DIETARY ANTIOXIDANTS AND THE EFFICIENCY OF OXYGEN TRANSPORT AND UPTAKE DURING ENDURANCE EXERCISE

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ABSTRACT

This thesis aimed to determine whether various dietary antioxidant supplementation protocols could enhance the efficiency of oxygen (O_2) transport during endurance cycling, and subsequently improve exercise performance. Two naturally-sourced supplements (keratin; KER and pomegranate extract; POMx) were selected for investigation, based on their rich content of either thiols (KER) or polyphenols (POMx).

Study One was a crossover study which compared the effect of chronic KER intake compared to a sodium caseinate placebo (CAS) of equal protein content. Fifteen endurance-trained males consumed the supplement on six days per week, for a period of four weeks (0.8 $g.kg^{-1}d^{-1}$), while participating in endurance cycling training. Blood samples collected throughout each intervention period were unchanged by either supplement for any parameter measured (all $p > 0.05$). Likewise, neither the O_2 consumption (VO_2) required to sustain a given level of submaximal cycling exercise, nor the maximal VO_2 attained during a graded exercise test to exhaustion were affected by KER or CAS (submaximal VO_2, $p = 0.13$; VO_{2max}, $p = 0.25$). Further, the maximal power output obtained in the VO_{2max} test was not significantly different between treatments ($p = 0.51$). Consequently, KER was not recommended as an ergogenic aid for athletes.

Study Two investigated the effects of acute POMx supplementation on VO_2 during submaximal and maximal cycling exercise, in normoxic (sea-level; SEA) and hypoxic (1657m altitude; ALT) environments. In a randomized, double-blinded, crossover study design, eight highly-trained cyclists ingested 1000 mg of POMx or a placebo (PLAC), 2.5 hours prior to completing three stages of submaximal cycling at 50%, 65% and 80% of maximal O_2 consumption (VO_{2max}), followed by a time trial to exhaustion at a workload calculated to

elicit 100%VO_{2max} (TTE100%). The protocol was completed on four occasions: in SEA and ALT, with a POMx, and a PLAC trial in each environment. POMx did not alter VO_2 during submaximal exercise in either environment (p = 0.67), or during the TTE100% in SEA (p = 0.46). However, its intake allowed maintenance of SEA VO_2 values during intense exercise in hypoxic conditions, as indicated by the VO_2 measured five minutes into the TTE100% (+3.8 $ml.min^{-1}kg^{-1}$, 95% CI, -5.7, 9.5, p = 0.001). However, despite this, POMx did not significantly affect TTE100% performance in either environment (p = 0.41), possibly due to the highly-trained nature of participants, who may have required a longer supplementation period for an ergogenic effect to be observed with POMx.

Study Three was based on the above findings, and aimed to determine whether an 8-day supplementation period with POMx would be sufficient to alter VO_2 and cycling performance at sea-level. Further, this study explored the benefits of combining thiol and polyphenol antioxidants to take advantage of their theoretically complementary effects on erythrocytes and nitric oxide (NO). Eight trained cyclists completed four supplementation protocols in a randomized, blinded, crossover designed study: placebo (stevia, PLAC), POMx only, *N*-acetylcysteine (NAC) only and POMx + NAC (BOTH) for eight days (15 $mg.kg^{-1}d^{-1}$). On the eighth day, 2.5 hours after the final dose, the participants completed a submaximal cycling protocol, as described for Study Two, followed by a five-minute time trial. As opposed to the acute supplementation protocol in the previous study, short-term POMx supplementation decreased the VO_2 required to complete each stage of the submaximal part of the exercise test compared to all other supplement conditions (-2.1 $ml.min^{-1}kg^{-1}$, 95% CI, -2.8, -0.23, $p < 0.04$). In contrast, NAC significantly increased submaximal VO_2 (+1.9 $ml.min^{-1}kg^{-1}$, 95% CI, 0.26, 3.6, $p < 0.03$), negating the POMx-lowering effects on POMx when the two were co-supplemented. Regardless, none of the treatments significantly

altered performance in the subsequent time trial ($p > 0.05$). Thus, it is suggested that this test was too short for the increased VO_2 efficiency to show any meaningful effects on performance.

In conclusion, based on the lack of evidence for enhancement of performance, this thesis does not support the recommendation of the selected dietary antioxidant supplements for athletes, for performance-enhancing effects at least. However, because the primary outcome measure of this thesis, VO_2, appears to be altered by POMx, the intake of polyphenol-rich products warrants further investigation. Based on the results of Chapters 6 and 7, it appears that the benefits of POMx become more significant as demands on O_2 transport and utilization processes increase. Therefore, the suggested areas of future research would involve exercise of greater duration, and environmental conditions where O_2 availability and/or limitations to the various transport parameters differ.

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LIST OF ABBREVIATIONS

$A–aO_2$	Alveolar-arterial pressure of oxygen difference
ADP	Adenosine diphosphate
ALT	High-altitude environment (Study 2)
ATP	Adenosine triphosphate
BOTH	Supplementation with pomegranate extract and *N*-acetylcysteine (Study 3)
BP	Blood pressure
bpm	Beats per minute
BRJ	Beetroot juice
BW	bodyweight
CAS	Sodium caseinate placebo (Study 1)
CAT	Catalase
cGMP	Cyclic guanosine monophosphate
^oC	Degrees celsius
CI	Confidence interval
CIT	L-citrulline
cm	Centimetre
CO_2	Carbon dioxide
CYS	Cysteine
d	Per day
DBP	Diastolic blood pressure

DEXA	Dual-energy x-ray absorptiometry
dL	Decilitre
EDTA	Ethylenediaminetetraacetic acid
EIAH	Exercise induced arterial hypoxaemia
ENV	Environment, used in statistical analyses of Study 2
EPO	Erythropoietin
ES	Effect size
ET	Ellagitannin
ETC	Electron transport chain
EX	Pre or post exercise test, used in statistical analyses of Study 1
Fe^{2+}	Iron moiety
Fisher's LSD	Fisher's Least Significant Difference
fL	Femtolitre
g	Gram
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GSSG	Glutathione disulfide
h	Hour
Hb	Haemoglobin
Hct	Haematocrit
HHb	Methaemoglobin
HR	Heart rate
INT	Intensity, used in statistical analyses of Study 2 and 3

INT1	Intervention 1, Study 1
INT2	Intervention 2, Study 1
KER	Novel keratin supplement
kg	Kilogram
L	Litre
Lin	Linear factor
$La^-]_b$	Blood lactate concentration
m	Metre
MAP	Mean arterial pressure
MCH	Mean cell haemoglobin concentration
MCHC	Mean cell haemoglobin concentration relative to erythrocyte volume
MCV	Mean erythrocyte cell volume
mg	Milligram
min	Minute
µL	Microlitre
ml	Millilitre
MLSS	Maximal lactate steady state
mm	Millimetre
mmHg	Millimetre of mercury
mmol	Millimole
NAC	*N*-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
nm	Nanometre

NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthases
O_2	Oxygen
OxyHb	Oxygenated haemoglobin
pg	Picogram
PO	Power output
PO_2	Partial pressure of oxygen
POMJ	Pomegranate juice
POMx	Pomegranate extract
PLAC	Placebo
PREPOST	Pre or post-intervention, used in statistical analyses of Study 1
PUFA	Polyunsaturated fatty acid
Q	Cardiac output
Q_{max}	Maximal cardiac output
RBC	Total erythrocyte count
RNS	Reactive nitrogen species
RONS	Reactive oxygen/nitrogen species
ROS	Reactive oxygen species
RPE	Rating of perceived exertion
rpm	Revolutions per minute (pedaling cadence)
s	Seconds
SaO_2	Arterial oxygen saturation

SBP	Systolic blood pressure
SD	Standard deviation
SEA	Sea-level environment (Study 2)
-SH	Sulfhydryl group
SmO_2	Local muscle oxygen saturation
SNO	*S*-nitrosothiol
SNO-Hb	*S*-nitrosohaemoglobin
SOD	Superoxide dismutase
St	Stage, used in statistical analyses of Study 1
STPD	Standard temperature and pressure dry
SV	Stroke volume
TAU	Taurine
TBARS	Thiobarbituric acid
THb	Total haemoglobin
TIME	Time-point in the intervention, used in statistical analyses of Study 1, 2 and 3
TR	Treatment, used in statistical analyses of Study 1 and 2
TTE	Time trial to exhaustion
TTE100%	Time to exhaustion at 100% VO_{2max} (Study 2)
V_E	Expired ventilation
V_I	Volume of inspired air
VCO_2	Volume of expired carbon dioxide
VCO_{2max}	Volume of carbon dioxide expired at VO_{2max}
VO_2	Rate of whole-body oxygen consumption

VO_{2max}	Maximal rate of oxygen consumption
W	Watts
WBC	Total leukocyte count

CHAPTER 1. INTRODUCTION

To sustain myocellular energy charge during contractile activity, adenosine triphosphate (ATP) is produced through predominantly oxidative (oxygen-dependent) metabolic pathways (Costill, Thomason, & Roberts, 1972). During these processes, oxygen (O_2) is an integral part of the electron transport chain (ETC), acting as the terminal electron acceptor to drive the flow of electrons, and generate 'energy potential' as an electrochemical proton gradient across the inner mitochondrial membrane (Wikström & Saraste, 1984). However, in order to perform this role, O_2 must first be transported from the point of pulmonary gas exchange through the vasculature to the muscle mitochondria, a pathway containing a number of variables which limit its efficiency (Bassett & Howley, 2000).

The components of the O_2 transport pathway are greatly influenced by the actions of the reactive oxygen/nitrogen species (RONS) compounds, which are produced in large quantities during exercise. These RONS are associated with cellular damage when they accumulate (Darley-Usmar & Halliwell, 1996). In particular, the highly-reactive compounds, superoxide, hydrogen peroxide and peroxynitrite, are associated with the peroxidation of erythrocyte (red blood cell) wall components (Çimen, 2008). On the other hand, increased production of the RONS nitric oxide (NO) stimulates greater vasodilation and blood flow (Stamler & Meissner, 2001). Overall, it appears that various species of RONS can either limit or enhance O_2 transport, and therefore interventions that alter the concentrations of these species may impact O_2 transport and uptake.

The concentrations of various types of RONS are influenced by antioxidant compounds which have the ability to delay or prevent the oxidation of other substrates. The body

contains an endogenous thiol antioxidant system, which is highly effective in limiting the build-up of damaging species under conditions of rest or low levels of exercise (Brosnan & Brosnan, 2006). However, athletes who complete large volumes of exercise and are exposed to greater levels of oxidative stress, may benefit from exogenous antioxidants, to prevent RONS accumulation and thereby enhance the efficiency of the O_2 transport system (Reid, Stokić, Koch, Khawli, & Leis, 1994). In particular, exogenous thiols and polyphenol antioxidants can be provided through dietary sources, and ingestion of these has been associated with protective effects on erythrocytes, and improvements to elements of the O_2 transport cascade (Bonarska-Kujawa, Cyboran, Żyłka, Oszmiański, & Kleszczyńska, 2014; Stoclet et al., 2004; Zembron-Lacny, Slowinska-Lisowska, Szygula, Witkowski, & Szyszka, 2010). In addition, these antioxidants can enhance the bioavailability of NO and prevent its transformation into more reactive forms of RONS (Gago, Lundberg, Barbosa, & Laranjinha, 2007; Ignarro, Byrns, Sumi, de Nigris, & Napoli, 2006; Peri et al., 2005).

While much previous research has investigated the provision of exogenous antioxidants through pharmaceutical supplements, the consumption of 'superfoods' or compounds extracted from natural sources are becoming increasingly more popular (Nielsen, 2016). Although there have been a number of new products being developed in this category, little scientific evidence exists to encourage their use by athletes. The aim of the current thesis is to investigate two dietary antioxidant products: poultry feather-sourced keratin protein (KER) which is rich in thiols, and pomegranate extract (POMx) which is rich in polyphenols, to determine whether either enhances the efficiency of O_2 transport and uptake during endurance exercise, and subsequently, improves exercise performance.

