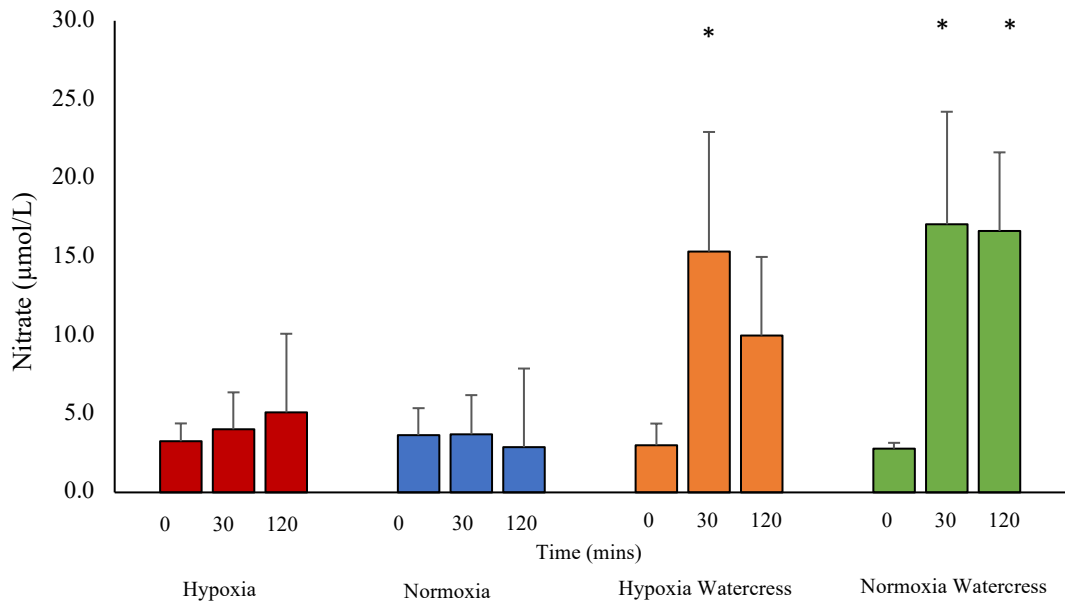


Figure 5.4.1 Plasma nitrate concentrations in all four conditions at 0 (baseline), 30 and 120 minutes (n=9). Values are mean \pm SD. * Significant difference between conditions ($P < 0.05$).

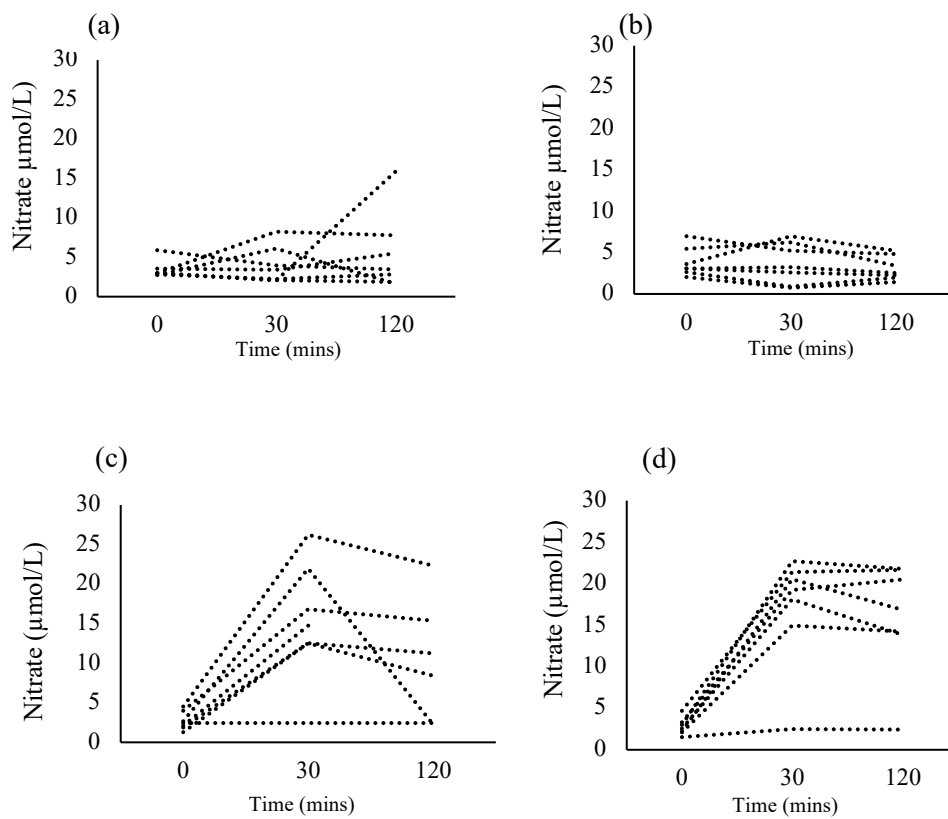


Figure 5.4.2. Individual nitrate concentration in plasma at 0(baseline), 30 and 120 minutes, (a) hypoxia control, (b) normoxia control, (c) hypoxia watercress, (d) normoxia watercress (n = 9).

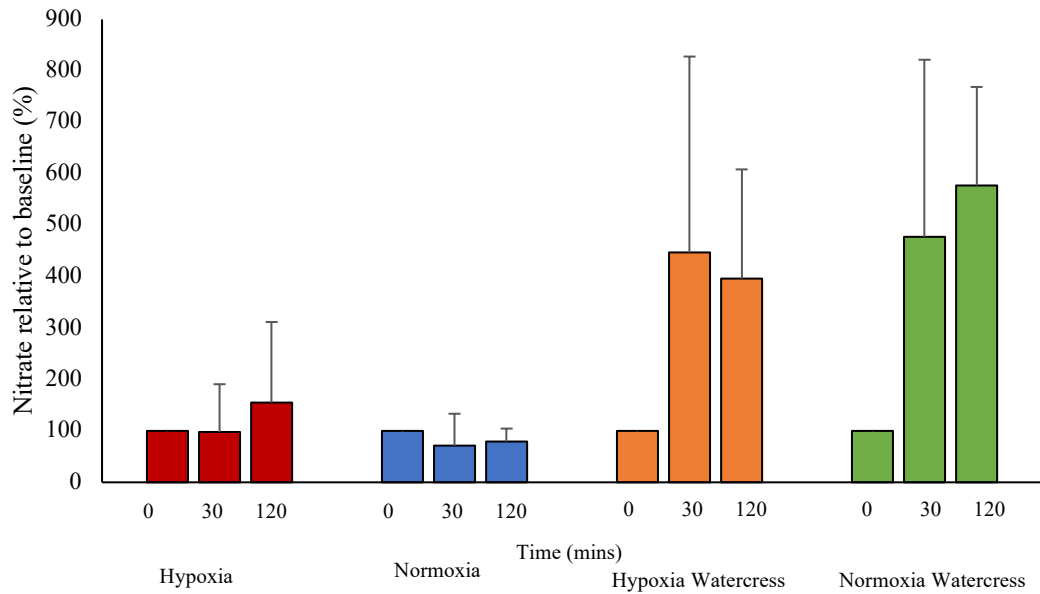


Figure 5.4.3. Percentage change for nitrate concentration relative to baseline (0) in all four conditions at 0, 30 and 120 minutes (n = 9). Values are mean \pm SD.

5.3.6. Nitrite

Nitrite concentration was not normally distributed ($P < 0.05$), as assessed by Shapiro-Wilk's test of normality on the studentized residuals. Baseline values for plasma nitrite concentration did not significantly differ between the four conditions (Hypoxia 0.18 (0.18 – 0.26) nmol/L, Normoxia 0.22 (0.22 – 0.38) nmol/L, Watercress Normoxia 0.22 (0.17 – 0.25) nmol/L, Watercress Hypoxia 0.21 (0.19 – 0.27) nmol/L,) ($F(3, 24) = 0.876$, $P = 0.467$); Figure 5.5.1). Nitrite concentration showed no time, condition or two-way interaction effects ($P > 0.05$), Figure 5.5.3. Friedman test analysis was found to be largely comparable to a two-way repeated measure ANOVA, with both tests reporting consistent results.

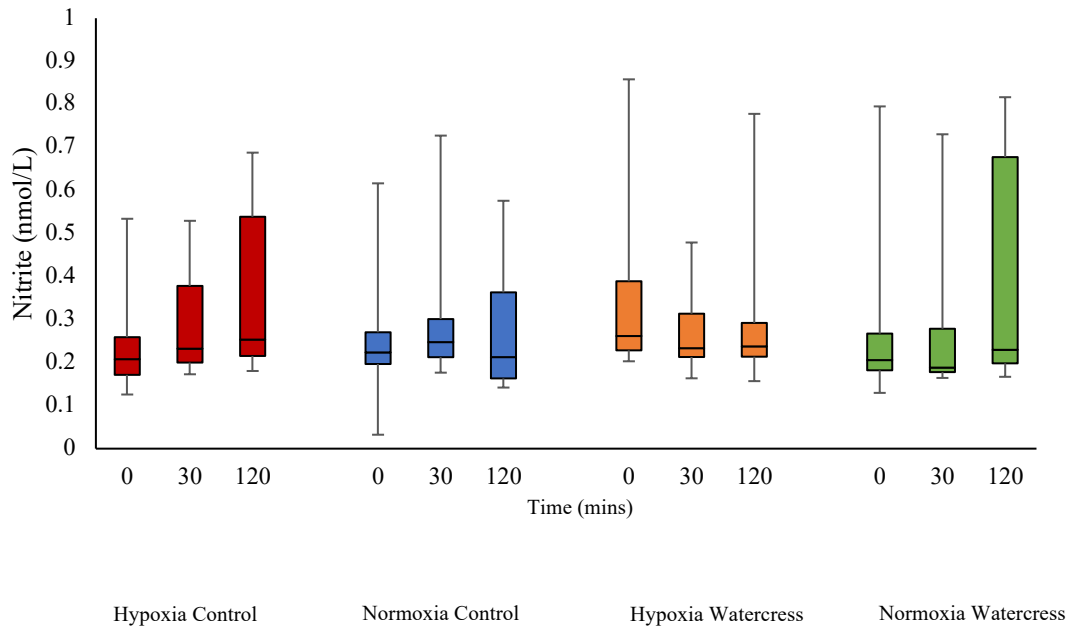


Figure 5.5.1. Plasma concentration of nitrite (nmol/L) at baseline, 30 minutes and 120 minutes post ingestion for all four conditions (n = 9). Boxes represent 50% of all data with the median in between: upper and lower whiskers show the rest of the data.

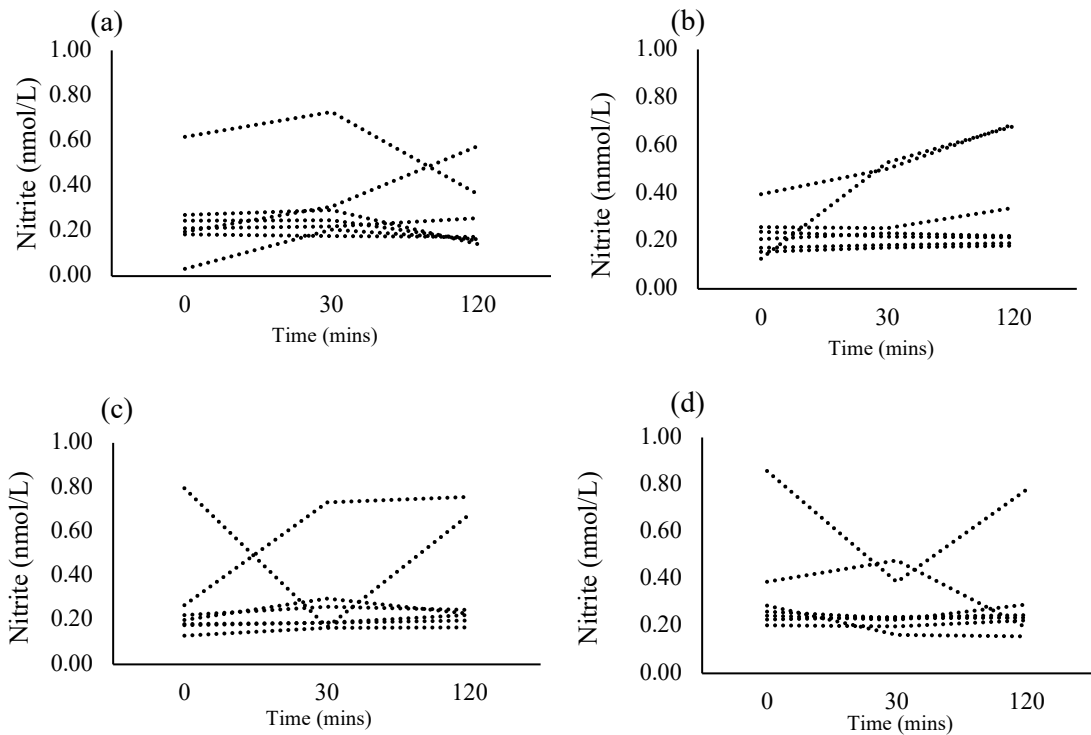


Figure 5.5.2. Individual nitrite concentration in plasma at 0 (baseline), 30 and 120 minutes, (a) hypoxia control, (b) normoxia control, (c) hypoxia watercress, (d) normoxia watercress (n = 9).

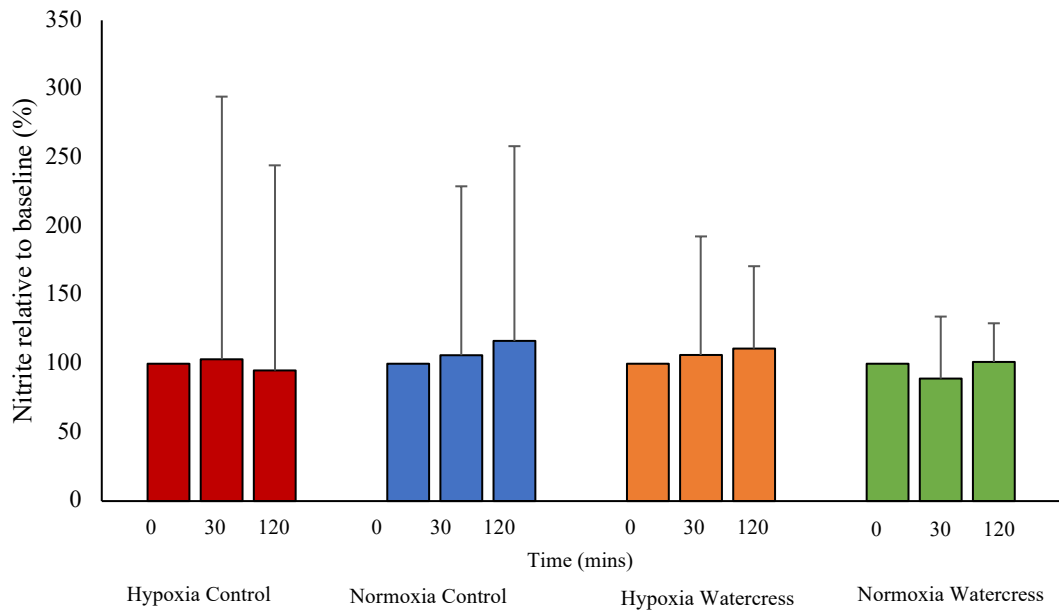


Figure 5.5.3. Percentage change for nitrite concentration relative to baseline (0) in all four conditions at 0, 30 and 120 minutes. Values are median and interquartile ranges.

Oxidative and nitrosylative stress markers

Oxidative stress is characterized by excessive production of reactive oxygen and nitrogen species (e.g., hydrogen peroxide, hydroxyl radical, nitric oxide and peroxynitrite) (Dhalla, Temsah, & Netticadan, 2000). In the previous chapter, using young healthy individuals we observed no statistically significant interaction between treatment and time in all oxidative stress markers.

5.3.7. Nitrotyrosine

There were no outliers, as assessed by examination of studentized residuals for values greater than ± 3 . Nitrotyrosine concentration was not normally distributed ($P < 0.05$), as assessed by Shapiro-Wilk's test of normality on the studentized residuals. A Friedman test was run to determine if there were time point differences in nitrotyrosine concentration in each supplementation group. Nitrotyrosine concentration was

significantly different at different time points in the Watercress hypoxia supplementation group $\chi^2(2) = 9$, $P = .011$, see Table 5.5. Post hoc Wilcoxon signed rank test determined a statistically significant median increase from baseline (Median = 112.5 ng/mL) to 30 minutes (Median = 324.1 ng/mL), $z = 2.20$, $P = 0.028$ and from baseline (Median = 112.5 ng/mL) to 120 minutes (Median = 345.3 ng/mL), $z = 2.52$, $P = 0.012$.

5.3.8. Lipid hydroperoxides

Lipid hydroperoxide concentration was assessed to measure lipid oxidation, to demonstrate oxidative stress. Lipid hydroperoxide concentration was not normally distributed ($P < 0.05$), as assessed by Shapiro-Wilk's test of normality on the studentized residuals. There was no statistically significant interaction between condition and time on lipid hydroperoxide concentration in serum ($F(6, 36) = 539$, $P = 0.775$). Additionally, there were no time or condition effects observed ($P > 0.05$). Substantial variation was observed in all conditions in the range of 3.20 – 293.1 $\mu\text{mol/l}$, as shown in Table 5.6. Friedman test analysis was found to be largely comparable to a two-way repeated measure ANOVA, with both tests reporting consistent results.

Table 5.5. Nitrotyrosine concentration at 0 (baseline), 30 and 120 minutes.

	0 mins	30 mins	120 mins
Nitrotyrosine (ng/mL)			
Hypoxia Control	121.6 (32.6 – 340.9)	141.2 (37.2 – 288.3)	150.0 (104.5 – 246.0)
Relative to baseline (%)	x	↑ 16.5	↑ 24.0
Normoxia Control	146.0 (98.3 – 175.5)	153.9 (128.7 – 327.3)	157.5 (127.3 – 241.6)
Relative to baseline (%)	x	↑ 5.4	↑ 7.9
Hypoxia Watercress	112.5 (72.7 – 149.9)	324.1 (199.3 – 415.3) *	345.3 (166.4 – 411.6) *
Relative to baseline (%)	x	↑ 189.3	↑ 208.0
Normoxia Watercress	168.8 (110.8 – 187.2)	110.8 (55.9 – 214.5)	205.64 (133.5 – 413.1)
Relative to baseline (%)	x	↓ 34.6	↑ 168.8

Values are median and interquartile ranges (n = 9). ↑ = increase, ↓ = decrease. * Significant difference from baseline (0 mins), P < 0.05.

Table 5.6. Lipid hydroperoxide concentration at 0 (baseline), 30 and 120 minutes.

	0 mins	30 mins	120 mins
LOOH ($\mu\text{mol/L}$)			
Hypoxia Control	23.6 (14.3 – 46.0)	21.2 (18.9 – 32.7)	20.1 (18.3 – 32.6)
Relative to baseline (%)	x	↓10.17	↓14.8
Normoxia Control	17.0 (13.2 – 46.0)	13.4 (10.2 – 15.3)	27.6 (15.3 – 29.8)
Relative to baseline (%)	x	↓21.2	↑62.4
Hypoxia Watercress	19.1 (13.8 – 53.4)	16.2 (10.7 – 33.2)	17.4 (13.2 – 52.1)
Relative to baseline (%)	x	↓15.2	↓8.9
Normoxia Watercress	30.1 (17.4 – 44.5)	12.8 (8.9 – 54.8)	19.5 (14.2 – 53.7)
Relative to baseline (%)	x	↓57.5	↓35.2

Values are median and interquartile ranges (n = 9). ↑ = increase, ↓ = decrease.

5.3.9. Protein carbonyls

Protein carbonyls concentration was assessed to measure protein oxidation, to demonstrate oxidative stress. Protein carbonyls were normally distributed ($P > 0.05$), as assessed by Shapiro-Wilk's test of normality on the studentized residuals. Protein Carbonyls (see Table 5.7) showed no time, condition or two-way interaction effects ($P < 0.05$). The watercress normoxia control was the only condition to observe a moderate effect size (ES 0.76) with an increase from 30 mins post supplement ($27.4 \pm 17.2 \mu\text{M}/\text{mg}$ protein) to 120 minutes ($45.9 \pm 31.6 \mu\text{M}/\text{mg}$ protein). A small effect size was observed a decrease from baseline to 120 minutes post supplementation for the normoxia control (48.8 ± 15.1 to $42.9 \pm 34.4 \mu\text{M}/\text{mg}$ protein). Similarly, a small effect was observed (ES 0.26), however the hypoxia watercress condition increased from baseline ($23.8 \pm 31.7 \mu\text{M}/\text{mg}$ protein) to 120 minutes post supplementation ($32.1 \pm 35.5 \mu\text{M}/\text{mg}$ protein).

Table 5.7. Protein carbonyl concentration for the four conditions at 0 (baseline), 30 and 120 minutes.

PC ($\mu\text{M}/\text{mg protein}$)	0 mins	30 mins	120 mins
Hypoxia Control	35.9 \pm 23.6	28.6 \pm 20.9	38.7 \pm 24.2
Relative to baseline (%)	x	↓ 20.3	↑ 7.8
Normoxia Control	48.8 \pm 15.1	54.1 \pm 14.9	42.9 \pm 34.4
Relative to baseline (%)	x	↑ 10.8	↓ 12.1
Hypoxia Watercress	23.8 \pm 31.7	43.8 \pm 42.4	32.1 \pm 35.5
Relative to baseline (%)	x	↑ 84.1	↑ 34.9
Normoxia Watercress	40.6 \pm 27.3	27.4 \pm 17.2	45.9 \pm 31.6
Relative to baseline (%)	x	↓ 32.5	↑ 13.1

Values are mean \pm SD. PC = protein carbonyls concentration (n = 9). The arrows indicate ↑ = increase, ↓ = decrease from baseline

5.3.10. Platelets

To quantify platelet reactivity to dietary nitrate platelet aggregation was measured. Fifty percent of platelet aggregation induced by ADP concentration for each condition was calculated. A two-way repeated measure ANOVA found no statistically significant platelet aggregation for time, condition or two-way interaction ($P > 0.05$; Figure 5.6.1).

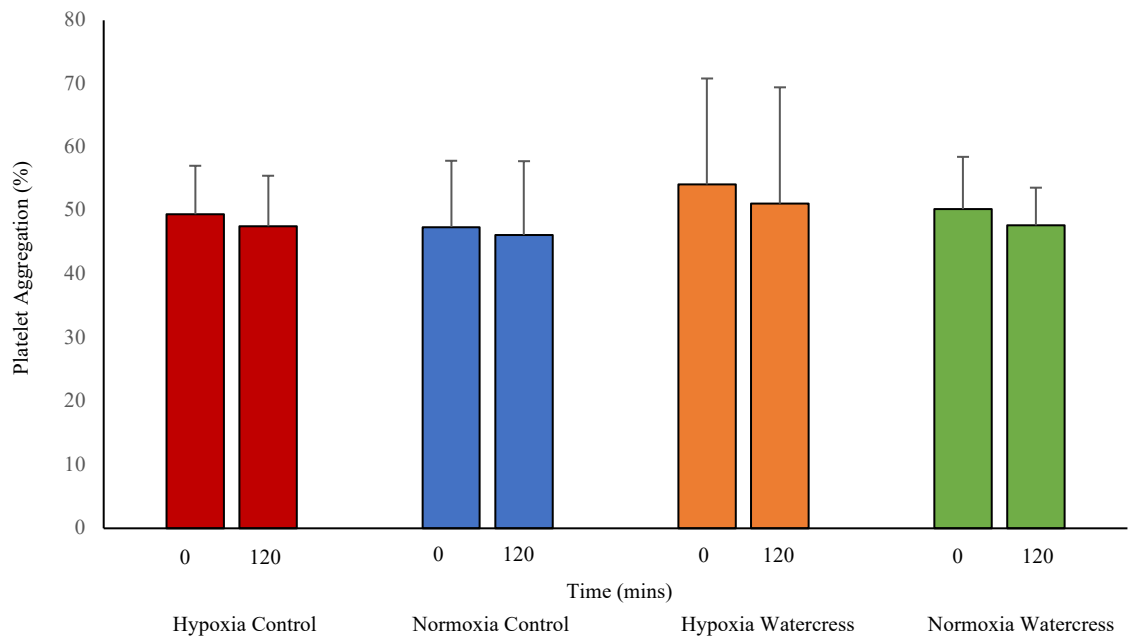


Figure 5.6.1. Platelet aggregation in all four conditions at 0 and 120 minutes ($n = 5$). Values are mean \pm SD.