CHAPTER 2. REVIEW OF LITERATURE

2.1 OXYGEN REQUIREMENTS DURING EXERCISE

2.1.1 Introduction

The process of O_2 transport from the oral-nasal cavity to the mitochondria involves a number of factors which can limit the rate and efficiency by which O_2 can be used in the muscle to produce ATP (Bassett & Howley, 2000). As exercise intensity increases, whole-body O_2 uptake (VO_2) must increase sufficiently to meet the greater requirements of the active muscles for ATP. Thus, the rate that O_2 is transported to, and taken up by, the active muscles, is a major factor in endurance sport performance (Bassett & Howley, 2000). The capacity for O_2 transport is improved through training adaptations such as greater muscle capillarization, mitochondrial density and haemoglobin (Hb) production, an enlarged plasma volume and an increased cardiac output capacity (Q), secondary to a rise in stroke volume (SV) (Blomqvist & Saltin, 1983; Lundby, Montero, & Joyner, 2016). Thus, generally, highly-trained athletes have developed their maximal oxygen uptake capacity (VO_{2max}) to its genetic limit (Arrese, Ostariz, Mallen, & Izquierdo, 2005). However, by improving the efficiency of the O_2 transport system, the VO_2 required to maintain a given level of submaximal exercise may decrease, allowing exercise to be performed with a lower metabolic cost, and VO_{2max} to occur at a higher power output or speed. Further, more efficient O_2 transport may increase the percentage of VO_{2max} at which the 'lactate

threshold' (maximal lactate steady state; MLSS) occurs, allowing a higher relative VO_2 to be sustained for a given period of time (Joyner, 1993; Coyle, 1995).

The current section presents a brief overview of the O_2 transport process and describes the three key phases of VO_2 kinetics at the onset of exercise. Subsequently, the elements in this pathway which may present limitations to efficient O_2 transport are discussed, and areas which may be targeted by dietary supplements are identified.

2.1.2 Oxygen transport from the inspired air to the muscles

Following inhalation, O_2 travels along a pathway which is driven by a series of decreasing O_2 pressure gradients between the inspired air (Pressure of O_2; PO_2: ~160 mmHg at sea-level) and the muscle mitochondria (PO_2: ~1–2 mmHg) (di Prampero, 2003; Wagner, 1996). The first of these pressure gradients is situated between the alveoli of the lungs and the pulmonary capillaries and provides the place of O_2 entry into the bloodstream. Once in the vasculature, O_2 binds to Hb proteins, which travel to the heart to be be pumped around the body during cardiac systole. Being the site of O_2 binding, Hb proteins are a principle component of blood and comprise approximately 42% of whole blood in females and 46% in males (Retzlaff, Tauxe, Kiely, & Stroebel, 1969)

The movement of blood through the vasculature is moderated by local, nervous and hormonal regulatory mechanisms which narrow (vasoconstrict) or widen (vasodilate) the arterioles, depending on the relative demands of the bodily systems they service. Accordingly, during exercise, blood flow to the active muscles and cardiovascular system increases, while blood flow to inactive muscle groups and the digestive and renal systems decreases (Castenfors, 1977; Duncker & Bache, 2008; Laughlin & Armstrong, 1984).

Following their transport to the extramuscular vicinity, another partial pressure gradient between the extra and intramuscular capillaries promotes O_2 diffusion into the muscles themselves. Subsequently O_2 moves down a final pressure gradient into the mitochondria, the site of ATP production. This process is assisted by myoglobin, a cytoplasmic hemoprotein, located in oxidative skeletal muscle fibres and cardiac myocytes, with a similar structure and functionality to Hb. During resting conditions, myoglobin acts as a storage site for O_2 in the muscle. Then, at the onset of exercise, it rapidly desaturates to provide an immediate source of O_2, while also increasing the PO_2 gradient between the extra- and intra-muscular capillaries (Ordway & Garry, 2004). O_2 that does not diffuse into the muscles returns via the venous system and heart to the pulmonary system, where it is exhaled, along with carbon dioxide (CO_2), which is produced in the tricarboxylic acid cycle and pyruvate dehydrogenase reaction (di Prampero & Ferretti, 1990; Wagner, 1996).

2.1.3 Oxygen uptake kinetics

The term VO_2 kinetics describes the characteristics of the VO_2 response to the onset of exercise. For submaximal steady-state exercise, below the maximal lactate steady state (MLSS), the point at which lactate production exceeds its removal, three phases have been identified (Burnley & Jones, 2007; Poole & Jones, 2012) (Figure 2.1). These phases correspond to adjustments being made by components of the O_2 transport pathway to the increased energy requirements. Phase I, also known as the cardio-dynamic component, represents an initial rapid increase in VO_2, and is enabled through a rise in the quantity of venous blood returning to the heart with the increase in muscular activity, and a sudden increase in Q to maintain mean arterial pressure (MAP) and distribution of Q. In this initial phase, the PO_2 in blood returning to the pulmonary system is similar to resting conditions,

because it passes by the muscles prior to an exercise-induced increase in O_2 extraction. In contrast, phase II, the fundamental, primary or fast component of the response, is marked by a decrease in venous pulmonary PO_2, as greater O_2 extraction starts to occur. In addition, VO_2 continues to rise exponentially until a steady-state has been attained. Phase III, is reached approximately three minutes after the initiation of exercise and represents the attainment of a relative steady-state VO_2 (Davies, Di Prampero, & Cerretelli, 1972; Whipp, Ward, Lamarra, Davis, & Wasserman, 1982; Whipp & Wasserman, 1972). In exercise performed above the MLSS, rather than achieving a steady-state, there is an additional component to the VO_2 response. This VO_2 "slow component" is a gradual, continuous increase in VO_2, which becomes apparent above MLSS (Whipp, 1994).

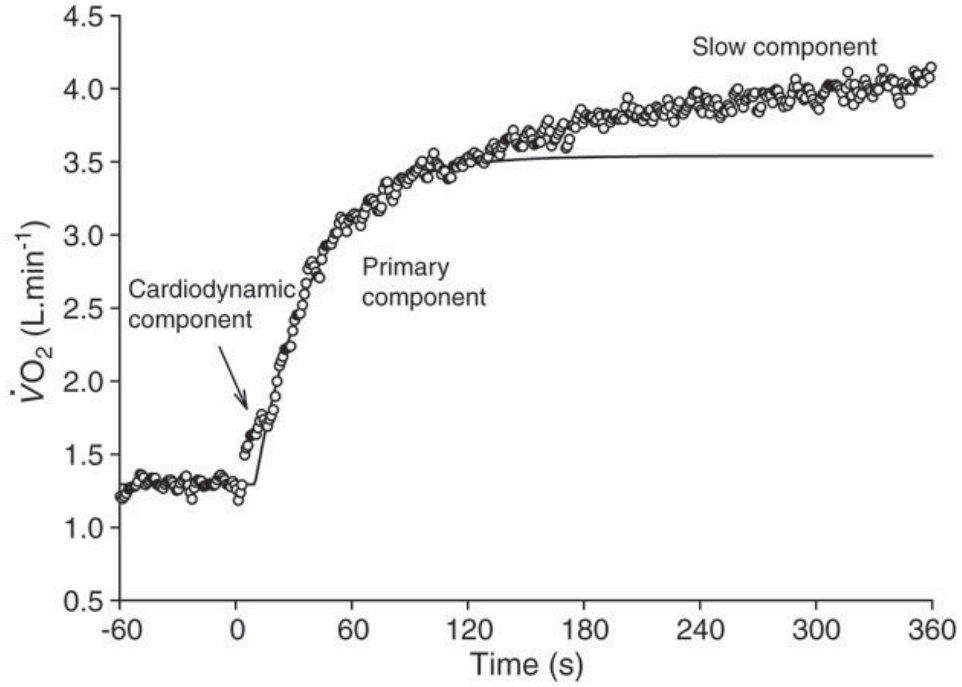

Figure 2.1 The VO_2 response to heavy-intensity exercise in a healthy individual (Burnley & Jones, 2007)

2.1.4 Limiting factors in oxygen transport and uptake during exercise

2.1.4.1 The pulmonary system

2.1.4.1a Exercise in normoxic conditions

Being the site of O_2 entry into the bloodstream, the pulmonary system is an integral part of O_2 delivery. As previously mentioned, the rate of O_2 diffusion from the alveoli to the pulmonary capillaries is dependent on the alveolar-arterial PO_2 difference ($A–aO_2$), which in turn is determined by the ratio of ventilation (V_I) to pulmonary capillary perfusion (Bassett & Howley, 2000). Under resting conditions, the $A–aO_2$ is very small (~0–5 mmHg), indicating that complete gas exchange is occurring. However, during exercise, the $A–aO_2$ widens to >20 mmHg, as O_2 is removed at faster rates due to an increased blood flow (Dempsey & Wagner, 1999). Thus, to maintain efficient gas exchange, V_I must increase sufficiently. In healthy low-moderately-trained individuals ($VO_{2max} \leq 55$ ml.min.kg^{-1}), under normobaric conditions, VO_2 is generally not limited significantly by the pulmonary system (Dempsey et al., 1977; Dempsey & Wagner, 1999). However during heavy or severe exercise trained athletes, particularly those with high maximal Q (Q_{max}) values, have been shown to experience notable decreases in arterial O_2 saturation (SaO_2) of up to 15% (Dempsey, Hanson, & Henderson, 1984; Dempsey & Wagner, 1999). This is significant because a 4-5% desaturation from resting SaO_2 values corresponds to a ~1% decrease in VO_{2max} (Powers, Lawler, Dempsey, Dodd, & Landry, 1989). This condition, known as exercise induced arterial hypoxaemia (EIAH), is attributed to a combination of factors which reduce the efficiency of pulmonary gas exchange or excessively widen the $A–aO_2$ (≥35 mmHg) (Hopkins 2006, Dempsey 1984, Dempsey and Wagner 1999). As exercise increases in intensity or duration,

V_I may be inadequate to cater for the increased blood flow and O_2 requirements (ventilation/perfusion inequality) (Rice et al., 1999). Further, the large blood flow which accompanies high rates of Q reduces the erythrocyte transit time through the pulmonary capillaries, shortening the time period for complete O_2 saturation (Hopkins, 1996).

However, while it is clear that some highly-trained athletes do experience a pulmonary limitation to VO_2, the nature of the limitation (increased blood flow) does not provide an area for improvement with nutritional supplements, unless Hb content or the percentage of erythrocytes in whole blood (haematocrit; Hct) can be increased. In most athletes, maximal ventilation rates can be increased with respiratory muscle training, but this is not associated with changes in VO_{2max} or VO_2 at the ventilatory threshold (Amonette & Dupler, 2002).

2.1.4.1b Exercise in hypoxic conditions

Gas exchange in the pulmonary system limits VO_2 more substantially when exercise is performed in hypoxic conditions, (e.g., in high-altitude) which are characterized by a lower atmospheric PO_2 (e.g., ~619 mmHg at 1700 m) (Gillam, 2012). The resultant decrease in alveolar PO_2 reduces the $A–aO_2$, and therefore the drive for O_2 movement into the circulation. Previous research has demonstrated that for every 100m increase in altitude, VO_{2max} decreases by ~0.6% which corresponds to a decrease in exercise performance of ~1.4% (Wehrlin & Hallén, 2006). Therefore, interventions which are aimed at improving VO_2, are likely to have a greater impact in hypoxic conditions.

2.1.4.2 The cardiovascular system

2.1.4.2a Cardiac output

Given that the greatest difference between highly-trained athletes and untrained healthy individuals in any area of limitation to VO_2 is Q, it is clear that this is a major factor in O_2 transport capacity (di Prampero, 2003). As stated by Andersen and Saltin (1985), VO_{2max} is limited by O_2 delivery during exercise which engages a muscle mass greater than ~15kg, such as during cycling or running, because the peripheral blood requirement exceeds cardiac pumping capacity. According to an extensive review of VO_2 research by Bassett and Howley (2000), Q accounts for 70–85% of VO_{2max} capacity, with 5.9–7.5 $L.min^{-1}$ needed per litre increase in VO_2 (Saltin & Calbet, 2006). Q is determined by heart rate (HR) and SV, with the capacity of the latter being the predominant determinant in Q_{max}. However, while Q_{max} can be improved greatly with endurance training, elite athletes are have highly-developed Q capacities with little potential for further increases (Blomqvist & Saltin, 1983). Thus, while Q is a key determinant of VO_{2max}, it is unlikely to be improved through nutritional or training interventions in these individuals.