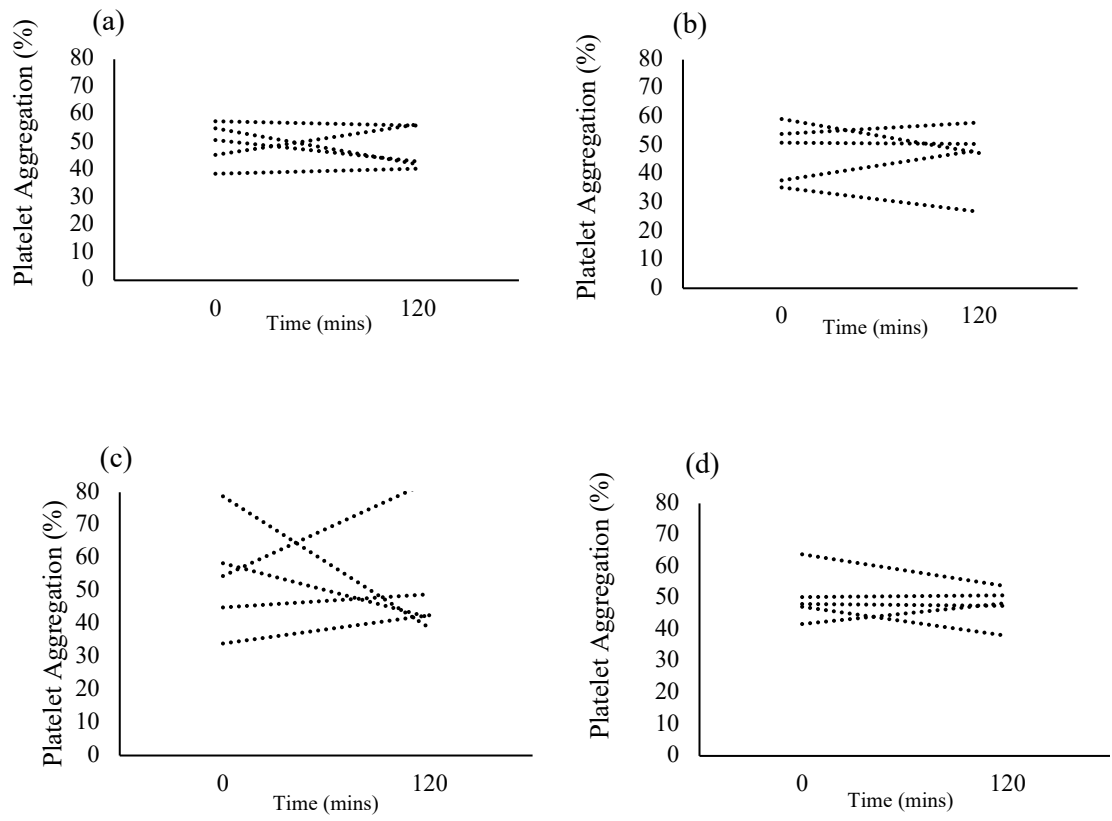


Figure 5.6.2. Individual platelet concentration in plasma at 0 (baseline) and 120 minutes, (a) hypoxia control, (b) normoxia control, (c) hypoxia watercress, (d) normoxia watercress (n = 5).

5.4. Discussion

In this chapter we hypothesised acute watercress supplementation in both normoxic and hypoxic conditions will increase nitrate, nitrite concentration, improve cardiovascular parameters and alter oxidative/nitrosylative stress markers in comparison to the control conditions in a healthy, aged, physically active population. The present investigation findings were consistent with our hypotheses, acute hypoxia significantly reduced oxygen saturation and watercress supplementation increased plasma nitrate levels in a healthy aged population in a hypoxic and normoxic environment. However, in contrast to our hypotheses, acute watercress supplementation did not significantly alter plasma nitrite levels, vasodilatory responses or oxidative stress markers in the normoxic and hypoxic conditions.

Research has shown nitrate rich supplementation to follow a stepwise reduction from nitrate to nitrite and further to nitric oxide (Lundberg et al., 2008). In contrast to previous studies, the watercress supplementation in the normoxic environment did not observe a significant increase in plasma nitrite from baseline to the two-hour time point in comparison to other conditions, similarly this was also observed in Chapter 4 (Kelly et al., 2014; Rammos et al., 2014; Webb et al., 2008). The reduction of nitrite is potentiated in hypoxic conditions, and therefore nitric oxide production from this pathway can be seen as a reserve system to guarantee continual nitric oxide generation when the endogenous, oxygen dependent, NOS pathway may be dysfunctional, possibly due to a hypoxic setting, the process of ageing or disease (Castello, McClure, Crook, & Poyton, 2006; Lundberg et al., 2008; Modin et al., 2001). The watercress supplement in the hypoxic environment did not observe an augmented nitrite response by the two-hour time point in comparison to the other conditions. This may be due to the age-related changes in bacteria in the oral cavity (Stanaway et al., 2017). However, plasma nitrite levels have been shown to peak between 2 – 3 hours after nitrate consumption, perhaps a blood

sample one hour later would have indicated a significant increase in nitrite levels in the watercress conditions and potential physiological responses (Jones et al., 2018; Webb et al., 2008). Practically speaking, participants found the two hours restricted bathroom access to be problematic, a three-hour blood sample would have proven to be difficult due to lack of facilities in the chamber.

Based on earlier nitrate research, we hypothesised that the nitrate rich watercress supplement would lower resting blood pressure compared to the control interventions (Kapil et al., 2010; Lansley et al., 2011; Vanhatalo et al., 2010). The current investigation did not observe a supplement effect on blood pressure in either normoxia or hypoxia. This may have been due to the variability in blood pressure measures, the small sample size or the dose. Research has observed chronic and acute ingestion of nitrate, to increase plasma nitrite and for this increase to be associated with a reduction in systolic and diastolic blood pressure (Bailey et al., 2010; Bailey et al., 2009; Lansley et al., 2011; Lansley et al., 2010; Larsen et al., 2010; Lundberg & Govoni, 2004; Vanhatalo et al., 2010; Webb et al., 2008). Studies have reported systolic and diastolic blood pressures to be reduced by up to 10 mmHg after nitrate daily doses ranging from 5.2 to 22.5 mmol, although these studies were mainly in young males (Bailey et al., 2009; Kapil et al., 2010; Lansley et al., 2011; Webb et al., 2008). A recent review indicated varied findings for the effects of acute and chronic dietary nitrate on blood pressure in older adults (Stanaway et al., 2017). The review suggested reasons for these inconsistent findings on efficacy of nitrate supplementation on blood pressure may be due to variances in the participant's health statuses. Our sample of older adults were healthy and physically active, this may have blunted the effect of nitrate treatment.

A study reported after three weeks of beetroot juice (300-400 mg of nitrate) in an old overweight population, systolic blood pressure reduced by 7 mmHg (Jajja et al., 2014). Interestingly, one week after the intervention blood pressure scores returned to pre-

intervention baseline (Jajja et al., 2014). Although the nitrate dose was the same (400 mg), our sample of older adults were healthy, with participants reporting endurance-based exercise three times a week, for over an hour each session. This may have blunted the effect of the watercress supplementation as research has indicated plasma nitrite to be directly related to exercise capacity (Rassaf et al., 2007). Resting plasma nitrate and nitrite levels are elevated in endurance-trained individuals compared to untrained individuals, likely due to augmented training-related NOS activity (Bescós et al., 2011; Jungersten, Ambring, Wall, & Wennmalm, 1997; McAllister & Laughlin, 2006; McConell et al., 2007; Rassaf et al., 2007).

In the current investigation, reactive hyperaemia indices did not differ significantly between watercress conditions and water treatments or over time in healthy older participants. This would suggest that an acute dose of watercress had no effect on vascular function in healthy, aged adults. A higher dose of nitrate may be required for this cohort due to the association of ageing and the decreased sensitivity of vascular smooth muscle cells to the vasodilation effects of nitric oxide (Lyons, Roy, Patel, Benjamin, & Swift, 1997). This diminished sensitivity causes a reduced nitric oxide -dependent dilation of vessels as shown by a decreased in vitro response of vascular smooth muscle cells to nitric oxide with ageing (Minamino & Komuro, 2007). This may in part explain the reduced vascular responses in this specific cohort (Minamino & Komuro, 2007).

The present study observed no inhibition of platelet aggregation, however, a study attributed inhibition of *ex vivo* platelet aggregation to be associated with the increase in nitrite concentrations (Webb et al., 2008). This association was demonstrated by a separate group, by purposefully interrupting the entero-salivary circulation by spitting and avoiding and swallowing saliva for 3 hours following beetroot ingestion (34.0 mmol/L) (Webb et al., 2008). The expectoration and avoidance caused a diminished increase in plasma nitrite but not nitrate concentration, and inhibited the reduction in

blood pressure blood pressure and no inhibition of platelet aggregation was observed (Webb et al., 2008). Therefore, in the present study, the lack of increase in plasma nitrite may have also influenced the absence of inhibition on platelet aggregation.

Nitrate is reduced to nitrite by facultative anaerobic bacteria in the oral cavity (Siervo et al., 2018). The ageing process is associated with changes in oral microflora which may have affected the capacity of bacterial reductase activity in the conversion of nitrate into nitrite in this cohort (Percival, Challacombe, & Marsh, 1991). Furthermore, with ageing, gastric acid production decrease, this process may impact on the generation of nitric oxide in the stomach from the acid mediated disproportionation of nitrite (Britton & McLaughlin, 2013; Zweier, Samouilov, & Kuppusamy, 1999). Thus, it remains unclear whether a larger dose of watercress may be required for aged populations to compensate for the decline in redox potential and to enhance nitric oxide bioavailability.

The watercress supplementation in the present study showed no significant alteration in oxidative stress markers in either condition, at any time point. Previous research has observed a decrease in DNA damage and lipid peroxidation following watercress supplementation compared to control (Fogarty et al., 2013; Gill et al., 2007). Research has compared physically fit older adults to unfit age matched control group and found the active older adults to have less oxidative stress than unfit age-matched controls (Traustadóttir et al., 2012). The authors suggested this was due to differences in activity of antioxidant enzymes (Traustadóttir et al., 2012). Although, Margaritelis et al., 2014 has also shown oxidative stress markers to vastly differ among individuals, as was certainly observed in this study.

Interestingly, a study observed an acute consumption of vegetables smoothie, high in nitrate and low in thiocyanate, to lower systolic blood pressure, whereas the same dose of nitrate co-ingested with thiocyanate rich vegetables did not decrease systolic blood pressure and reported a smaller increase in salivary nitrite (Dewhurst-Trigg et al., 2018).

From this, the authors suggested vegetables high in thiocyanate to compete with nitrate into the salivary glands and potentially inhibit nitrate metabolism (Dewhurst-Trigg et al., 2018). Watercress is rich in glucosinolates, when glucosinolates are consumed they are hydrolysed by the enzyme myrosinase to form isothiocyanates (Gill et al., 2007). Although watercress was not included by Dewhurst-Trigg et al., (2018), in the vegetable smoothie, further studies are needed to examine the effect of watercress on salivary nitrite and thiocyanate (Dewhurst-Trigg et al., 2018).

5.4.1. Limitations

Limitations in the present work include the small sample size in the study; however, this was chosen based on previous sample sizes used in interventional studies in this area of research. The lack of controlled diet over the course of this research may have contributed to the difference in the studies, as the plasma nitrite levels in the current investigation showed a large variance. In the present investigation to enhance “ecological validity,” we allowed participants to consume their “regular” diet before the initial testing session and to replicate this identical diet prior to all subsequent sessions. Given the absence of rigid control of diet, the possibility of nondisclosed poor adherence prior to the sessions may have influenced our results. Diets high in vegetables may also contribute to the high variation observed in this thesis.