2.1.4.2b Blood flow

Although Q sets a limit on the maximal potential for blood volume, it is the efficiency by which this flows through the vasculature that determines the rate of O_2 delivery to the periphery of the active muscles (Saltin & Calbet, 2006; Wagner, 1996). Further, an increased blood flow augments the PO_2 in the vasculature compared to that inside the muscles, thus improving the drive for O_2 diffusion into the muscles (Calbet & Joyner, 2010).

The rate of vascular blood flow for a given arterial pressure is largely facilitated by alterations in flow resistance due to vasoconstriction and vasodilation, which promotes the appropriate distribution of blood flow into the areas of highest requirement. As such, during exercise, blood flow to the myocardium and active muscles is enhanced through the dilation of their associated blood vessels, while those pertaining to the inactive muscle groups and the digestive and renal systems constrict (Saltin, Rådegran, Koskolou, & Roach, 1998). Vasodilation is largely controlled by the signaling compound NO, which catalyzes the formation of the major vasodilatory compound, cyclic guanosine monophosphate (cGMP) in the smooth muscle cells of the endothelium (Mónica, Bian, & Murad, 2016). Thus, interventions which target the production of NO have the potential to influence blood flow, and thereby alter O_2 transport and VO_2. This idea will be explored further in section 2.2.5

It is important to note that although the active muscles have a large O_2 requirement during exercise, the proportion of blood flow directed to them is also dependent on the requirements of other systems. For example, during very heavy exercise, the respiratory muscles may require greater blood flow to drive greater amounts of O_2 into the bloodstream. Subsequently, a larger proportion of blood is directed to the pulmonary system, potentially decreasing locomotor muscular perfusion (Harms et al., 1997). Similarly, the proportion of blood flow to the muscles may be compromised during exercise in hot temperatures, due to the relatively greater blood flow to the periphery for heat dissipation (Brengelmann, Johnson, Hermansen, & Rowell, 1977). In this regard, the pulmonary system or periphery becomes a limiter of muscle blood flow, which is in turn a limiter of muscle oxygen saturation and power at VO_{2max}.

2.1.4.2c Erythrocyte count

While the previously-mentioned cardiovascular limitations determine the quantity of blood transported to the muscles, the capacity of O_2 transport in the blood depends on the quantity of Hb in the blood stream. Hb constitute ~90% of total erythrocyte weight, and contain four proteins (two alpha and two beta globin chains), each with an iron moiety (Fe^{2+}) capable of binding to O_2 (Van Leeuwen, Poelhuis-Leth, & Bladh, 2013). The degree of Hb saturation is largely determined by the $A–aO_2$ difference, with a fully saturated Hb having a maximal capacity of ~1.34 $ml.g^{-1}$ O_2 (Gregory, 1974). The O_2 dissociation curve of Hb follows a sigmoidal curve, in which uptake of one molecule facilitates the uptake of subsequent molecules. However, once fully saturated, the dissociative tendency increases to promote the movement of O_2 into the tissues (Borrione, 2008). The contribution of Hb to the efficiency of O_2 transport was demonstrated by Burnley, Roberts, Thatcher, Doust, and Jones (2006), who measured a ~4.5% reduction in VO_2 following a ~7% decline in Hb, induced through the withdrawal of 450 ml of whole blood (Figure 2.2).

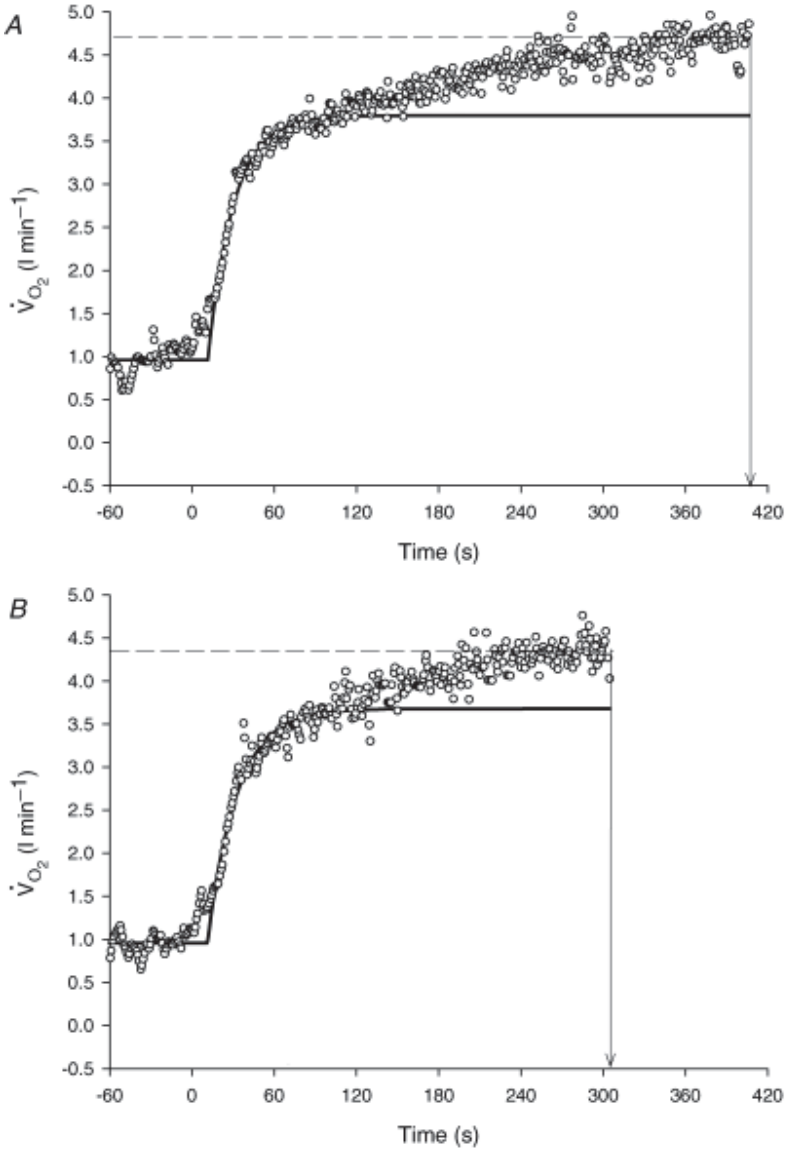

Figure 2.2 VO_2 response to severe-intensity exercise in a representative subject before (A) and after (B) the donation of ~450 ml of whole blood. The horizontal dashed line indicates the end-exercise VO_2 (Burnley et al., 2006).

Because higher volumes of Hb afford greater O_2 transport, much previous sport-performance research has explored methods of increasing erythrocyte or Hb count (Calbet, Lundby, Koskolou, & Boushel, 2006). Historically, endurance athletes have increased Hb via illegal doping with the erythrocyte-stimulating hormone, erythropoietin (EPO), or through

blood reinfusion. Audran et al. (1999) demonstrated that 26 days of treatment with EPO stimulated an increase of 5–7% in Hb, a 9% increase in VO_{2max} and a 5% increase in $\%VO_2$ at MLSS. However, there is also potential for dietary supplements to provide smaller increases in Hb by reducing its rate of breakdown. This concept will be explored further in 2.3.3.

2.1.4.3 The muscular system

2.1.4.3a Muscle oxygen uptake

Another limitation to the efficiency of O_2 transport is the rate of O_2 movement from the bloodstream into the mitochondria, the energy-producing area of the muscle cells. As with the $A–aO_2$, O_2 diffusion from the extramuscular capillaries to the interstitial space, across the plasma membrane, into the muscle cytoplasm, and finally the mitochondria, is driven by numerous O_2 pressure gradients (Honig, Connett, & Gayeski, 1992). In addition, an increase in intramuscular adenosine diphosphate (ADP) concentration in the muscle cells signals a need for more ATP production and promotes O_2 movement into the muscle. Thus, simply increasing the blood flow to the muscles does not allow an increased VO_2 if the mitochondria are not actively using and clearing the O_2 transported into them (Bassett & Howley, 2000). The rate of O_2 diffusion is greatest in the muscle cells closest to the capillaries, with those more distally placed being at a disadvantage (Honig et al., 1992). Thus, interventions which may allow more equal distribution of O_2 among the muscle fibres could improve the matching of O_2 delivery to muscular requirements.

In addition, the rate of O_2 diffusion into, and energy production from, the mitochondria is dependent on their quantity in the muscle. However, highly-trained endurance athletes have a large mitochondrial density as a key adaptation to endurance training (Hawley,

2002), so this is unlikely to be a limiting factor in VO_2 in these individuals, although the shape of the cristae may change to increase surface area. Further, the efficiency of mitochondrial ATP production is influenced to a small degree by the mitochondrial enzymes which drive the metabolic pathways. Yet, as demonstrated by Holloszy and Coyle (1984) while increasing mitochondrial enzyme quantity greatly improves endurance performance due to improvements in metabolic flexibility, the improvement in VO_{2max} through improved O_2 extraction is relatively small.

2.1.5 In summary

During aerobic exercise, limitations to VO_2 can occur at all stages during the movement of O_2 from the pulmonary system into the mitochondria. While Q appears to be the principal determinant of VO_{2max}, highly-trained athletes have generally developed their Q_{max} and VO_{2max} to the extent of their genetic ability, and are unlikely to improve these parameters significantly further. Instead, areas such as vascular conductance, blood Hb content and the efficiency of capillary to muscle O_2 diffusion appear to offer greater potential for improvements to the efficiency of O_2 transport with nutritional interventions.

2.2 FREE RADICAL SPECIES AND OXYGEN TRANSPORT

2.2.1 Introduction

One of the major contributors to the previously-described limitations to VO_2 is the exposure of O_2 transport pathway components to large quantities of free radical or oxidant species. These are biologically active compounds, generally containing either O_2 (reactive oxygen species, ROS) or nitrogen (reactive nitrogen species, RNS), which are collectively referred to as RONS and are characterized by the presence of one or more unpaired electron(s) in their outer shells (Gutteridge, 1995). Due to their unstable configuration, these compounds tend to react with other cellular components, leading to perturbations in the cellular environment, and a state termed 'oxidative stress', defined as 'an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage' (Sies & Jones, 2007, p.45).

The risk of developing a state of oxidative stress is increased during exercise, because RONS generation is exacerbated by a greater flux of O_2 through metabolic pathways (Vollaard, Shearman, & Cooper, 2005). Thus, athletes completing large amounts of exercise training are subject to a significant degree of oxidative stress, which may hinder their ability to transport and uptake O_2 (Reid et al., 1994).

On the other hand, because RONS can rapidly alter the form of other compounds, they also function as vital signaling molecules, which are necessary in low to moderate quantities for normal cell function and adaptation to exercise (Reid et al., 1994). In particular, NO, the parent RNS compound is also a major stimulator of vasodilation (Mónica et al., 2016). Thus, it appears that supplementation protocols which stimulate NO production may be

beneficial, rather than limiting, to VO_2 (Bailey et al., 2009; Larsen, Weitzberg, Lundberg, & Ekblom, 2007).

The following section will explore the positive and negative ways that RONS may influence O_2 transport, to identify how alterations in their concentrations may influence VO_2.

2.2.2 Classification of reactive oxygen/nitrogen species

2.2.2.1 Reactive oxygen species

ROS are formed through the reduction of O_2 during aerobic metabolic processes. The loss of a single electron from O_2 forms the highly-reactive compound, superoxide, which is sequentially converted to hydrogen peroxide, hydroxyl radical and hydroxide ion in subsequent reducing reactions (Gutteridge, 1995) (Figure 2.3). The major contributor to ROS-induced cell damage is the hydroxyl radical, which is formed from metal-catalyzed reactions with hydrogen peroxide (Halliwell, 1990). Under resting conditions, excess ROS are neutralized either through conversion to water by the enzyme catalase (CAT), or following the donation of an electron by the amino acid antioxidant, glutathione (GSH) (see 2.3.2.1 for further detail). However, if the rate of ROS production exceeds the antioxidant removal capacity, ROS accumulation and oxidative stress may occur (Reid et al., 1994).