Although, the oxygen saturation was significantly reduced in the both hypoxic environments from the 30 minutes post ingestion time point, signifying the efficacy of the hypoxic environment. Mean oxygen saturation in healthy aged adults is around 95%, with hypoxaemia, conventionally defined as an oxygen saturation < 90% (Beasley et al., 2017; Majumdar, Eurich, Gamble, Senthilselvan, & Marrie, 2011). Although both hypoxic conditions observed a significant reduction in mean oxygen saturation, neither conditions scored below 90% over the two-hour period. Due to ethical restrictions the

chambers oxygen setting was not permitted to be reduced any further. Perhaps due to the restricted oxygen setting, time constraint and sedentary nature of this protocol, the participants vascular system was not over stressed to observe an induced vasodilatory response.

5.4.2. Conclusion

Watercress supplementation does not improve vascular function or attenuate oxidative stress biomarkers in a healthy aged population at rest under normoxia or hypoxia. The current investigation showed plasma nitrate to significantly increase in both watercress conditions, two hours after consuming watercress. However, plasma nitrite levels did not significantly increase two hours after ingestion followed by no physiological effects on the parameters analysed in this study. Further research is required using a larger sample size and a higher dose acutely and/ or chronically to elucidate the effect of watercress on healthy aged adults.

**Chapter 6:
Repeatability of 16.1 km time trial
performance, blood flow and oxidative
stress markers.**

6.1. Introduction

The two most important aspects of measurement error are validity and repeatability (Hopkins, 2000). Validity represents the accuracy of an observation in terms of its proximity to the true value, whereas, repeatability relates to the capacity to reliably reproduce similar values when a measurement is repeated, with minimum error. Repeatability is essential in human science, as there are many technical and biological factors that may contribute to the error of measure.

Hopkins (2000) maintains that within-individual variation is the most important factor of repeatability, as it affects the accuracy of estimations of change in the variable. When observing performance or health parameters, the smaller the within-individual variation, the easier it is to identify a true change (Hopkins, 2000). Many aspects can affect the within-individual variation, including the use of different equipment, different operatives of equipment, however, the primary source of error stems typically from biological variations. For example, oxidative stress associated with exercise has been extensively investigated, however, the repeatability of these markers is not well defined. Various factors can affect the oxidative stress balance, including diet, lifestyle and environmental conditions (Carraro et al., 2018). These factors can cause a day-to-day variation and may modify the observed value of measure.

While many factors can affect the observed values of a measure, the present study will focus on the repeatability of 16.1 km time trial performance (TT), blood flow and oxidative stress blood markers after a 16.1 km TT. Research has shown that time trials are reliable, as they have been shown to have a CV of < 5% (Currell & Jeukendrup, 2008). This makes TTs useful for measuring the efficacy of an ergogenic aid in a controlled trial and offers insight on Chapter four's TT study (Hopkins, 2000). The repeatability of a 16.1 km TT distance has been demonstrated in previous research. However, there is a need to

measure the repeatability of a testing method in one's laboratory and in the specific participant population (Currell & Jeukendrup, 2008; Sparks et al., 2016).

In light of the substantial variation seen for a range of endpoints in Chapters four and five, the purpose of this study is to assess the error of measurement in chosen parameters to help interpret the previous findings. Therefore, the aim of the study was to investigate the repeatability of forearm blood flow, the change in oxidative/nitrosylative stress markers after the 16.1 km TT and 16.1 km TT performance in a physically active male cohort.

6.1.1. Hypotheses

We hypothesised the healthy, physically active cohort would demonstrate a repeatable performance over the 16.1 km time trial performance one week apart. We hypothesised the venous occlusion plethysmography occlusion results and, the oxidative and nitrosylative stress markers measures one week apart would not be repeatable and to have a high variation.

6.2. Methods

6.2.1. Participants

Eleven healthy active males ($n = 11$) volunteered to participate in the present investigation and were recruited using by email and poster advertisement based on the University of Hull. All participants completed a physical activity readiness questionnaire and as a minimum requirement volunteers were required to be physically active as defined by the World Health Organisation (engage with 150 minutes of moderate-intensity aerobic physical activity throughout the week or, at least 75 minutes of vigorous-intensity aerobic physical activity throughout the week, or an equivalent combination of moderate- and vigorous-intensity activity). Further inclusion/ exclusion criteria are outline in Table 6.1. Participants were informed the purpose of the investigation was to study the repeatability of the performance times, venous occlusion plethysmography and biochemical parameters measured in blood. Prior to participation the protocol and the potential risks were outlined with verbal and written informed consent granted. This study was approved by the University of Hull Ethics Committee and conformed to the Declaration of Helsinki.

Table 6.1. Inclusion/exclusion criteria

Inclusion criteria	Exclusion criteria
Between 18 – 50 years old	Smoking
Physically active	History of cardiovascular/ metabolic disease
Male	Medication
Healthy	Supplements

6.2.2. Study design

Participants attended the laboratory three times altogether. All TTs were completed one week apart, at the same time of day (± 1 h) to minimise diurnal variation over the course of three weeks. The same equipment was used for all visits and operated by the same test administrator. Prior to each testing participants were requested to fast for a minimum twelve hours (water only permitted). Participants were not permitted to consume food or fluids during trials. Participants were requested to refrain from alcohol twenty-four hours before each test. For all TTs, participants were asked to cover the 16.1 km distance as quickly as possible on the SRMTM ergometer (Schoberer Rad Messtechnik (SRM), Germany).

In an attempt to reduce the influence of diet and physical activity on the outcome measures the participants were requested to fill out diet and physical activity diary for 2 days prior to their first visit. Participants were then asked to duplicate the diet and physical activity diary prior to returning for the following visits. In addition, participants were reminded to maintain their regular diet.

6.2.3. Visit 1

The first trial was carried out to familiarise the participants with the testing procedure and the cycle ergometer. Blood flow was measured prior to TT in all three visits. In the following two sessions venous blood samples were collected pre- and post-exercise.

6.2.3.1. Anthropometric measurements

Anthropometric measurements were performed (see Table 6.2.) as previously described in chapter (General Methods Chapter 3, 3.2.1).

Table 6.2. Anthropometric measures.

Characteristic	Mean \pm SD
Body Mass (kg)	79.5 \pm 8.7
Age (yr)	21.2 \pm 6.5
Height (cm)	179.0 \pm 5.0
BMI (kg/m ²)	23.4 \pm 2.0

All data are presented as mean \pm standard deviation. kg = kilograms, yr = years, cm = centimetres, BMI = body mass index, kg/m² = kilograms per metres squared.

6.2.3.2. Venous occlusion plethysmography

Blood flow volume measurements were recorded at baseline, 30 minutes and 120 minutes post supplementation. Please see the General Methods Chapter, section 3.2.5. for protocol.

6.2.3.3. Time Trial

Participants completed 16.1 km TT on the SRMTM ergometer with integrated heart rate monitoring (Polar Electro, Finland, General Methods Chapter 3, section 3.5.2.2). The cycle ergometer seat height, handlebar position and height were adjusted to replicate each of the participant's bicycle per trial. Before each trial participants performed 5 minutes warm up. The ergometer SRM (Schoberer Rad Messtechnik (SRM), Germany), was programmed in the mode "open end test". Participants started the test in "gear 9" and were allowed to change gear. In this mode, power output varies with pedal rate and/ or a gear change. Participants were asked to cover the 16.1 km distance as quick as possible. To simulate a time trial, no blood samples or cardiorespiratory measurements were taken

during the time trial. TT began after a numerical countdown from a stationary start. Power, heart rate, distance and time were recorded by the SRM software. To avoid experimental bias, the only feedback available to cyclists during the trial was the distance covered. No verbal encouragement was given so to ensure every participant had the same setting.

6.2.4. Visit 2 and 3

6.2.4.1. Venous occlusion plethysmography

Prior to exercise blood flow measurements. Please see General Methods Chapter 3, section 3.5.2.4 for protocol.

6.2.4.2. Blood collection

Participants fasted for twelve hours overnight prior to provision of a venous blood sample. This protocol restricts the dietary effect of blood borne metabolites such as antioxidants and lipids (Pronk, 1993). Samples were obtained for serum and plasma, as described in general methods (General Methods Chapter 3, 3.2.8.) at baseline, thirty minutes post supplementation and one hundred twenty minutes post supplementation.

After blood collection, EDTA vacutainers were placed on ice whilst SST's clotted in the dark at room temperature (ten minutes) before centrifugation at 1300 x g (Heraeus Labofuge 400R, Kendro Laboratory products, Bishops Stortford, UK) for ten minutes. Plasma and serum were removed using a 1 ml pipette and transferred to a 2 ml cryovial and immediately frozen (- 80 °C) prior to analysis. Whole blood was drawn by venepuncture into evacuated tubes containing 3.2% sodium citrate (Greiner-Bio One Ltd, Gloucestershire, United Kingdom) and inverted 4 times at baseline (prior to entering the chamber) and one hundred twenty minutes post supplementation for measurement of platelet aggregation.

6.4.2.3. Time trial

Participants completed 16.1 km TT on the SRMTM ergometer, protocol was followed as described in the above section 6.2.3.3.

6.2.5. Biochemical analysis

6.2.5.1. Lipid hydroperoxides

Lipid hydroperoxides were measured spectrophotometrically using the method of Wolff (1994) (General Methods Chapter 3, section 3.3.1). The intra-assay COV was < 4%.

6.2.5.2. Protein Carbonyls

Protein Carbonyls derivatives were measured according to the method of Levine (2002) (General Methods Chapter 3, section 3.3.3). The intra-assay COV was < 3%.

6.2.5.3. Nitrotyrosine

Nitrotyrosine was measured using a commercially available Enzyme-Linked Immunosorbent Assay kit (ab210603, Abcam, Cambridge UK) (See section 3.3.5). The intra-assay COV was < 4%.

6.2.5.4 Bradford Assay

Protein concentration was measured spectrophotometrically using the Bradford assay (General Methods Chapter 3, section 3.3.2). The intra-assay COV was < 5%.

6.2.6. Statistical analysis

Results are presented as mean \pm standard deviation unless otherwise stated. Data was analysed using a purpose made Excel spread sheet for reliability analysis (Hopkins, 2006). Repeatability of measures are reported as the coefficient of variation (CV), 90% confidence intervals (CI), intraclass correlations (ICC), typical error (TE) and Pearson correlation (r).

6.3. Results

The TTs were performed in a temperature-controlled environment (23.8 ± 1.0 °C; humidity $60 \pm 5\%$). Several biochemical markers have a reduced number of participants reported due to the limited availability of sample.

6.3.1. Time trial performance parameters

The largest difference between TT times was with trial one and trial three, with 39 seconds mean difference (see Table 6.3.), all individual times are presented in Figure 6.1. Mean TT times from visit two to three decreased by 7 seconds (1678 ± 146 vs 1671 ± 157 seconds). Comparison of the two trials observed a CV of 1% (95% CI; 0.7 – 1.9%), see Table 6.4. This indicates the time trial performance had an acceptable level of repeatability across the two events.

Table 6.3. Mean TT times (seconds) for each visit (n=11). TT 1, time trial one, TT 2, time trial two and TT 3, time trial 3.

	TT1	TT2	TT3
Times (seconds)	1710 ± 155	1678 ± 146	1671 ± 157

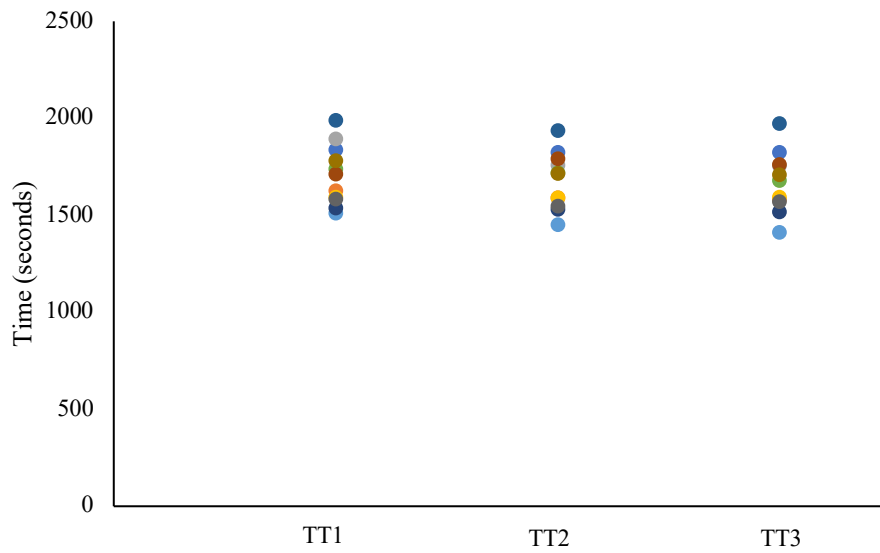


Figure 6.1.1. Individual time trial times (seconds) in all three visits ($n = 11$). Each colour dot represents the participant across the three visits. TT 1, time trial one, TT 2, time trial two and TT 3, time trial 3.

Table 6.4 The reliability of TT performance parameters between visit 2 and 3 (n =11).

Dependent Variable	N	COV (90% CI)	ICC	r
Time Trial (seconds)	11	1 (0.7 – 1.9)	0.9	0.987
Average Heart Rate (beats per minute)	11	4 (2.7 – 8.3)	0.7	0.717
Average Power Output (watts)	11	2.6 (1.8 – 4.8)	0.9	0.987

COV (90% CI), Coefficient of variation and 90% confidence interval; ICC, Intraclass coefficient; r, Pearson Product Moment Correlation

6.3.2. Blood flow volume

Mean (SD) blood flow values are displayed in Table 6.5.; individual data are displayed in Figure 6.2.1. Blood flow for visit 2 to 3 observed a CV of 39.3 % (CI (31.6 – 99.5%), (Table 6.6).

Table 6.5. Mean blood flow for each visit (n=11).

	TT 1	TT 2	TT 3
Blood flow (%/min)	0.038 \pm 0.01	0.047 \pm 0.02	0.046 \pm 0.01

Values are mean \pm SD. TT 1, Time trial one, TT 2, time trial two and TT 3, time trial 3.

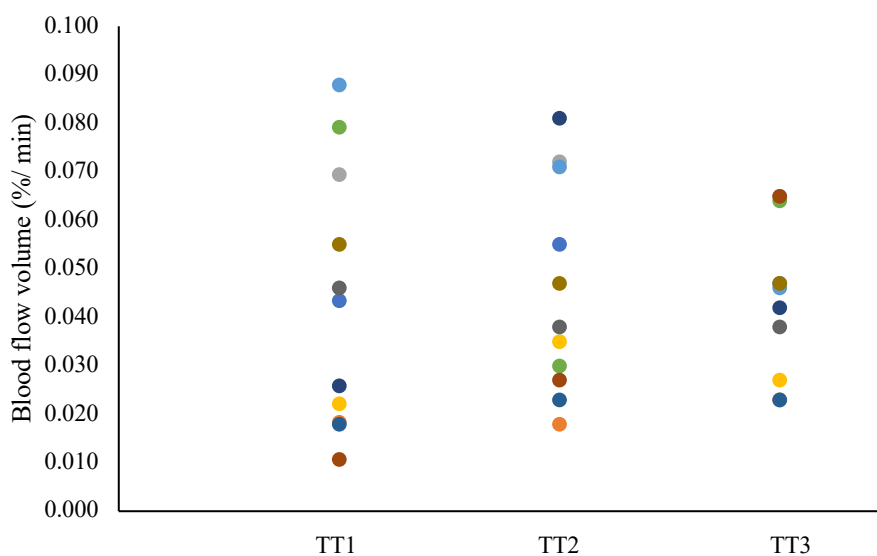


Figure 6.2.1. Individual blood flow (% / min) in all three visits (n = 11). TT 1, Time trial one, TT 2, time trial two and TT 3, time trial 3.

Table 6.6. The reliability of blood flow between visit 2 and 3 (n =11).

Dependent Variable	N	COV (90% CI)	ICC	r
Blood flow (% / min)	11	39.3 (31.6 – 99.5)	0.5	0.530

COV (90% CI), Coefficient of variation and 90% confidence interval; ICC, Intraclass coefficient; r, Pearson Product Moment Correlation

Oxidative and nitrosylative stress markers

6.3.3. Nitrotyrosine

Baseline values from TT2 to TT3 observed a CV of 85.2 (90% CI:55.7 – 187%). The mean (SD) plasma nitrotyrosine concentration percentage change for TT2 and TT3 were 166.0 ± 101.6 and $126 \pm 81.0\%$. The compared two values observed a CV of 95.8 (90% CI: 62 -215.7%). Nitrotyrosine showed a large variation ($r = 0.05$; see Figure 6.3.1.).

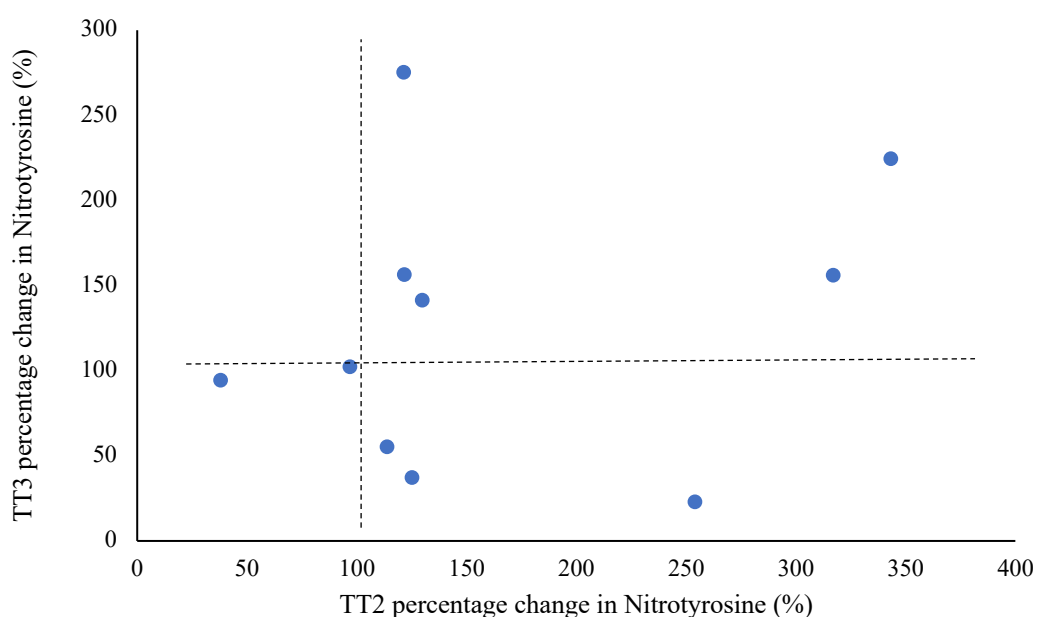


Figure 6.3.1. Nitrotyrosine percentage change plotted for TT2 and TT3 (n = 10). Each dot represents a participant. TT 2, time trial two and TT 3, time trial 3.

6.3.4. Lipid hydroperoxides

Baseline values from TT2 to TT3 observed a CV of 116.1 (90% CI: 70.9 – 338.8%). The mean (SD) plasma lipid peroxidation percentage change for T2 and TT3 were 79.4 ± 38.5 to $78.5 \pm 50.2\%$, respectively. The compared two values observed a CV of 272.9 (90 % CI: 157.3 -850.5%). Lipid peroxidation showed a large variation ($r = - 0.77$; see Figure 6.4.1.).

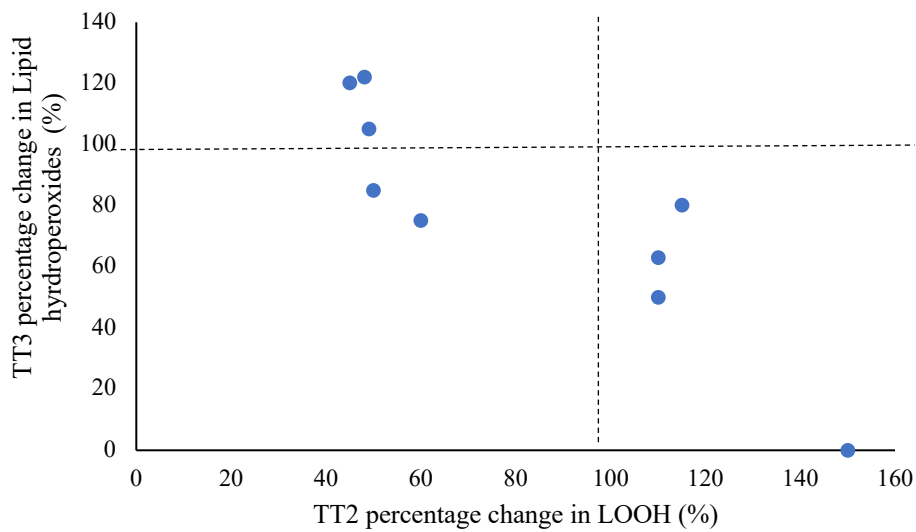


Figure 6.4.1. Lipid hydroperoxides percentage change plotted for TT2 and TT3 (n = 9). Each dot represents a participant. TT 2, time trial two and TT 3, time trial 3.

6.3.5. Protein carbonyls

Baseline values from TT2 to TT3 observed a CV of 65.5 (90% CI:43.6 - 136.7%). The mean (SD) plasma protein carbonyls concentration percentage change for TT2 and TT3 were $178.5 \pm 91.5\%$ and $148.5 \pm 47.3\%$, respectively. The compared two values observed a CV 60.6 (90% CI: 40.5 – 124.8%). Protein carbonyls showed a large variation ($r = 0.005$; Figure 6.5.1).

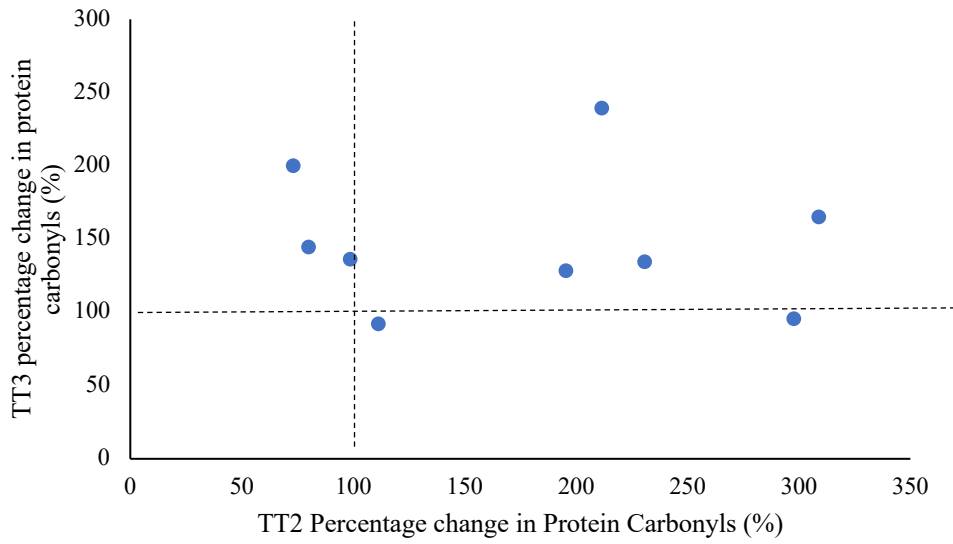


Figure 6.5.1. Protein Carbonyls percentage change plotted for TT2 and TT3 for each participant (n = 9). Each dot represents a participant. TT 2, time trial two and TT 3, time trial 3.

6.4. Discussion

The aim of the present experiment was to determine the repeatability between week two and three of baseline forearm blood flow, 16.1 km TT, oxidative and nitrosylative stress parameters at baseline and after the TT performance. The purpose of this study is to enhance the comprehension and value of the findings in the previous experimental chapters.