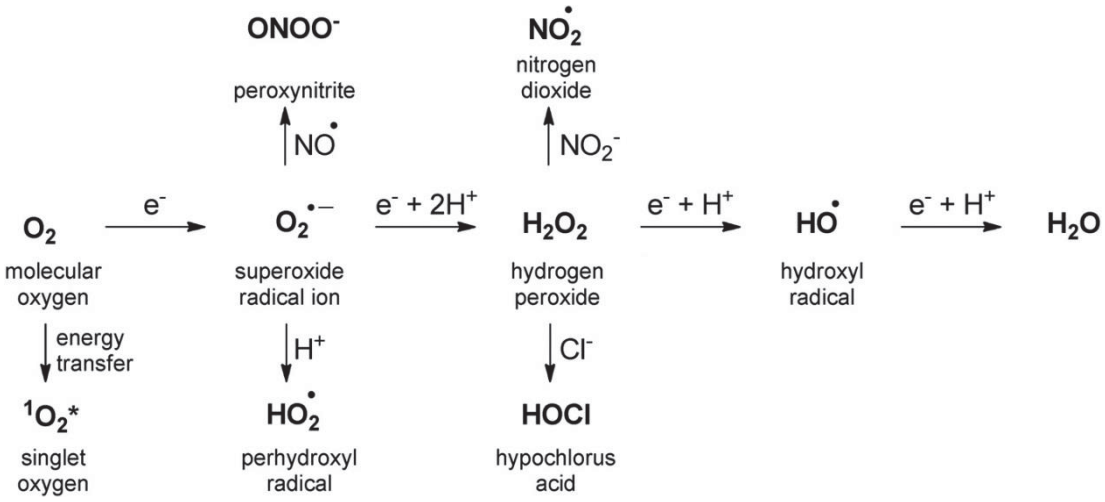

Figure 2.3 Formation of ROS through energy- and electron-transfer reactions (Krumova & Cosa, 2016)

2.2.2.2 Reactive nitrogen species

The RNS cascade encompasses several nitrogen-containing compounds of varied reactivity, which are linked through NO. Although NO is classified as a free radical, it is uncharged, and only reacts readily with a select group of compounds, primarily other free radicals and transition metals (Beckman & Koppenol, 1996). NO is chiefly formed through the endogenous oxidation of L-arginine by three types of NO synthases (NOS)—endothelial, neuronal and inducible (inflammatory) (Moncada & Higgs, 1993). However, under hypoxic (low O_2) or acidic conditions, such as those present in the muscles during exercise, activity of the endogenous aerobic pathway is limited and increased production via a secondary anaerobic pathway, involving the conversion of nitrates (NO_3^-)–nitrites (NO_2^-)–NO, becomes necessary (Figure 2.4). This pathway is of interest to the current thesis because ~25% of NO_3^- consumed in the diet is excreted in the saliva post-swallowing and converted to NO_2^- by commensal bacteria on the tongue (Benjamin et al., 1994; Lundberg, Weitzberg,

Lundberg, & Alving, 1994). Subsequently, following absorption, NO_2^- can be converted to NO through acidic reduction (Lundberg & Weitzberg, 2005) or catalyzation by a reducing agent in the plasma, such as methaemoglobin (Doyle, Pickering, DeWeert, Hoekstra, & Pater, 1981) or xanthine oxidase (Millar et al., 1998). Therefore, this pathway provides a method by which supplementation may increase plasma NO

However, although NO is a major contributor to efficient O_2 transport, other components of the RNS cascade are associated with oxidative stress. NO has a very short half-life (~1–2 ms) and rapidly combines with superoxide and O_2, to produce peroxynitrite and nitrogen dioxide respectively (Beckman & Koppenol, 1996; Lundberg & Weitzberg, 2005). Such compounds are highly reactive and are associated with cellular damage (see 2.2.4 for further detail). Thus, the RNS cascade both enhances and limits the O_2 transport pathway, depending on the relative concentration of its components.

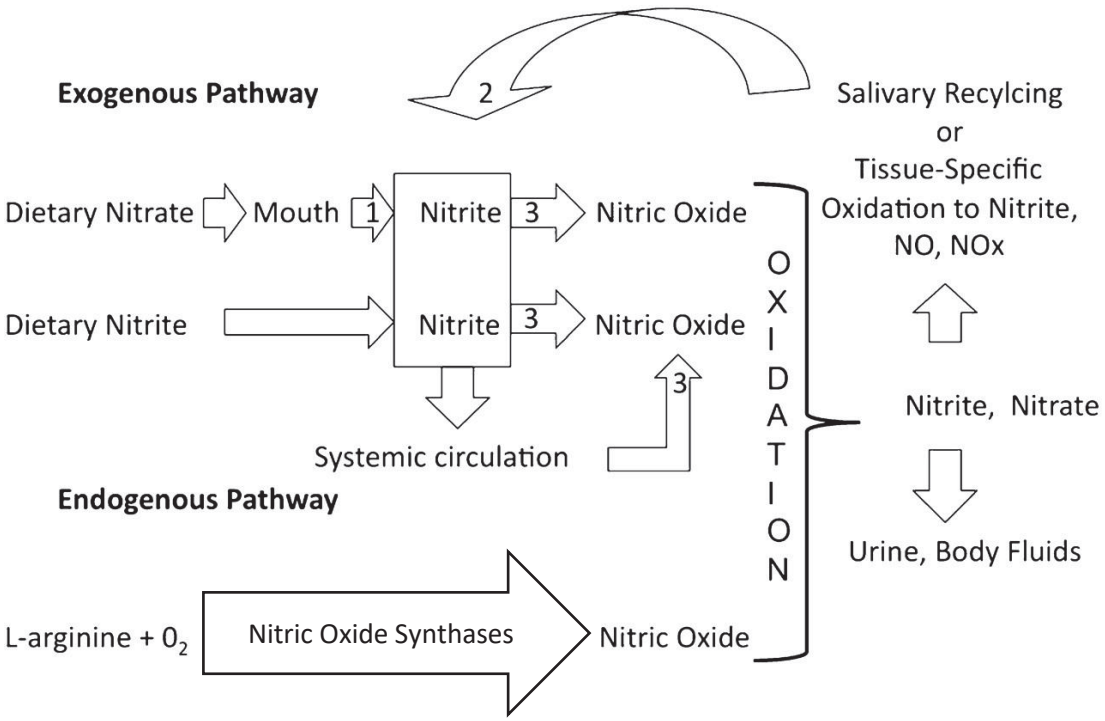

Figure 2.4 The production of NO from endogenous and exogenous (dietary) sources. 1: The action of bacterial nitrate reductases on the tongue and mammalian enzymes that have nitrate reductase activity; 2: Mammalian enzymes with nitrite reductase activity; 3: Conversion of NO_2^- to NO by reducing agents, e.g., methaemoglobin, xanthine oxidase (Hord, Tang, & Bryan, 2009)

2.2.3 Production of reactive oxygen/nitrogen species during exercise

Although the increase in RONS production during exercise has been demonstrated indirectly since the late 1970's (Brady, Brady, & Ullrey, 1979; Davies, Quintanilha, Brooks, & Packer, 1982; Dillard, Litov, Savin, Dumelin, & Tappel, 1978), it was not until 2007 that post-exercise intramuscular RONS accumulation was shown directly using electron paramagnetic spectroscopy (Bailey et al., 2007). Today, the exercise-induced rise in RONS is attributed to a greater O_2 flux through cellular components, a rise in blood temperature, larger amounts of shear stress on the vasculature and increased formation from oxidases (e.g. xanthine oxidase, NADPH oxidase) due to inflammation and ischaemia (Sakellariou et al., 2013). In

erythrocytes, the auto-oxidation of oxyhaemoglobin (oxyHb) to (non-O_2 containing) methaemoglobin (HHb) also increases during exercise, producing superoxide. While the primary location of RONS production is unclear, it is likely that a large portion are released from muscle tissue, where the increased O_2 flux through structures such as the ETC, sarcoplasmic reticulum, transverse tubules and plasma membrane has been demonstrated to increase RONS production by two to four fold (Liu, Fiskum, & Schubert, 2002). In addition, RONS may diffuse into the plasma from the endothelium and smooth muscle surrounding the vasculature, where the rates of production are greatly influenced by vascular shear stress. As well as being produced externally to the blood, RONS are produced within erythrocytes themselves during intracellular auto-oxidation reactions, which also occur with increased frequency during exercise (Davies et al., 1982).

2.2.4 Negative effects of reactive species on oxygen uptake

A state of oxidative stress has the potential to limit O_2 transport due to the high vulnerability of erythrocytes to RONS-induced damage. As described in 2.1.5.2c, erythrocytes play a key role in O_2 transport as blood O_2 carriers. While RONS can cause damage to numerous components of cells (e.g. protein, DNA, lipids), erythrocyte membranes are particularly susceptible to lipid peroxidation due to their large polyunsaturated fatty acid (PUFA) content (~28–37% total fatty acid weight), one of the primary targets of RONS (Nikolaidis & Jamurtas, 2009). During lipid peroxidation, hydroxyl radicals remove hydrogen atoms from the methylene carbon side chains of PUFAs, forming secondary lipid-containing RONS, initiating a chain peroxidation reaction, and causing structural damage and the loss of fluidity to the cell membrane. This decreases or removes the functionality of intrinsic membrane enzymes and transporters, which among other roles

facilitate O_2 delivery to tissues. The breakdown of outer membrane lipids also exposes proteins within the membrane to RONS (Gutteridge, 1995). Total breakdown of the erythrocyte (haemolysis) can occur when substances penetrate deeply into the membrane weakening the inner structure and causing water which is being transported to swell the cell and permanently damage the membrane (Bonarska-Kujawa et al., 2014). Subsequently, this causes further oxidative stress in the bloodstream because the release of Hb reacts with hydrogen peroxide and forms toxic iron ions (Gutteridge, 1994).

The post-exercise increase in lipid peroxidation was first demonstrated by Dillard et al. (1978) who measured a 1.8-fold increase in exhaled pentane levels following 60 minutes of cycling at 50% VO_{2max}. Ensuing research has demonstrated significant increases in the more precise marker of lipid peroxidation, malondialdehyde, as measured by thiobarbituric acid (TBARS), following a range of endurance exercise modes, durations and intensities, for example a cycle or run VO_{2max} test (+~14%) (Ashton et al., 1998; Hartmann, Nieβ, Grünert-Fuchs, Poch, & Speit, 1995) and during 40–90 minutes cycling at 60%VO_{2max} (~+65–85%) (Børsheim, Knardahl, & Høstmark, 1999; Laaksonen et al., 1999). In addition, the susceptibility of erythrocytes to oxidative stress or lipid peroxidation has been shown to increase following a half marathon running race (Duthie, Robertson, Maughan, & Morrice, 1990), treadmill running at 75%VO_{2max} and cycling at 60%VO_{2max} (Smith, Kolbuch-Braddon, Gillam, Telford, & Weidemann, 1995). Thus, RONS accumulation in the blood may significantly influence the quantity and functionality of erythrocytes and Hb available for O_2 transport.

Besides damaging cells directly, RONS create further potential for oxidative stress when the two free radical cascades combine, because superoxide and NO react to form peroxynitrite. The reaction rate between the two compounds is so rapid (6.7×10^9 $m.s^{-1}$) that almost every

collision between them results in the formation of this strong oxidizing agent, which is cytotoxic when present in large amounts (Beckman, Beckman, Chen, Marshall, & Freeman, 1990). In addition to lipid peroxidation, peroxynitrite converts oxyHb into HHb (Schmidt, Klatt, & Mayer, 1994), and contributes to mitochondrial damage (Ballinger et al., 2000) and endothelial dysfunction (Milstien & Katusic, 1999), all of which decrease the efficiency of O_2 movement through the blood or into the muscles. Further, the production of peroxynitrite via this reaction reduces the availability of NO for signaling activities, potentially impacting the efficiency of blood flow. The effect of a limited NO concentration is shown in patients with hypertension, hypercholesterolemia and endothelial dysfunction, who along with low plasma NO, are characterized by increased blood pressure (BP), platelet aggregation and vascular damage (Cooke & Dzau, 1997). Unsurprisingly, individuals with these conditions have extremely lowered VO_2 capacities due to limitations to blood flow. While, these individuals represent an extreme example of limited NO, their blood characteristics indicate that a decrease in NO during chronic periods of oxidative stress due to heavy training could negatively affect blood values and O_2 transport capacity.

2.2.5 Positive effects of reactive species on oxygen uptake

The body's response to RONS follows the principles of hormesis (Calabrese & Baldwin, 2002), whereby low to moderate quantities are necessary for cellular signaling processes and regulation of hormones, while large amounts contribute to oxidative stress (Ji, Gomez-Cabrera, & Vina, 2006; Radak, Chung, Koltai, Taylor, & Goto, 2008). Recently, an exception to this has emerged, with increases in plasma NO concentrations through dietary supplementation appearing to benefit O_2 transport (Bailey et al., 2009; Larsen et al., 2007).

As mentioned in 2.2.2.2, endogenous NO production cannot be enhanced through supplementation with components of the aerobic NOS synthase pathway, but dietary NO_3^- can be converted to NO via the anaerobic pathway (Larsen et al., 2007). Accordingly, acute and short-term (≤6 days) supplementation with sodium nitrate (~5.5 mmol NO_3^-) or high-NO_3^- beetroot juice (BRJ) (~5–11 mmol NO_3^-) increases plasma NO_2^- by ~90-140% and decreases systolic BP (SBP) by ~4-5%, indicating the occurrence of an increase in plasma NO (Bailey et al., 2009; Bailey, Winyard, et al., 2010; Larsen et al., 2007; Vanhatalo et al., 2010). In low to moderately-trained individuals (VO_{2max} ≤57 ml.min.L^{-1}), these changes are associated with a decrease in the VO_2 required to exercise at a given submaximal intensity, and improvements in exercise performance during knee extension, cycling and running time trial to exhaustion (TTE) protocols, and cycling time trials (Bailey et al., 2009; Bailey, Winyard, et al., 2010; Lansley, Winyard, Bailey, et al., 2011; Lansley, Winyard, Fulford, et al., 2011; Porcelli et al., 2015). Conversely, NO_3^- supplementation via BRJ does not generally improve VO_{2max} (Bailey et al., 2009; Bailey, Winyard, et al., 2010) and is largely ineffective in altering VO_2 values in highly-trained individuals (VO_{2max} ≥60 ml.min.L^{-1}) despite inducing an increased plasma NO_2^- concentration (Boorsma, Whitfield, & Spriet, 2014; Nyakayiru et al., 2016; Porcelli et al., 2015; Wilkerson et al., 2012). Nonetheless, these results indicate the potential for supplements to influence VO_2 during exercise via targeting changes in RONS concentrations.

2.2.6 In summary

While small to moderate levels of RONS are necessary for optimal cell function, their accumulation causes oxidative stress, leading to erythrocyte damage and limitations to O_2 transport. However, NO appears to be an exception, with recent studies indicating a link

between increased NO and more efficient O_2 usage. Consequently, supplementation protocols which reduce the overall build-up of RONS, or enhance NO production may improve some of the previously-described limiting factors in O_2 transport, namely Hb concentration and blood flow.

2.3 ANTIOXIDANTS, REACTIVE SPECIES AND OXYGEN UPTAKE

2.3.1 Introduction

The term 'antioxidant' encompasses a wide range of substances with the ability to delay or prevent the oxidation of other substrates (Halliwell, 2007). Through endogenous methods, the body manufactures sufficient antioxidants to prevent the build-up of RONS during resting conditions or following low levels of exercise training. However, athletes who complete large amounts of training, and produce greater quantities of RONS, may benefit from ingesting antioxidants exogenously to prevent the development of oxidative stress (Reid et al., 1994). Exogenous antioxidants include compounds, or precursors to compounds that are already produced in the body, and unique dietary components with antioxidant properties, which complement or enhance the endogenous system. To function effectively, exogenous antioxidants must be well absorbed in the digestive system so that they can enter the cells and carry out their antioxidant actions (Hackman et al., 2008; Hollman, 2016).

This section will describe how endogenous antioxidants limit the build-up of RONS and introduce the various types of exogenous antioxidants which may be used in conjunction with this system during times of increased oxidative stress. In addition, the use of

antioxidants to enhance the actions of NO will be discussed. Understanding how the different types of antioxidants function will help to identify how groups of dietary compounds may influence VO_2 during exercise.

2.3.2 Endogenous versus exogenous antioxidants

2.3.2.1 Endogenous antioxidants

The major group of endogenous antioxidants are the thiol family, a collection of compounds which are characterized by the presence of a sulfhydryl group (-SH) in their makeup. Thiols act in conjunction with antioxidant enzymes to prevent or delay the accumulation of RONS, by donating electron(s) during redox reactions to neutralize their unstable outer shells. Thiols are described as being redox buffers, because they are able to cycle between states of varying electron number without becoming highly reactive themselves. Although the majority of thiol compounds are produced endogenously, the parent thiol, methionine, is an essential amino acid which is absorbed from the diet. Through the transsulfuration pathway (Figure 2.5), methionine is converted into cysteine (CYS) which combines with glycine and glutamate to form the tripeptide amino acid glutathione (GSH), the primary thiol involved in ROS neutralization (Brosnan & Brosnan, 2006; Stipanuk, 2004). Because GSH cannot be absorbed in the diet, the concentration of its constituents is a major determinant of total antioxidant status of the blood (Wu, Fang, Yang, Lupton, & Turner, 2004). In addition to its role in GSH formation, CYS is also a precursor to the non-protein amino acid, taurine (TAU), which also appears to have antioxidant properties, although the specific nature of these is undetermined (Shimada, Jong, Takahashi, & Schaffer, 2015).

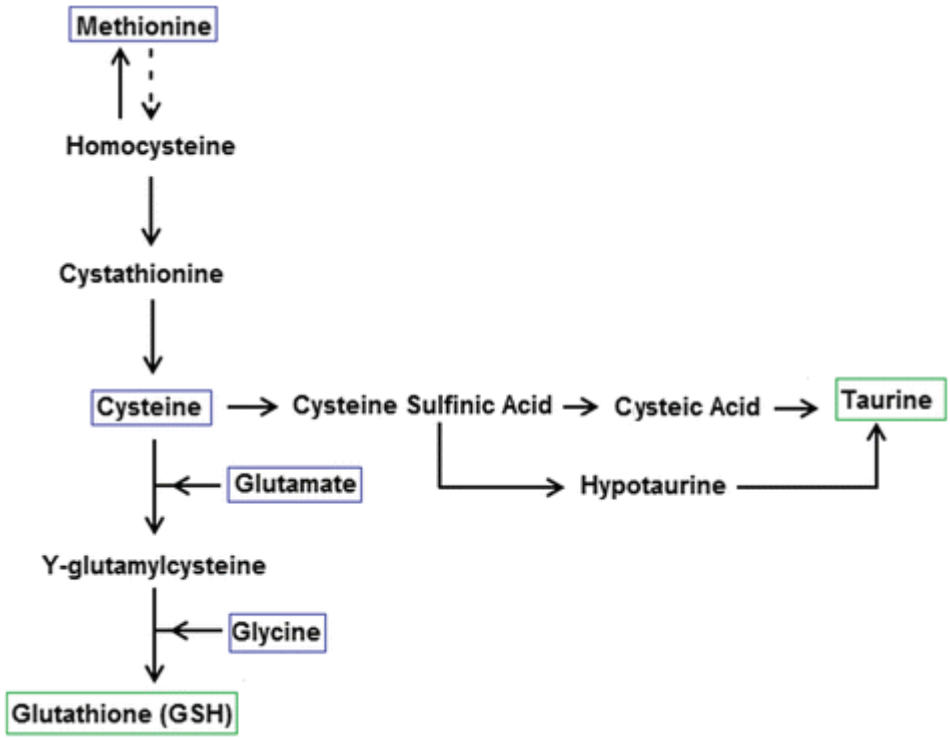

Figure 2.5 Summary of thiol metabolism and resulting thiol antioxidants. Methionine is metabolized to CYS which further forms endogenous antioxidants TAU and GSH via two distinct pathways. Supplementing with TAU and/or cysteic acid may 'spare' CYS, upregulating GSH synthesis, thus further boosting endogenous antioxidant defense(McLeay, Stannard, Houltham, & Starck, 2017)

2.3.2.2 Exogenous antioxidants

This thesis will discuss two major groups of exogenous antioxidants: exogenous thiols and polyphenols, which both have the potential to influence O_2 transport. Exogenous thiols are rarely found in large quantities in food, but may be taken in supplement form to increase the capacity of the endogenous thiol system. Although GSH and CYS are poorly absorbed when ingested orally (Meister, 1991), CYS-containing compounds, such as *N*-acetylcysteine

(NAC) have greater absorbability and can be converted into CYS in the liver prior to secretion into the circulation (Medved et al., 2004).

Polyphenols are a group of phytochemicals that are characterized by the presence of one or more aromatic rings and at least two hydroxyl groups and are found in high concentrations in green tea, wine, cocoa and numerous fruits and vegetables (Bravo, 1998). There are currently >500 dietary polyphenolic compounds identified on the United States Department of Agriculture Phenol-Explorer database. These are classified into four main classes: flavonoids (e.g. red and blue fruits), phenolic acids (e.g. grains, seeds), stilbenes (e.g. red wine) and lignans (e.g. flax, grains), each incorporating numerous subclasses (Bhagwat, Haytowitz, & Holden, 2008, 2014; Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Of these, the flavonoid class appears to have the greatest potential to limit oxidative stress and improve O_2 transport (Myburgh, 2014).

2.3.3 Antioxidants limit the negative actions of reactive species on the oxygen transport pathway

2.3.3.1 Neutralization of excess reactive species

2.3.3.1a Endogenous antioxidants

Endogenous antioxidants carry out the majority of ROS neutralization through the GSH cycle, using the antioxidant enzymes, superoxide dismutase (SOD), CAT, glutathione peroxidase (GPx) and glutathione reductase (GR) as catalysts (Figure 2.6). During this cycle, SOD converts superoxide into hydrogen peroxide, and CAT and GPx reduce hydrogen peroxide to water, with GPx using GSH as an electron donor. Subsequently, the oxidized

dimer form of GSH, glutathione disulphide (GSSG) is reduced back to GSH in a coupled reaction with nicotinamide adenine dinucleotide phosphate (NADPH) to prevent cytotoxicity and to regenerate the antioxidant for future use. In addition to its role in the GSH cycle, GSH can also directly donate a hydrogen atom to, and neutralize, RONS (Dröge, 2002).

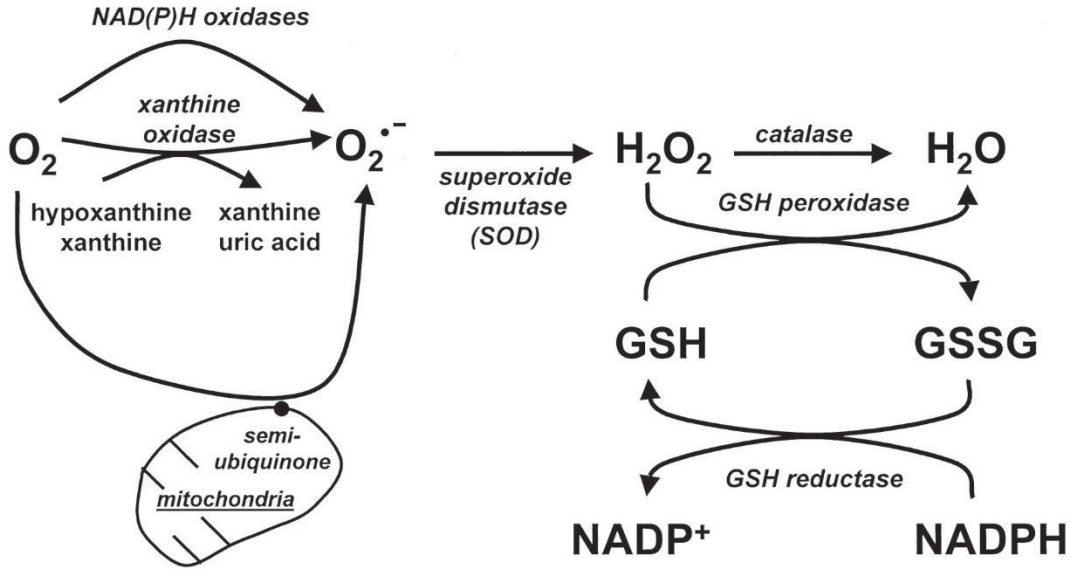

Figure 2.6 Pathways of reactive oxygen species production and clearance (Dröge, 2002)

The role of antioxidant enzymes in reducing erythrocyte oxidative stress has been demonstrated by measuring changes in relative GSH and GSSG concentrations pre and post-exercise. Sastre et al. (1992) demonstrated that following a treadmill graded exercise test to exhaustion, GSSG was 72% greater than pre-exercise levels, while GSH was 63% lower post-exercise, indicating increased activity of the GSH cycle. Similarly, Gohil, Viguie, Stanley, Brooks, and Packer (1988) measured an 100% increase in GSSG and a 60% decrease in GSH following 90 minutes of cycling at a moderate intensity (65% VO_{2max}). In addition, increases

in post-exercise antioxidant enzyme activity have been observed following moderate intensity cycle or running exercise (Marzatico, Pansarasa, Bertorelli, Somenzini, & Della Valle, 1997; Ohno et al., 1986). However, following prolonged and exhaustive exercise (e.g., 90 minutes cycling at 80%VO_{2max}), the post-exercise activity of antioxidant enzymes decreases, which suggests that the capacity for ROS removal has been overwhelmed (Tauler et al., 2005).