6.4.1. Repeatability of time trial performance

The primary findings from this investigation suggest that a 16.1 km TT on a SRM cycle ergometer in a physically active, male cohort, is highly repeatable. Data shows a mean decrease of seven seconds from TT2 to TT3, with a low CV of 1% for the performance time, and a CV of 2.6% for power output. Previous research has assessed similar exercise protocol durations, and have reported reliability CV's ranging from 1.1 - 1.9% for TT performance (Clark, Paton, & O'Brien, 2014; Driller, 2012; Sporer & McKenzie, 2007; Zavorsky et al., 2007). Laboratory based TTs have been widely used as exercise performance criteria and to evaluate the efficacy of an intervention. This study adds values to the findings in Chapter four, given the low typical error of measurement reported in the 16.1 km TT performance. As reported in Chapter four, beetroot juice improved TT performance by 0.2% and watercress supplementation enhanced TT performance by 1.4%. This 0.4% increase from the watercress condition in comparison to the CV reported in the current investigation, adds value to Chapter four's findings. This further suggests, watercress juice may enhance 16.1 km TT performance in physically active, young males.

6.4.2. Repeatability of blood flow

In the previous chapters, we have observed large variability when reporting forearm blood flow data. This purpose of this investigation was to observe how repeatable the measures can be under controlled conditions.

The data reported a large CV of 39.3% from trial two to trial three in baseline forearm blood flow. Previous research have assessed similar venous occlusion plethysmography protocols, and have reported blood flow reliability CV's ranging from 8.3 – 31% (Altenkirch, Fransson, & Koch, 1989; Petrie et al., 1998; Roberts, Tsao, & Breckenridge, 1986; Thijssen, Bleeker, Smits, & Hopman, 2005). The CV in the current study is found to be higher compared to other investigations, this variation may be due to several mechanisms. Forearm blood flow continuously changes in response to the sympathetic nervous system, the ambient temperature or mental arousal. Although the laboratory's temperature was controlled and the environment quiet, the participants were aware the next test was the TT after the blood flow measurement. This protocol was set up to evaluate and emulate the technique used in Chapter four and may have caused the high CV. Another likely day to day cause of variation may have been the positioning of the strain gauges on the forearm. Although, on each occasion great care was taken with positioning.

The results from the current study suggest that blood flow measurement in our laboratory are not repeatable and have a high variability, making it challenging to further our comprehension of results in Chapters four and five.

6.4.3. Repeatability of oxidative and nitrosylative stress markers

Many factors can influence oxidative stress such as dietary habits, health status, physical activity and the environment (Muñoz & Costa, 2013). However, a few studies have reported daily changes in oxidative stress biomarkers at baseline, even when confounding factors are considered (time of day, physical activity status and diet) (Goldfarb, Bloomer, & McKenzie, 2005; Margaritelis et al., 2014). Goldfarb et al., (2014) reported large daily fluctuations over a four-week period in young and healthy participants at rest. The current investigation observed a varied baseline for both oxidative and nitrosylative stress markers. For example, examining the biomarker with the lowest coefficient of variation at baseline; protein carbonyls concentration across the two visits showed a mean concentration of 50.64 $\mu\text{M}/\text{mg}$ in the range of 19.31 to 100.06 $\mu\text{M}/\text{mg}$. This highlights the importance of baseline measurements as there can be a fivefold difference of the concentration of protein carbonyls. This variation may potentially be due to diet, inflammation, fitness status or normal physiological fluctuations (Traustadóttir et al., 2012). Future research should aim to investigate the seasonal, monthly and daily fluctuations of RONS, as this may determine if a normal threshold of oxidative and nitrosylative stress biomarkers exists.

Oxidative and nitrosylative stress markers showed a larger variation when comparing the change in markers in week two to week three after completing the 16.1 km TT. Perhaps, in the current investigation it would have been beneficial to include a control session. In the control session participants would have attended the laboratory and had blood samples taken at the same time points (pre-exercise and end of previous TT time) but did not exercise. This could have possibly reduced individuality and the artefact of sampling time in the oxidative and nitrosylative stress markers. These findings were in line with Margaritelis et al., (2016). The study reported oxidative stress to significantly vary among recreationally active young males ($n = 100$) after the same bout of exercise, with some

participants experiencing elevated levels whilst others experiencing low levels of oxidative stress (Margaritelis et al., 2016). In the broader context of this thesis, the oxidative and nitrosylative stress values were also found to be highly variable in Chapter 4 and 5 with this, making it difficult to infer any effect of supplementation on oxidative and nitrosylative stress markers.

6.4.4. Limitations

In line with the majority of experiments, blood samples were collected immediately after exercise (Bloomer, Goldfarb, & McKenzie, 2006; Miyazaki et al., 2001). This narrow time window may not have reflected the change in stress markers (Michailidis et al., 2007). Blood samples collected over a longer period of time after the exercise may have seen delayed increase in stress measures. Studies have reported inconsistent results concerning the effect of exercise on levels of oxidative and nitrosylative stress markers (Bloomer, Davis, Consitt, & Wideman, 2007; Bloomer et al., 2006; Margaritelis et al., 2016). These differences may be due to the type of test protocol and sampling times and assays. Future research should consider performing serial measurements during a 24-hour period to understand the time course changes of stress measures. The present investigation was only performed in adult male participants, this should be repeated in adult female participants to assess the repeatability of the measures.

6.4.5. Conclusion

The present study provides evidence that forearm venous occlusion plethysmography and oxidative / nitrosylative stress measures are varied in the laboratory. These findings have clear implications for the design of future studies, specifically the calculation of sample

size, the timing of measurements, and thorough consideration that is required when anticipating confounding variables.

In conclusion, the protocol in the current investigation demonstrates the repeatability of a 16.1 km TT performance on a SRM cycle ergometer in young physically active male cohort. The low CV suggests that this test can enable sports scientists and trainers to better comprehend factors (e.g. ergogenic aids) that may influence cycling performance. As seen in Chapter four, the results suggest watercress supplementation may be an effective ergogenic aid for physically active young males.

Chapter 7: General Discussion

7.1. Introduction

The consumption of dietary nitrate has been one of the most discussed topics in exercise and nutrition communities over the past few years. Initially, it was thought nitrate and nitrite were carcinogens however, long-term toxicological findings in rats have not confirmed that nitrate and nitrite are carcinogenic (Forman, Al-Dabbagh, & Doll, 1985). The carcinogenic risks of dietary nitrate were disregarded as no epidemiological research could provide a causal link between nitrate consumption and cancer (Al-Dabbagh, Forman, Bryson, Stratton, & Doll, 1986; Nathan S Bryan & Ivy, 2015). In comparison to this, in recent years there has been reports of positive effects of dietary nitrate ingestion on human health. The reports of reduced blood pressure in healthy adults via a reduction of ingested nitrate to nitrite to nitric oxide alongside the emerging evidence of enhanced exercise performance has roused a great deal of scientist's interests.

Most of this positive evidence is from complex mixtures such as, beetroot juice, which is full of active constituents (fibre, antioxidants and minerals). Therefore, the link with nitrate intake is not fully understood. Thus, the aim of this thesis is to provide novel insight into the efficacy of an alternative nitrate rich juice, watercress juice, compared to a well-established, commercially available, dietary nitrate supplement; beetroot juice, both compared to sodium nitrate in order to gain mechanistic insights into the mechanism of action of nitrate rich supplements. Cardiovascular performance variables were observed in a young and old, healthy population.

7.2. Research questions addressed

Study 1. Can nitrate-rich supplements enhance vasodilatory variables, time trial performance and alter oxidative stress markers, in comparison to sodium nitrate or water in a young, healthy, physically active males?

Study 2. Can watercress juice enhance vasodilatory variables, influence oxidative stress biomarkers in normoxic or hypoxic environment, in healthy, physically active, older cohort?

Study 3. What is the repeatability of measures used in this thesis in order to understand the impact of inter and intra-individual variation in the studies in this thesis including; forearm blood flow prior to exercise using venous occlusion plethysmography, 16.1 km TT performance and the change in oxidative stress markers after the TT, in a healthy, physically active, young male cohort?

7.3. Summary of findings

Chapter 4. The effect of acute nitrate supplementation on vasodilatory responses, oxidative stress biomarkers and a 16.1 km time trial

The primary aim of this study (Chapter 4) was to compare the acute effects of nitrate matched supplements on vascular and oxidative stress parameters at rest and following a 16.1 km cycling time trial (TT). The study was a randomised crossover design, with sodium nitrate and water acting as positive and negative controls, respectively. The results of this study showed no significant difference between vasodilatory responses, oxidative/nitrosylative stress biomarkers or performance enhancement of a 16.1 km TT. An increase in plasma nitrite prior to exercise was only observed in beetroot juice and watercress juice, however these increases were not significant. Watercress supplementation was the only supplement to demonstrate an increase in forearm blood flow volume ($+0.18 \pm 0.03$ %/min), from baseline to two hours post-ingestion, however this was not significant ($P = 0.73$). Water significantly decreased (-0.61 ± 0.01 %/min) ($P = 0.03$), whereas beetroot ($P = 0.249$) was equivocal and sodium nitrate decreased ($P = 0.07$). The results of this study showed that oxidative stress was not significantly increased in any of the conditions for the biomarkers analysed. Numerous investigations have reported increased reactive oxygen and nitrogen species production following exercise (Fisher-Wellman & Bloomer, 2009). The lack of oxidative/nitrosylative stress observed in Chapter 4 may be due to the cohort (young, healthy, physically active) and the type of exercise completed was not strenuous enough to incur damage. Sodium nitrate reported the slowest time trial performance in comparison to the other three conditions. Watercress supplementation had the faster 16.1 km TT with a 1.4 % improvement compared to the water condition, while the beetroot juice was faster by 0.2 %. A critical improvement in performance in competitive sport is classed as small as 0.5 - 1.5 %,

although statistically non-significant, in concert with this, the small improvements in the watercress condition would potentially have a meaningful improvement in cycling TT performance in sub elite cyclists. These results suggest that there is potentially another bioactive component that is not nitrate or there is a synergistic effect between the nitrate content and other bioactive compounds in watercress which may have enhanced cycling performance in a young healthy active population.

Although statistically non-significant, having observed the watercress supplementation to exhibit a potential to improve TT performance, in young, physically active males, amongst all four conditions, we wanted to establish whether hypoxic setting would alter the efficacy of the supplementation in a healthy, aged population.

Chapter 5. The effect of acute watercress supplementation on cardiovascular and oxidative stress parameters in normoxic and hypoxic environments at rest with a healthy, aged population.

The purpose of this study (Chapter 5) was to investigate the efficacy of watercress juice on vasodilatory parameters and oxidative stress biomarkers in a healthy, aged population, in hypoxic and normoxic environments compared to a control (water) condition in hypoxic and normoxic environment. The study was a randomised, crossover design, the hypoxic environment acted to mimic some disease states. In the hypoxic, but not normoxic conditions, oxygen saturation significantly decreased, indicating the environmental effect. Results demonstrated plasma nitrite to not significantly increase in either watercress condition. Evidence suggests dietary nitrate supplementation provides a therapeutic influence by reducing the risk of hypertension (Kapil et al., 2010; Kelly et al., 2013; Vanhatalo et al., 2010). Novel data from this study indicated that watercress does not reduce resting systolic and diastolic pressure compared to control, in an older

population. The results of the study showed no significant difference in forearm blood or the reactive hyperaemia index when comparing the control conditions to the watercress conditions. There are plausible reasons for this finding. Firstly, it is postulated the lack of therapeutic effect or vasodilatory response is due to the high isothiocyanate content in watercress which may inhibit nitric oxide metabolism (Dewhurst-Trigg et al., 2018). Oxidative stress biomarkers were not significantly increased in any of the conditions. However, substantial variation was observed between the different conditions for lipid hydroperoxides (3.20 – 293.1 $\mu\text{M}/\text{mg}$ protein) protein carbonyls (1.72– 129.4 $\mu\text{M}/\text{mg}$ protein) and nitrotyrosine (4.01 – 983.9 ng/mL). Secondly, although the population were older, the participants were all physically active and not on any medication. Previous research has compared physically fit older adults to unfit age matched control group and found the active older adults to have less oxidative stress than unfit age-matched controls (Traustadóttir et al., 2012). The authors suggested this was due to differences in activity of antioxidant enzymes (Traustadóttir et al., 2012).