2.3.3.1b Exogenous antioxidants

Although the body produces sufficient endogenous thiols to maintain ROS levels at a non-stressful level during resting conditions and low to moderate levels of exercise, the increase in RONS production during intense or prolonged exercise may overwhelm its capacity. To a certain extent, the body adjusts to greater RONS production by upregulating the production of endogenous thiols and antioxidant enzymes as a training adaptation. However, this may still not be sufficient to cope with the greater exposure to oxidative stress when large amounts of training are being completed (Gomez-Cabrera, Domenech, & Viña, 2008). Thus, to prevent the previously-mentioned limitations to VO_2 occurring, it could be beneficial to ingest supplemental thiol compounds exogenously. On the other hand, previous research has demonstrated that chronic antioxidant supplementation has the potential to slow training responses and adaptations (Slattery, 2015).

As previously mentioned, CYS-containing compounds are better sources of exogenous thiols than GSH or CYS themselves, due to their greater absorbability (Wu et al., 2004). Orally delivered NAC is readily absorbed in the stomach and gut and sent to the liver where it is almost entirely converted to CYS, which is then incorporated into GSH (Atkuri, Mantovani,

Herzenberg, & Herzenberg, 2007). Accordingly, acute (~5300–6300 mg, 1h prior to exercise) and short-term (1200 $mg.d^{-1}$, 8–9 days) supplementation with oral NAC increase GSH concentrations by 25–35% and decrease markers of lipid peroxidation by 18–40% (Corn & Barstow, 2011; Slattery, Dascombe, Wallace, Bentley, & Coutts, 2014; Smith et al., 2016; Zembron-Lacny et al., 2010). In addition, research by Zembron-Lacny et al. (2010) indicated that NAC may have the potential to improve VO_2. Specifically, eight days of NAC supplementation (1200 $mg.d^{-1}$) in 15 untrained males significantly increased Hb (+10%) and EPO (+26%). However, although these results suggest an increase in O_2 transport capacity, there were no differences in VO_{2max} values obtained in a subsequent graded cycling test to exhaustion. Similarly, Kelly, Wicker, Barstow, and Harms (2009) measured an increase in the mean maximal inspiratory pressure of the lungs during cycling at 85% VO_{2max} following acute NAC supplementation (1800 mg), which indicated a reduction in respiratory muscle fatigue, but this did not correspond to any significant changes in VO_2. Nonetheless it is possible that such changes would have had a greater impact on VO_2 during prolonged exercise, as opposed to a short maximal test, where the efficiency of the aerobic system has a greater effect on performance. Thus, further research involving CYS-containing compounds is required to determine whether this is a viable way of improving erythrocyte parameters and O_2 transport.

2.3.3.2 Direct protection of erythrocytes

While thiols reduce the effects of oxidative stress through limiting the build-up of plasma RONS, flavonoid polyphenols directly protect erythrocytes, thereby increasing the availability of cells for O_2 transport. Based on a spectrophotometric assessment of erythrocytes following the addition of polyphenols, Bonarska-Kujawa et al. (2014)

attributed this protective effect to the integration of polyphenols into erythrocyte cell membranes, which forms a defensive coat and prevents ROS-induced degradation. In addition, polyphenols contain structural elements, namely aromatic hydroxyl groups and catechol or dihydroxylated B-rings, which can act as RONS scavengers (Rice-Evans, Miller, & Paganga, 1996).

Unlike thiols, flavonoids are not produced endogenously and can only be ingested through the diet, where they are found abundantly in fruits and vegetables. Dietary flavonoid sources which have been associated with protective effects on erythrocytes include juices or extracts from chokeberry (Pilaczynska-Szczesniak, Skarpanska-Steinborn, Deskur, Basta, & Horoszkiewicz-Hassan, 2005), cranberries (Labonté et al., 2013), grapes (Barona, Aristizabal, Blesso, Volek, & Fernandez, 2012; Labonté et al., 2013), Ecklonia cava (Oh et al., 2010), lychee fruit (Kang et al., 2012), purple sweet potato leaves (Chang, Hu, Huang, Yeh, & Liu, 2010) and pomegranate (Roelofs, Hirsch, Trexler, Mock, & Smith-Ryan, 2015; Roelofs, Smith-Ryan, Trexler, Hirsch, & Mock, 2016). Intake of flavonoids from these foods (e.g. 200 $g.d^{-1}$ purple sweet potato leaves for 7d, 46 $g.d^{-1}$ grape extract for 30d or 1000 mg pomegranate extract, 30 min before exercise) has been associated with decreased markers of oxidative stress and lipid peroxidation, and increased vasodilation and blood flow (Barona et al., 2012; Chang et al., 2010; Roelofs et al., 2016). However, few studies have investigated whether such changes influence VO_2. Oh et al. (2010) showed a trend towards an increased VO_{2max} (6.5%) in untrained males during a treadmill graded test to exhaustion, following supplementation with 180 ml Ecklonia cava (~72 mg flavonoids), which was accompanied by an improved exercise performance. Conversely, thirty days of supplementation with 200 $mg.d^{-1}$ lychee fruit extract (~66$mg.d^{-1}$ polyphenols) in low-trained individuals had no effect on VO_{2max} during a treadmill graded exercise test to exhaustion,

but increased the running speed reached at which VO_{2max} occurred (+15%) and the VO_2 at MLSS (+7%) (Kang et al., 2012). Thus, further research is required to determine whether the effects of flavonoids on erythrocytes can improve O_2 transport.

2.3.4 Antioxidants enhance the effects of nitric oxide

2.3.4.1 The effect of thiols on nitric oxide concentration

Thiols enhance the availability of NO because their neutralization of ROS reduces the concentration of superoxide available to react with NO and form peroxynitrite (Arteel, Briviba, & Sies, 1999). Accordingly, the addition of GSH or NAC to rabbit or rat aortic rings has been shown to improve endothelial vasodilation (Goldschmidt & Tallarida, 1991; Lopez, Snyder, Birenbaum, & Ma, 1998), while the treatment of endothelial cells with thiol-depleting agents reduces NO production (Ghigo et al., 1993).

In addition, thiols can stabilize NO by donating their -SH group to the compound thus forming a thiol adduct known as an *S*-nitrosothiol (SNO). SNOs are suggested to have the same NO-bioactivity as NO itself, but in a form that is less vulnerable to degradation, with a half-life of several hours longer. Accordingly, McKinley-Barnard, Andre, Morita, and Willoughby (2015) demonstrated that the combination of the endogenous precursor to NO, L-citrulline (CIT), with GSH was more effective at increasing markers of NO production than supplementation with CIT alone. Of particular interest is *S*-nitroso Hb (SNO–Hb) which is formed through the binding of NO to a CYS residue on Hb. The binding of NO to Hb is favoured in high O_2 conditions, while a low O_2 environment stimulates NO release, suggesting that this reaction allows the delivery of NO to less oxygenated tissues where it is most useful (McMahon, Leveritt, & Pavey, 2016). Conversely, some authors have suggested

that if SNOs do not release sufficient NO when it is needed, they may not be a beneficial method for extending the life of NO (Bailey et al., 2011). However, no previous research has investigated whether increased SNO production improves O_2 status or exercise performance.

2.3.4.2 The effect of polyphenols on NO concentration

One of the major ways that polyphenols are likely to enhance O_2 transport is through their promotion of increased plasma NO concentration, both through enhancing NO production and protecting it from degradation. Specifically, flavonoids have been shown to stimulate the conversion of NO_2^- to NO. For example, the consumption of 125 ml red wine increases the amount of NO formed in the stomach following the intake of sodium nitrate by >40-fold (Gago et al., 2007). Similarly, the addition of apple extract to human saliva (18 $mg.ml^{-1}$) increases the formation of NO following a NO_3^--rich meal by 9-fold (Peri et al., 2005).

Further, flavonoids augment the plasma NO concentration by preventing its reaction with ROS. Ignarro et al. (2006) demonstrated that the addition of pomegranate juice (POMJ) (3 µL of a 30-fold dilution of concentrate), blueberry juice (300 µL), Concord grape juice (300 µL) or red wine (300 µL) to a solution containing NO and superoxide, significantly reduced the rate of disappearance of NO. Further, the addition of flavonoid-containing juices to NO increased its inhibitory effect on the proliferation of rat smooth muscle cells, indicating that the antioxidants had enhanced the biological activity of NO.

2.3.5 In summary

The human body contains an endogenous antioxidant system which is sufficient for preventing RONS accumulation during rest or low levels of exercise. However, when large

amounts of exercise training are being completed, athletes may benefit from supplementing with exogenous thiols or polyphenols to reduce the negative impact of RONS on VO_2. Further, endogenous antioxidants may enhance O_2 transport by increasing the bioavailability of NO.

2.4 KERATIN AND POMEGRANATE EXTRACT AS DIETARY SUPPLEMENTS

2.4.1 Introduction

Based on the potential of exogenous antioxidants to influence the activity of RONS and enhance O_2 transport, this thesis will investigate two dietary antioxidant supplements, one rich in thiols and the other rich in flavonoid polyphenols. In accordance with the recent trend towards the consumption of natural products rather than pharmaceutical substances (Nielsen, 2016), both products are sourced from the environment. The thiol-rich supplement is a novel keratin protein (KER) sourced from chicken feathers, which can be consumed in protein powder or bar form. The flavonoid-rich supplement is pomegranate extract (POMx), a powdered form of pomegranate skin and arils. This section will introduce the two supplements and describe the effects of their use in previous research.

2.4.2 Keratin

Keratins are fibrous, high protein (85–99%) compounds, which act as structural components in epidermis, hair, nails, wool, fur, feathers, horny tissues and the organic matrix of tooth enamel (Papadopoulos, 1985). The rigid configuration of keratin-based materials is due to their large thiol component, which forms a tight network of disulfide crosslinks throughout

the compound (Kornitłowicz-Kowalska & Bohacz, 2011). This rich thiol content also suggests that keratins have the potential to be used as exogenous antioxidant supplements if the protein can be converted to a digestible form for humans. Specifically, because keratins are high CYS, a precursor to both GSH and TAU (Figure 2.5), their consumption has the potential to increase the concentration of either or both of these compounds, and subsequently influence O_2 transport (Wolber, McGrath, Jackson, Wylie, & Broomfield, 2016). However, although feather meal is used as a component of pig, sheep and chicken feed, it is poorly digested, (Wang & Parsons, 1997), and has been shown to significantly impair growth, weight gain and food intake when consumed in quantities >8% in animal diets (Van Heugten & Van Kempen, 2002). Keratins are also consumed by humans in animal products, such as skin, chicken feet and pigs' trotters, but based on their low digestibility (~5% *in vitro*), have not previously been suitable for consumption in significant amounts in humans (Kelly & Marsh, 2010; Kornitłowicz-Kowalska & Bohacz, 2011).