Dietary nitrate in the form of watercress juice did not enhance nitric oxide bioavailability and vascular function or modify oxidative/nitrosylative stress biomarkers in healthy, physically active, older adults. The results were surprising, as it is thought the efficacy of nitrate are likely greater when endogenous nitric oxide synthesis from NOS is compromised such as ageing, CVD or under specific conditions such as hypoxia or high altitude. This data led us to investigate whether forearm blood flow and oxidative/nitrosylative stress markers were repeatable. This would offer further insight and determine the methods used in this thesis to be repeatable, which is important for rigorous experimental research.

Chapter 6. Repeatability of 16.1 km time trial performance, blood flow and oxidative/nitrosylative stress markers.

The primary aim of the study (Chapter 6) was to investigate the repeatability of forearm blood flow using venous occlusion plethysmography prior to exercise, 16.1 km TT performance and the change in oxidative/nitrosylative stress markers after the TT, one week apart, in a recreationally active, male cohort. The results of the study indicated that the 16.1 km TT performance test on the SRM ergometer in the laboratory is a reliable endurance performance measure, as used in chapter 4. From this, it could be postulated watercress supplementation may be an effective ergogenic aid for recreationally active young males. Forearm blood flow observed high variability prior to exercise, in a controlled environment. The investigation demonstrated considerable variability in oxidative stress biomarkers after completing a 16.1 km TT comparing week one and week two. This suggests interventional studies observing oxidative/nitrosylative parameters need to pay particular attention to controlling confounding variables and caution should be given when reporting findings. As there are currently no reference values for oxidative/nitrosylative stress biomarkers, it is difficult to comment on the degree of variation (Davison et al., 2012). With this variability, it can considerably decrease statistical power (i.e., the probability that a test will identify an effect that exists). Chapter 6 highlights the efficacy of the watercress supplementation from Chapter 4.

7.4. Discussion

Investigational evidence in the present thesis highlights a discordance in nitrate literature. Findings from this thesis did not support the theory that dietary nitrate is capable of enhancing vasodilatory parameters (Chapters 4 and 5). Acute dietary nitrate

supplementation did not increase nitric oxide bioavailability, inferred from measures in plasma nitrite and had no effect on cardiovascular markers (blood pressure, heart rate, cardiac output) in young or older volunteers even when older cohort undertook hypoxic stress. An important, novel observation in the current thesis is that pure inorganic sodium nitrate did not alter cardiovascular function either (Chapter 4).

Experimental evidence in the present thesis did not support the theory dietary nitrate is capable of reducing systemic blood pressure. Data from Chapter 4 and 5 assessed in a healthy, young and old cohort are inconsistent with previous research (Jonvik et al., 2016; Muggeridge et al., 2014; Thompson et al., 2016; Wylie et al., 2013). The absence in reduction of blood pressure may be due to the health and activity status of the cohort (V. Kapil et al., 2010). In normotensive participants and highly trained individuals, the efficacy of nitrate may be less evident/absent due to optimum expression and activity of NOS, reduced inflammation/oxidative stress, decreased acidosis/ systemic hypoxia (Jones et al., 2018). It is postulated that crosstalk between endothelial NOS and nitrate-nitrite-nitric oxide pathway occurs, limiting nitric oxide generation when its availability is already optimal, thus reducing the risk of hypotension (Bondonno et al., 2015; Kerley et al., 2017).

An important, novel observation in the Chapter 4 is that sodium nitrate was observed to be ineffective at improving TT performance or altering cardiovascular function. Studies have investigated the effects in cycling (Cermak, et al., 2012; Cermak, et al., 2012; Lansley et al., 2011), running (Murphy, Eliot, Heuertz, & Weiss, 2012; Peacock et al., 2012) and rowing (Bond, Morton, & Braakhuis, 2012). With some reporting an increase in exercise performance after nitrate consumption (Bailey et al., 2009; Bailey et al., 2010; Lansley et al., 2011; Masschelein et al., 2012), while others reported no significant effects (Bescos et al., 2012; Christensen, Nyberg, & Bangsbo, 2013; Peacock et al., 2012). Most of these studies with no significant effects used sodium nitrate or potassium nitrate as a

supplement, while the positive findings used complex extracts. This thesis supports watercress juice and beetroot juice supplementation may have an ergogenic effect; however, our findings suggest nitrate may not be the bioactive species. Our data supports this theory with the watercress supplementation and to a lesser extent the beetroot supplement ergogenic effect on TT performance, as seen in Chapter 4. Watercress juice observed the fastest 16.1 km TT, with a 1.4 % improvement compared to the water condition, while the beetroot juice was faster by 0.2 %. A critical improvement in performance in competitive sport is classed as small as 0.5 - 1.5 %, although statistically non-significant, in concert with this, the small improvements in the watercress condition could be expected to have meaningful improvement in cycling TT performance in sub elite cyclists. From this evidence, it is indicated nitrate-rich supplements exhibit potential performance improvement (Cermak, et al., 2012; Cermak, et al., 2012; Lansley et al., 2011). Whether this is due to effective species independent of nitrate, or, in line with previous research, additional bioactive constituents may have an important role in nitrate metabolism and may have synergistic effect through an increased conversion of nitrite to nitric oxide (Lansley et al., 2011; Stanaway et al., 2017). This cannot be verified in this thesis.

Research has attempted to determine whether or not the main mechanism of action is nitrate driven by use of nitrate depleted beetroot juice. Interestingly, Gilchrist et al., (2014) described little ionic (sodium, potassium, calcium, magnesium and chloride) and ascorbate difference in nitrate-rich beetroot juice compared to nitrate-depleted beetroot juice. However, there was loss of pigment in the production of the placebo (nitrate-depleted beetroot juice), suggesting damage to its phytochemical content (Gilchrist et al., 2014). Although the nitrate-depleted beetroot juice is an effective blind placebo supplement, other bioactive compounds have not been controlled for (e.g., antioxidants and polyphenols), making it is possible that other compounds found in beetroot juice may

contribute to the physiological outcomes (Gilchrist et al., 2014). Nevertheless, Webb et al., observed (2013) observed a no elevation in plasma nitrite and negation of reduction in blood pressure after participants were instructed to expectorate for 3 hours immediately after nitrate supplementation, suggesting nitrate content is a main contributor to blood pressure reduction.

There have been numerous studies suggesting the concept of responders and nonresponders to nitrate supplementation (Coggan et al., 2018; Jones et al., 2014; Jonvik et al., 2015; Wilkerson et al., 2012). Research has suggested this response may be related to the training status of the participants; that is, aerobically trained participants seem to benefit less from nitrate supplementation than participants who are less well trained (Wilkerson et al., 2012). In comparison to less well-trained individuals, endurance athletes are identified to have higher baseline plasma nitrite, superior training-related NOS activity, a higher proportion of type 1 fibres, and greater mitochondrial and capillary density, all of which may restrict the potential for nitrate supplementation to benefit performance (Bescos et a., 2011; Jensen et al., 2004; McAllister et al., 2006; McConnell et al., 2007; Wilkerson et al., 2012). However, the participants in the present study were recreationally active, with a mean maximal oxygen consumption of 46.2 ml/kg/min. Furthermore, the baseline plasma nitrite levels were similar to recreationally active participants (Lansley et al., 2011; Cermak et a., 2012). The other mechanistic bases behind differences in the effectiveness of nitrate supplementation, or responsiveness of the participant are not fully understood and obligate further research.

The term responder or nonresponder is often incorrectly used to define whether an athlete does or does not show superior performance capacity following a period of nitrate supplementation. Jonvik et al., (2015) stated for an individual to be classified as a responder to nitrate supplementation, plasma nitrate and nitrite concentrations should show a substantial increase after nitrate supplementation. Nitrite has been found to be a

more sensitive biomarker of nitric oxide synthesis / availability, as it is the main precursor of nitric oxide and other bioactive nitrogen oxides (Lundberg et al., 2008). Throughout this thesis plasma nitrite concentration did not significantly increase and the increase in concentration was to a lesser magnitude in comparison to previous research (Betteridge et al., 2016; Larsen et al., 2010; Vanhatalo et al., 2010; Vanhatalo et al., 2011). In Chapter 4, a greater number of participant's nitrite levels were observed to increase at the 120 minutes time point in comparison to the baseline in the watercress condition (7 of the 9 participants) and the beetroot condition (6 of the 9), of which 5 were the same participants for both supplementation groups. Although the mean time trial performance was not significantly improved by acute ingestion of watercress, the same 5 participants observed an increase in plasma nitrite concentration above the group mean and recorded their fastest TT performance in comparison to the rest of the supplementation groups. It evident that scientific evaluation of ergogenic benefits of nitrate supplementation does not always provide insight in the extent of response in the individual athlete when using a conventional study design. For individual's whom exercise performance is improved after nitrate supplementation, it is proposed they should be tested multiple times over a series of placebo-controlled nitrate supplementation trials to ensure a true response.

Chapter 5 observed varied responses across all four conditions, this may be due to the low dose of acute nitrate, the change of oral microbiome or more time was required to observe a peak in plasma nitrite in the healthy aged population. Interestingly, baseline nitrite concentration in older adults in Chapter 5 were similar to that reported in young adults in Chapter 4. This was unexpected as research has reported reduced nitrite concentrations in an older adult population, however, as previously mentioned this may be due to the health and activity status of the selected cohort (Sindler et al., 2011). Studies have reported a significant increase in plasma nitrite concentrations with increasing nitrate dosages (Flueck et al., 2015; Wylie et al., 2013). These studies observed inter-

individual variability in the response of nitrite concentration after low nitrate ingestion, with the number of non-responders decreasing after the dose was increased (Flueck et al., 2016; Wylie et al., 2013). Although, the dosage was selected in line with the commercially available beetroot juice (400 mg of nitrate) (James White, Suffolk, United Kingdom), the nitrate dose may have been too low (~ 400 mg) to observe any significant physiological effects in the current thesis. Most studies show ergogenic effects of nitrate dose of 300 – 520 mg (Domínguez et al., 2017). In the thesis, the acute effects of nitrate supplementation were investigated rather than chronic ingestion, this was chosen with consideration to financial cost and time commitment, allowing for a more user-friendly protocol to be adopted by athletes / recreational athletes.

Individual's time to peak blood concentration for nitrite can vary, causing wide variability. Research has reported nitrite levels to peak between 2 – 3 hours after nitrate supplementation (Jones et al., 2018; Webb et al., 2008). This variation is dependent, in part on gut bacteria, salivary flow rate and the reduction of nitrite to nitrate by the oral bacteria (Kapil et al., 2013; Tiso & Schechter, 2015; Webb et al., 2008). The oral and intestinal microbiome are integral for the reduction of nitrate to nitrate and nitric oxide and may partly explain the lack of increase in plasma nitrite concentration (Tiso and Schechter, 2015). If particular nitrate-reducing bacteria are not present in certain individuals, this may result in a lack of any beneficial effects. Participants were restricted of antibacterial mouthwash to control the variability of oral bacteria, it was not possible to control the gut bacteria in the current investigation (Tiso and Schechter, 2015). The oral microbiome is known to change over the lifespan and should be further explored with regards to the effects of increased nitrate consumption. This would provide valuable information about the effectiveness of nitrate supplementation over the life span but may also provide imperative information for public health bodies in charge of devising nutritional intake recommendations. For future nitrate research it may be that prior to

cardiovascular parameters being recorded, all participants are pre-screened to ensure a positive response to the nitrate supplementation, if not an increase in nitrate dosage. Therefore, elucidating the optimum nitrate dose and long-term effects of dietary nitrate on cardiovascular parameters and exercise performance may be beneficial for healthy and diseased cohorts.

Based on existing literature it is clear that nitrate supplementation can elicit both advantageous health and performance effects in some individuals. Individual's responses are likely variable responses due to a combination of the training status, oral microbiome and dosing. Coaches, clinicians, athletes and the general public should be aware of these potential issues and should customise the use of nitrate supplementation appropriately.