Previously, hydrolysis, chemical or enzymatic treatments have been used to destroy the CYS disulfide bonds to improve the bioavailability of keratin from poultry feathers, but such processes tend to also reduce the concentration of amino acids, particularly CYS (Kornitłowicz-Kowalska & Bohacz, 2011; Papadopoulos, 1985). Recently, a proprietary minimal hydrolysis process (patent application number US 13/381,766) has been developed, providing a means of breaking down the keratin proteins from feathers into fragments of lower molecular weight, while still maintaining the fibrous structure and high degree of integrity of the original proteins. Briefly, this process involves oxidizing insoluble keratin at a low pH with heat, and then raising the pH to form a solution of high molecular weight proteins in a low salt solution (Kelly & Marsh, 2010). The process results in ~97% of the protein's CYS content being converted to cysteic acid, which cannot be incorporated

into GSH but is a direct precursor to TAU (Houlthham, 2014). The novel product has an 84–90% digestibility *in vitro* (Wolber et al., 2016) and has been shown to be safe and palatable when consumed by humans in quantities up to 40 g (Houlthham, 2014).

An initial study conducted on Sprague–Dawley rats, provided evidence that KER may indeed have the potential to influence O_2 transport through the provision of thiols (Wolber et al., 2016). Over a four-week period, the replacement of 50% of a casein protein diet with KER (92 g KER per 1 kg feed) maintained body weight, was sufficiently digestible (~78%), and improved total CYS status. The diet had no detrimental effect to rat health and there were no adverse or toxic effects observed. Significantly, consumption of KER was also associated with increases in Hct (+19 $g.L^{-1}$) and Hb (+7 $ml.L^{-1}$), indicating that KER may have had a protective effect on erythrocytes. However, while these changes indicate that KER may improve O_2 transport capacity in rats, it has not yet been investigated whether the same effects can be induced by KER in humans.

2.4.3 Pomegranate extract

The pomegranate (*Punica granatum*) is a seeded, red, fleshy fruit of Middle East origin, which has been used historically and in modern research to treat a range of inflammatory conditions (Adams, Zhang, Seeram, Heber, & Chen, 2010; Aviram et al., 2004; Sergent, Piront, Meurice, Toussaint, & Schneider, 2010; Shukla, Gupta, Rasheed, Khan, & Haqqi, 2008). More recently, pomegranate products have been proposed as ergogenic aids with the potential to improve O_2 transport due to their high concentration of polyphenols. As described in 2.3.3.2 and 2.3.4.2, supplementation with polyphenol-rich foods is associated with a protective effect on both erythrocytes and NO (Bonarska-Kujawa et al., 2014; Ignarro

et al., 2006). According to the Phenol-Explorer database, which describes the mean polyphenol content values for >450 foods, pure POMJ ranks as the 60th richest dietary source of polyphenols (mg per 100g or mg per 100ml), containing 80–90% ellagitannins and 8–15% anthocyanins, which are both from the flavonoid class (Pérez-Jiménez et al., 2010). Further, POMJ has been shown to have a greater polyphenol content (~3.8 mg.ml^{-1}) than other beverages that have been associated with beneficial effects on O_2 transport and exercise performance, for example, red wine (~3.5 mg.ml^{-1}), Concord grape juice (~2.6 mg.ml^{-1}) and cranberry juice (~1.7 mg.ml^{-1}) (Ignarro et al., 2006). Pomegranate can also be ingested as an extract, allowing the richest sources of pomegranate polyphenols, the arils (seeds) and skin, to be consumed in a concentrated, sugar-free form (Mattiello, Trifirò, Jotti, & Pulcinelli, 2009).

In addition to its high polyphenol content, POMx contains a significant concentration of NO_3^-. As described in 2.2.5, the ingestion of high-NO_3^- BRJ is associated with increased vasodilation, a decrease in the O_2 cost of exercise and improved exercise performance in low to moderately trained individuals, presumably because it increases plasma NO production (Bailey et al., 2009; Bailey, Winyard, et al., 2010; Wylie et al., 2013). Although the NO_3^- concentration of POMx (~1.8 mmol.L^{-1}), as reported by Roelofs et al. (2016) is much lower than that in BRJ (~11mmol.L^{-1}) (Bailey et al., 2009), its combination of compounds to stimulate NO production, and protect it from degradation once produced, may make POMx an ideal supplement for improving the efficiency of O_2 transport.

A number of studies have demonstrated changes in vascular parameters, such as increases in blood vessel diameter and blood flow and a decrease in SBP, following acute or chronic pomegranate intake. However, only two studies have investigated the effect of pomegranate supplementation prior to endurance exercise. Trexler, Smith-Ryan, Melvin,

Roelofs, and Wingfield (2014) found a significant association between acute POMx intake (1000 mg, 30 min prior to exercise) and increased brachial artery blood flow and vessel diameter in active individuals (VO_{2max} = 51.3 $ml.min^{-1}kg^{-1}$). These findings coincided with a significant increase in performance during two treadmill runs to exhaustion at 90 and 100% of peak velocity, although no significant changes in performance were measured at higher running intensities. However, while the combination of increased blood flow and improved endurance exercise performance indicates a potential increase in O_2 transport, neither VO_2, nor local muscle O_2 saturation (SmO_2), were directly measured. In contrast, Trinity, Pahnke, Trombold, and Coyle (2014) found no effect of short-term POMJ consumption (1000 $ml.d^{-1}$, 7d) on BP, SV, Q or HR during cycling exercise at 40–100% VO_{2max}, or during a ten minute time trial in hot conditions (31.5^{o}C, 55% relative humidity). In addition, this study showed no changes in performance during either the time trial (TT) or a TTE protocol at 100%VO_{2max} (Trinity et al., 2014). However, rather than using a protocol based on a linear factor, in which the participant increases their work output by increasing their pedaling cadence, this study used a set power output (90% VO_{2max}) which the participant could request to be increased or decreased every thirty seconds after the first two minutes of the test. Protocols of this type do not hold the same ecological validity as a time trial in which the participant can control their work rate at all stages during the test (Jeukendrup, Saris, Brouns, & Kester, 1996). Thus, it is possible that the use of such a protocol may have contributed to the lack of change observed between treatment conditions in that portion of the study.

Further evidence for the potential benefits of pomegranate products on O_2 transport has been shown by studies involving muscle-damaging resistance exercise. When consumed for 15 days prior to completion of eccentric elbow flexor and leg extensor exercises, POMJ was

associated with a faster isometric strength recovery and reduced post-exercise muscle soreness. Although no measurement of O_2 or erythrocyte parameters occurred in that study, these effects were suggested to result from faster transport of O_2 and nutrients to the fatigued muscles (Trombold, Barnes, Critchley, & Coyle, 2010). In support of this proposal, another study involving resistance exercise and repeated maximal cycling sprints found that an acute dose of POMx (1000 mg) prior to exercise was associated with an increase in post-exercise blood vessel diameter and blood flow. That study did not measure post-exercise strength recovery, but found a significant improvement in the fifth of six sprint repetitions. This provided weak evidence that the changes in blood flow had allowed faster recovery between sprints, allowing improved performance in the later repetitions, presumably by enabling a greater rate of creatine phosphate replenishment (Roelofs et al., 2016). However, given that no significant differences were observed between groups in the final sprint reduces the strength of this finding.

Based on these endurance and resistance exercise-based studies, it is clear that further research is required to determine whether POM products have an ergogenic effect on O_2 transport. In particular, research involving measurements of VO_{2max} and local muscle oxygenation are needed to determine whether any effects of pomegranate on blood parameters translate to changes in O_2 usage.

2.4.4 In summary

KER and POMx are two antioxidant-containing supplements with the potential to improve O_2 transport by limiting the negative effects of RONS on erythrocytes, and enhancing the bioavailability of NO. KER supplementation has not previously occurred in humans, and only

two studies have investigated the intake of pomegranate products in endurance athletes. Thus, the current thesis aims to investigate whether either of these supplements can indeed be used as an ergogenic aid to improve VO_2 efficiency and subsequently increase endurance exercise performance.

2.5 HYPOTHESES

Study 1

Hypothesis 1: Chronic KER supplementation by highly-trained cyclists will increase plasma Hb concentration through a reduction in oxidative damage, thereby improving the O_2-carrying capacity of the blood.

Hypothesis 2: Chronic KER supplementation will improve the efficiency of O_2 transport during cycling exercise, resulting in increased VO_{2max} values, and reducing the VO_2 required to perform given intensities of submaximal cycling exercise.

Study 2

Hypothesis 3. Acute polyphenol supplementation by highly-trained cyclists will increase NO bioavailability and subsequently reduce the VO_2 required to perform given intensities of submaximal cycling exercise.

Hypothesis 4. The decreased O_2 cost of submaximal exercise will allow improved performance in a subsequent cycling time to exhaustion protocol.

Hypothesis 5. O_2 uptake and exercise performance will be more greatly influenced by polyphenol supplementation when taken in high altitude (low PO_2) conditions due to the reduced O_2 transport capacity in such environments.

Study 3

Hypothesis 6. Short term (eight days) polyphenol supplementation will be more effective in influencing VO_2 and exercise performance than acute supplementation.

Hypothesis 7. The effects of thiol and polyphenol supplements on VO_2 will be additive when taken together due to their complementary protective actions on NO.

CHAPTER 3. THESIS RESEARCH DESIGN

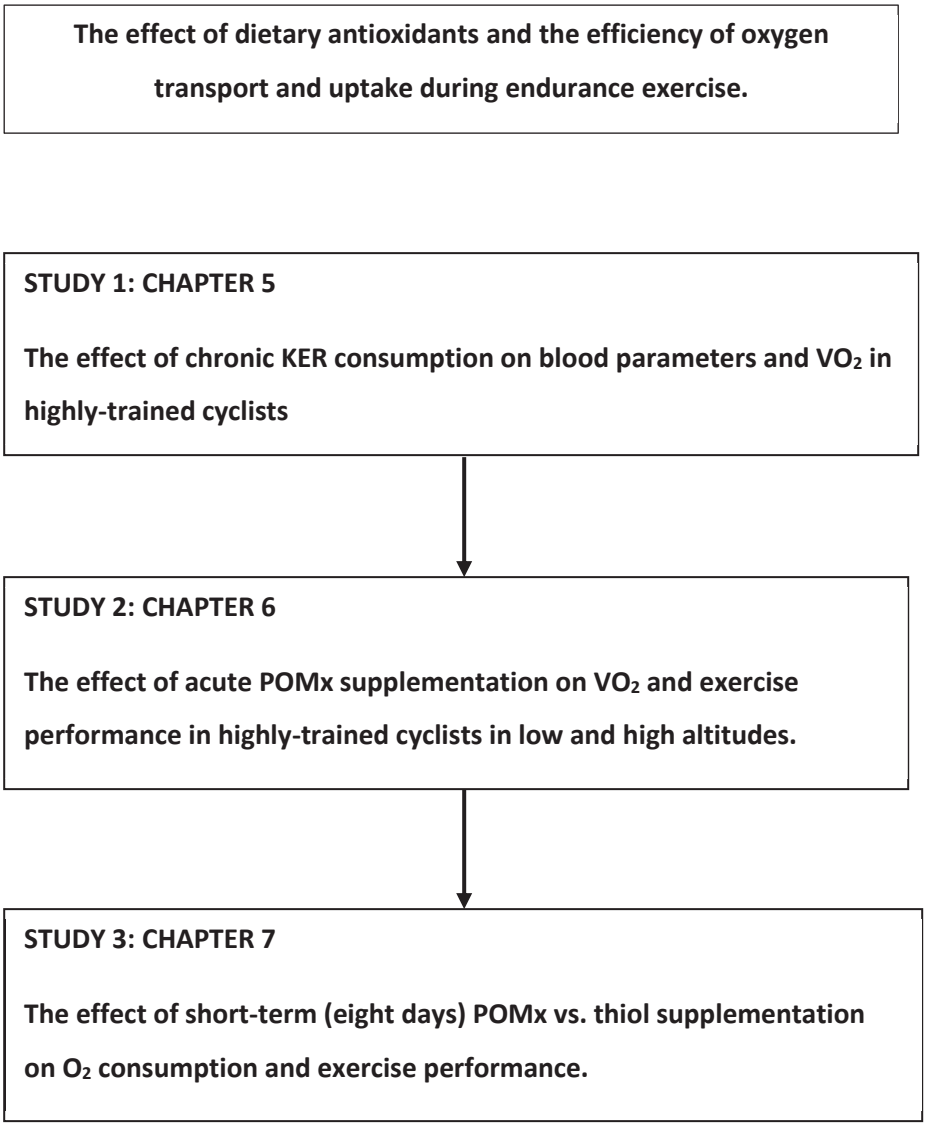

Figure 3.1 Thesis research design

CHAPTER 4. GENERAL METHODOLOGY

The purpose of this chapter is to describe the experimental methodology that was utilized throughout all of the subsequent experimental chapters. These methods were used in one or more experimental chapters. The recording intervals and additional procedures used within each individual study are outlined in the specific experimental chapter.