The current thesis observed a high variation in both oxidative and nitrosylative stress markers, as is quite common in this field. Research has shown oxidative stress markers to vastly differ among individuals, as observed in the current thesis (see Chapters 4, 5 and 6) (Margaritelis et al., 2014). Many factors can influence oxidative stress such as dietary habits, health status, physical activity and the environment (Muñoz & Costa, 2013). Research has also observed daily changes in oxidative stress biomarkers at baseline, even when confounding factors are considered (time of day, physical activity status and diet) (Goldfarb et al., 2005; Margaritelis et al., 2014). For example, in Chapter 5, the experiment demonstrated considerable variability in oxidative and nitrosylative stress biomarkers after completing 16.1 km TT, one week apart. Substantial variation was observed between all of the conditions, lipid hydroperoxides (3.20 – 293.1 $\mu\text{M}/\text{mg}$ protein), protein carbonyls (1.72– 129.4 $\mu\text{M}/\text{mg}$ protein) and nitrotyrosine (4.01 – 983.9 ng/mL). This variation may potentially be due to diet, inflammation or normal physiological fluctuations. Future interventional studies observing nitrosylative / oxidative stress parameters need to pay particular attention to controlling confounding variables. Caution should be given when reporting findings, as there are currently no

reference values for oxidative stress biomarkers, making it difficult to comment on the degree of variation (Davison et al., 2012). With this variability, it can considerably decrease statistical power (i.e., the probability that a test will identify an effect that exists). Future research should aim to investigate the seasonal, monthly and daily fluctuations of RONS, as this may determine if a normal threshold of oxidative and nitrosylative stress biomarkers exists.

7.5. Strengths and limitations

All experimental chapters included a crossover study design. This is advantageous as the effect of confounding covariates is decreased because each crossover participant acts as their own control, enabling confidence in the findings. Participants were administered a fixed dose of nitrate in Chapter 4 and 5. To elucidate whether non-responders would require higher quantities of nitrate, future research could administer dietary nitrate relative to plasma nitrite concentration increase. Due to the slow recruitment of male participants for study 5 and given the restricted time frame of the PhD process, it was decided that females would be welcome to partake in the study. No significant gender differences were observed in cardiovascular parameters. Participants were excluded if they were taking medications that may have contraindicated with the nitrate supplement, such as nitroglycerin or nitrate preparations used with angina, or proton pump inhibitors, or antihypertensive medications. We are confident that the strict criteria enabled us to screen out participants on medications that might have confounded the effect of the treatments on the nitrate-nitrite-nitric oxide path. Throughout all studies, participants were requested to maintain their normal dietary intake. Previous research instructed participants to exclude dietary nitrate rich foods during the experimental period (Bailey et al., 2010; Bailey et al., 2009; Larsen et al., 2007). While the unrestricted dietary method

applied in this thesis may have increased variability in dietary nitrate intake and impact markers, this approach allowed for ecological validity for consuming the watercress supplement and provided conclusions and implications to be made relevant to the general population. The primary purpose of administering dietary nitrate was to enhance the production and bioavailability of nitric oxide. Nitric oxide is exceptionally challenging to measure due to its very short half-life *in vivo* (less than 0.1s) (Kelm & Schrader, 1990). By contrast, nitrite and nitrate are stable metabolites of nitric oxide and are present in blood and urine. In this thesis nitrate and nitrite were quantified in plasma, this has been shown to provide the most appropriate and practical method to assess nitric oxide. It is acknowledged we could have measured the concentrations of nitrite and nitrate in saliva, muscle and urine and exhaled nitric oxide. This would have enabled the following of these metabolites from ingestion to exhalation/excretion. Future research may consider these measures and methods. Almost all oxidative stress biomarkers have been criticised for their repeatability (Urso & Clarkson, 2003). In present thesis there was a substantial amount of variation observed in the oxidative stress markers. Thus, future research should aim to investigate this variability to try to determine if a threshold exists after which oxidative stress occurs that is beyond the normative range.

7.6. Future research

While the studies in this thesis aimed to observe the efficacy of watercress supplementation in the context of a nitrate supplement, the research is limited in that the bioavailability of other potentially bioactive compounds were not assessed. This is especially relevant in light of the results in Chapter 4, which suggests there is potentially another bioactive component independent of nitrate or a synergistic role by bioactive compounds to help enhance exercise performance. The two-hour time point was chosen

in this thesis as research suggests nitrite begins to peak between 2 – 3 hours along with polyphenols normally peak in plasma then too (Ndhlala, Moyo, & Van Staden, 2010; Webb et al., 2008). Future research involving an adequately powered study investigating the pharmacokinetics, and pharmacodynamics of acute watercress supplementation would validate the preliminary results observed in Chapter 4.

Using data from the final experimental chapter power calculations for chapter 4 and chapter 5 were determined. Power calculations were performed using G* power v3.0. Based on the interaction data: partial eta squared (0.395), 80 % power and $\alpha = 0.05$, a sample size of 12 participants would provide an 81 % chance of correctly rejecting the null hypothesis of no significant effect of supplementation on time-trial performance. Similarly, based on variability data from the final study, partial eta squared (0.10), 80 % power and $\alpha = 0.05$, a sample size of 16 participants would be provide a 92 % chance of rejecting the null hypothesis of no significant effect of supplementation and blood flow volume.

Oral bacteria may be a major influence in regulating the efficacy of dietary nitrate, the concept of ‘responders’ and ‘non-responders’ requires further investigation. The essential function of the oral microbiome for the nitrate-nitrite-nitric oxide pathway emphasises an area that may prove vital in the efficacy of dietary nitrate supplementation. The oral microbiome plays a fundamental part in systemic nitric oxide homeostasis and the subsequent physiological benefits. The uptake mechanism after dietary nitrate consumption is not well understood along with inter-individual differences in oral bacteria (McDonagh, Wylie, Thompson, Vanhatalo, & Jones, 2019). Especially in the context of this thesis, due to the high content of glucosinolates in watercress, which converts to isothiocyanate. This conversion to isothiocyanates has been suggested to obstruct nitric oxide metabolism (Dewhurst-Trigg et al., 2018). There is a need to be

explore this area more to help assist with understanding the potential physiological effects of aging.

The work described in Chapter 4 suggests that dietary nitrate in comparison to the isolate sodium nitrate condition may have an ergogenic effect on performance. This may imply the driving mechanism may not be nitrate driven or other bioactive compounds have an important synergistic role in nitrate metabolism. It is important to note organic nitrate, commonly used as antihypertensive medication are limited by tachyphylaxis and the development of endothelial dysfunction (Daiber & Münzel, 2015). Therefore, the dose-response relationship and longer-term studies are required to validate the efficacy of watercress supplementation. These potential beneficial implications may benefit older healthy aged individuals or patients at risk of cardiovascular disease.

7.7. Conclusions

The effect of dietary nitrate is an exciting evolving topic in exercise and health research. From improved exercise performance, to possible therapeutic aid in the deterrence and treatment of pathological conditions, ongoing research aims to understand the full potential of nitrate supplementation. Current research demonstrates the efficacy of beetroot juice as an effective source of dietary nitrate. This thesis provides evidence inorganic isolate, sodium nitrate, is not an effective nitrate supplement. Whereas complex dietary nitrate supplements observed an ergogenic effect, although not significant, in a young, physically active, male cohort. This may stem from the facilitated nitrite reduction to nitric oxide, which may have been catalysed by vitamin C, polyphenols and additional antioxidants or from an effective different species independent of nitrate. The underlying mechanisms which influence the efficacy of watercress have yet to be fully understood

and so supplementation should be carefully considered for individuals and their requirements.

Based on the findings presented in this thesis, it can be concluded that while watercress supplementation may not always be effective, it may enhance exercise performance. As such it should be considered to have, and be utilized for, its ergogenic qualities.

7.8. Personal Development and Reflection

Throughout my time as a PhD student, I have developed as a researcher by building upon knowledge and learning from new experiences. At the outset, I was eager to dive straight into laboratory work without fully appreciating the logistical considerations required for undertaking such a large project to which I was not accustomed to. Leading up to Chapter 4, in hindsight, I can see I was naïve and keen to start collecting data. The supplement had arrived and there was no time to waste. Although an N of 10 is not a very large subject cohort, this study allowed me to appreciate the time required to execute a crossover design study. For Chapter 5, I found it challenging to adapt my protocol to meet the standards of the ethics committee and this cost time. Going forward, I have learnt and appreciated the payoff for early ethics submission and to allow more time for study completion. To execute this study, I required support from colleagues due to environmental conditions and specific markers being collected. This highlighted the importance of building relationships with staff members to allow for flexibility and adaptability. Another challenge I faced during the execution of this study was the unexpected flooding of the laboratory and the unforeseen closure for several weeks. This emphasised the importance of communication and building a good rapport with colleagues and participants.

I began analysing the blood flow volume data for Chapter 4 while collecting data for Chapter 5. Working closely with my supervisor in our biochemistry laboratory, I was taught how to analyse my blood samples using different techniques. It was during this time I realised there was a considerable variation in the participant's data. This was eye-opening and I felt it would be very helpful to investigate the repeatability of the blood flow volume measures, time trial performance and oxidative stress measures to understand my data better. Chapter 6 allowed me to analyse the repeatability of the chosen parameters and enhanced my understanding of the variation seen in the parameters.

Having conducted the final study, I now appreciate the significance of knowing/understanding the repeatability of your measures in your own laboratory. I can see now the importance of the final study and how it acts as a foundation for future research in terms of statistical power and choosing your main outcomes. This learning experience has been essential to me as a researcher going forward.

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Appendices

Appendix A: Ethical Approval

Ethics Independent Reviewer's Report

This form should be completed by a member of the Department of Sport, Health and Exercise Science Ethics Committee who has been assigned to review a particular ethics application by the chair of the committee. The front section of the Independent's Reviewer's Report should be printed, signed and dated, and attached to the back of the reviewed ethics application. The reviewed ethics application should be given to the Ethics Committee chair once all reviews have been completed. The checklist provided at this end of this form is to help the reviewer complete the review and guide the content of his or her written report, which should be typed into the relevant boxes that are given before the checklist. Any checkbox highlighted red that has been checked requires attention.

Please note that the checklist is for guidance only and reviewers should be aware of other ethical considerations relevant to the ethics application being reviewed.

An electronic copy of the completed report should be stored on the reviewer's computer.

Independent reviewer's name	Liam Sykes
Application title and number	Effect of nitrate supplementation on vasodilatory responses, oxidative stress biomarkers and a 16.1 km time trial. (1516013)
Principal investigator's name	Dr Andrew Garrett
Student investigator's name (if applicable)	Rachel Burke

Reviewer's recommended outcome			
Approve <input checked="" type="checkbox"/>	Revise <input type="checkbox"/>	Reject <input type="checkbox"/>	Refer <input type="checkbox"/>



Reviewers comments	
Section	Comment
Click here to enter text.	Click here to enter text.
Click here to enter text.	Click here to enter text.
Click here to enter text.	Click here to enter text.
Click here to enter text.	Click here to enter text.

Appendix B: Ethical Approval

Department of Sport, Health & Exercise Science



Ethics Independent Reviewer's Report

This form should be completed by a member of the Department of Sport, Health and Exercise Science Ethics Committee who has been assigned to review a particular ethics application by the chair of the committee. The front section of the Independent's Reviewer's Report should be printed, signed and dated, and attached to the back of the reviewed ethics application. The reviewed ethics application should be given to the Ethics Committee chair once all reviews have been completed. The checklist provided at this end of this form is to help the reviewer complete the review and guide the content of his or her written report, which should be typed into the relevant boxes that are given before the checklist. Any checkbox highlighted red that has been checked requires attention.

Please note that the checklist is for guidance only and reviewers should be aware of other ethical considerations relevant to the ethics application being reviewed.

An electronic copy of the completed report should be stored on the reviewer's computer.

Independent reviewer's name	James Hobkirk
Application title and number	The effect of acute watercress supplementation on cardiovascular and oxidative stress parameters in normoxic and hypoxic environments at rest with an aged population. Repeatability of 16.1 km time trial performance, blood flow and oxidative stress markers. (1617162)
Principal investigator's name	Dr Andrew Garrett
Student investigator's name (if applicable)	Rachel Burke

Reviewer's recommended outcome			
Approve <input checked="" type="checkbox"/>	Revise <input type="checkbox"/>	Reject <input type="checkbox"/>	Refer <input type="checkbox"/>



Reviewers comments	
Section	Comment
Click here to enter text.	Click here to enter text.