4.1 EQUIPMENT

4.1.1 Cycle ergometer

All exercise protocols were completed on an electronically-braked ergometer (Lode Excalibur, Groningen, The Netherlands). The Lode is considered to be the gold standard of ergometers, with previous research demonstrating its large reproducibility during cycling time trials and incremental exercise tests (Driller, 2012; Earnest, Wharton, Church, & Lucia, 2005). Two modes of ergometry operation were used during the studies in this thesis. Firstly, in hyperbolic mode, the Lode is set at a particular power output, and work done is independent of cadence. Secondly, in linear mode, the Lode acts like a mechanically-braked ergometer, in which a higher cadence results in a greater power output, as determined by the following equation (Jeukendrup et al., 1996).

$$\text{Work} = \text{Lin}*(\text{rpm})^2$$

where Lin is linear factor and rpm is pedaling cadence (revolutions per minute).

4.2 PRE-STUDY MEASURES

4.2.1 Consent form

Following expression of interest in a study, participants were provided with an information sheet, which detailed the procedures, measurements and requirements of the study, as well as their rights as a participant (see Appendix A.1 for an example). They were asked to read the information thoroughly, and ask questions if they did not fully understand any part of the study. They also completed a health questionnaire to determine whether they were eligible to complete the study (Appendix A.3). After this, they signed a consent form (Appendix A.2).

4.2.2 Anthropometric characteristics

At the beginning of each study, the weight and height of each participant was measured using a standard scale (Jadever, Taiwan; accurate to 0.01 kg) and stadiometer (Seca, Germany; accurate to 0.1 cm).

4.2.3 Body composition

In Study 1, Dual-energy X-ray Absorptiometry (DEXA, Discovery, Hologic, Massachusetts) was used to measure total body mass, lean muscle mass, fat mass and body fat percentage. The participant lay supine on a table, while a scanner emitting x-rays passed over them for approximately three minutes. As these beams pass into the tissue, they gradually lose energy in a process known as attenuation, with the amount of attenuation depending on the composition of the tissue they travel through. The DEXA divides the body into a series

of pixels and compares the amount of attenuation to known reference values for fat, bone mineral and lean soft tissue for each section using computer software (APEX Software Version 4.5.3) (Plank, 2005). This method is commonly accepted as the gold standard of body composition measurement and is used as a reference method for testing of other body composition systems (Svantesson, Zander, Klingberg, & Slinde, 2008). In addition, the Hologic Discovery model was shown to maintain precision and accuracy over a four year period when tested against a phantom of known bone mineral density (Hangartner, 2007). To ensure consistency between measurements, participants completed this test wearing underwear and a thin robe, and were positioned on the table using their first scan position as a reference.

4.2.4 Recording of diet and training

4.2.4.1 Training peaks

During periods where recording of training was required, participants used the online software, Training Peaks™ (Boulder, Colorado, USA) to detail the mode, duration and distance of any exercise that they completed. Where participants were required to replicate training from a previous intervention, this data could be copied to current calendar dates to give them a daily exercise schedule. An example of a typical recording is shown in Appendix A.4.

4.2.4.2 MyFitnessPal

The online software, MyFitnessPal™ (Under Armour, Baltimore, Maryland) was used for diet recording. Participants input each item of food that they consumed during the required periods, and an associated database determined the daily caloric and macronutrient intakes. Where participants were required to replicate their diet from a previous intervention, a summary of food intake from the previous intervention was printed off and given to them. An example of a typical recording is shown in Appendix A.5.

4.3 EXPERIMENTAL MEASURES

4.3.1 Four-stage, submaximal exercise test

This protocol measured oxygen (O_2) consumption at four submaximal workloads during cycling exercise. Each time this test was completed, it was done on an electronically-braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) in thermoneutral conditions (18–20°C) at sea-level. Following a five-minute warm-up at 100 Watts (W), participants completed four x seven-minute stages of increasing workload (females: 100, 150, 175, 200W; moderately-trained males: 100, 150, 200, 250 W; highly-trained males: 150, 200, 250, 300 W) at a cadence of 80–90 rpm.

Five minutes into each stage, heart rate (HR) was recorded, and a nose clip was applied to prevent nasal breathing. Expired air was collected via a mouthpiece into a Douglas Bag between minutes 5:30–6:30. Immediately after collection, the Douglas Bag was connected to a gas analyser, which had been calibrated immediately prior to the test. In Study 1, a metabolic cart with O_2 and carbon dioxide (CO_2) analyzers (AEI Technologies Inc, Pittsburgh,

USA) was used. This device required a one-point calibration using gas of a known composition (15.01% O_2, 5.01% CO_2). Studies 2 and 3 used a portable gas analyzing system (AD Instruments, Dunedin, New Zealand) which was required for use in the mobile laboratory in Study 2. This analyser required a two-point calibration with room air and a gas of known composition as previously described. Following gas analysis, the volume of air in the bag was determined using a dry gas meter, which used a vacuum to evacuate the air in the bag, while recording the quantity and temperature of the air removed.

Expired ventilation (V_E) and concentrations of O_2 and CO_2 values were used to calculate the volume of inspired air (V_I) using the Haldane transformation, where V_E was corrected for barometric pressure, ambient temperature and atmospheric water saturation. Subsequently, VO_2 and expired CO_2 (VCO_2) could be determined and are reported as standard temperature and pressure dry (STPD). VO_2 values were also adjusted for body weight (BW) to allow for better relative comparison between time points.

4.3.2 Incremental exercise test for maximal oxygen uptake determination

At the completion of the four-stage, steady-state exercise test, participants had a five-minute active rest period, in which they cycled lightly at 50 W. Subsequently, the ergometer was connected to a Lode-controlled 'ramp' protocol, which began at 100 W and then increased linearly with time (females: 20 $W.min^{-1}$; males: 25 $W.min^{-1}$). Participants continued the exercise for as long as possible and verbal encouragement was given to elicit maximal effort. Volitional fatigue was defined as a cadence of <50 rpm or voluntary cessation of exercise by the participant. As the participant's maximal O_2 uptake capacity

(VO_{2max}) was approached (as indicated by a change in breathing pattern), expired air was captured in Douglas bags until exhaustion. Expired gases were analysed for V_E, V_I, VO_2 and VCO_2 as described in 2.8.1. Attainment of VO_{2max} was confirmed by a respiratory exchange ratio ≥1.1.

4.3.3 Calculation of submaximal VO_2 values

Data from the exercise tests described in 2.8.1 and 2.8.2 was used to create an equation to allow estimation of the power output required to achieve a given percentage of the participant's VO_2. Using computer software (Microsoft Excel 2011, Redmond, Washington), VO_2 values for each submaximal stage were plotted against power output, to derive the following linear line equation

$$y = m.x + c$$

where $y = \%VO_{2max}$; m = gradient; x = power output; c = start point

4.3.4 Blood pressure

Systolic (SBP) and diastolic blood pressure (DBP) of the brachial artery were measured using an automated sphygmomanometer (Japan Precision Instruments, Gumma, Japan). This device measures BP by detecting the variations in pressure on the arterial wall which occur during systole and diastole. Thus, in all studies in this thesis, the average value of three readings was recorded, as recommended by Skirton, Chamberlain, Lawson, Ryan, and Young (2011).

4.3.5 Heart Rate

HR was recorded using a telemetric chest strap transducer and receiver watch. In such devices, a radio signal is transmitted from electrodes in the chest strap each time a heartbeat is detected (Achten & Jeukendrup, 2003). This method has been shown to record HR values which are within six beats of that measured with an electrocardiogram 95% of the time (Godsen, Carroll, & Stone, 1991). In Study 1, values were recorded from the receiver watch (Polar Electro, Kempele, Finland), at the time they occurred. In Studies 2 and 3, constant HR sampling every two seconds throughout the pre-exercise, exercise and post-exercise periods was recorded using a Garmin device (Garmin Edge 500, Kansas, USA).

4.3.6 Blood lactate

Blood was collected via fingerprick sample and analysed for blood lactate concentration ($[La^-]_b$) using a portable, battery-operated, blood lactate meter (Lactate Pro, Arkray KDK, Japan). The lateral aspect of the tip of the third or fourth finger was pricked with an automated lancet (Accu-Chek, Roche Diabetes Care Inc, Indiana, USA), and the first drop of blood discarded. Subsequently, a ~5 µL blood sample was collected in a coded reagent strip, containing the reagents potassium ferricyanide and lactate oxidase. The reagents react with lactate in the sample to form potassium ferrocyanide and pyruvate, and the lactate meter applies a voltage to the strip, which oxidises ferrocyanide, releasing electrons. The resultant current is measured amperometrically by the device to determine lactate content. In a test-retest protocol, the Lactate Pro was shown to have a high correlation and reliability between repeated measurements of a given blood sample for a wide range of $[La^-]_b$ concentrations (Tanner, Fuller, & Ross, 2010).

4.3.7 Haematocrit

To determine haematocrit (Hct), ~35 µL blood was collected from the fingertip capillaries in heparanized capillary microtubes and immediately spun in a microhaematocrit centifuge (Thermo IEC MB, Bellport, USA) at 14000 rpm for two minutes. The high *g* forces produced during spinning of the sample separates the comparatively heavier erythrocytes from the less dense plasma so that the length of each of the two components can be measured. The white cells (leukocytes), which are less dense than the erythyrocytes, form a 'buffy coat' between the erythrocytes and plasma columns. Hct is calculated as the length of red blood cells as a percentage of the total column of blood in the tube. While this method has low invasiveness and is inexpensive, underestimation of Hct can occur in high Hct samples due to damage of RBC as they move towards the bottom of the column of blood. This leads to the release of water from the cell which drifts upwards and contributes to the length of plasma in the tube, thus causing a slight decrease in Hct (Bull & Hay, 2001). Therefore, when this method was used, samples were taken in duplicate to allow calculation of an average value.

4.3.8 Venepuncture

Blood sampling by venepuncture was conducted according to the guidelines of the Mid Central District Health Board (MidCentral District Health Board, 2010). Standard precautions were followed to ensure the safety of both the participant and researcher, including the use of disposable gloves, alcohol swabs and proper disposal of needles and blood-contaminated products. The sample was collected from a vein in the area of the antecubital fossa of either arm using a needle and vacutainer (BD, New Jersey, USA). Where

whole blood was required, blood was collected in ethylenediaminetetraacetic acid (EDTA) vacutainers (~4 ml), and then immediately transferred to a fridge (~2^{o}C) and analysed within 48-hours as described in 2.9.1. Where plasma was required, blood was collected in heparanized vacutainers (~4–10 ml) and spun in a centrifuge (Eppendorf AG, Hamburg, Germany) within five minutes of collection at 2500 rpm for 12 minutes at 4^{o}C. Plasma was transferred into epindorphs and stored at -80^{o}C until analysis.

4.3.9 Muscle oxygen saturation and total haemoglobin content

A Moxy muscle O_2 monitor (Moxy, Fortiori Design LLC, Minnesota, USA) was used to measure local muscle O_2 saturation (SmO_2) and total haemoglobin (THb) in the capillaries of the *vastus lateralis* muscle. The validity and reliability of this device was measured as a side study to this thesis and is presented in Appendix F.1. The Moxy functions by sequentially sending light waves (630850 nm) from four light emitting diodes into the tissue and recording the amount of returning scattered light at two detectors. The penetration depth of the light received at each detector is half the distance between the light source and the detector. The scattered light is processed by an algorithm, which combines a tissue light propagation model, and the Beer-Lambert law to determine the amount of light absorbed at wavelengths pertaining to oxyHb and HHb. THb is the sum of oxyHb and HHb, and SmO_2 is the relative percentage of oxyHb compared to THb. The values obtained reflect the relative supply of O_2 to the muscle versus its uptake at a point in time. Because light passing into micro-vessels with a diameter greater than 1 mm is expected to be completely absorbed, it is assumed that the majority of reflected light comes from the capillaries, and thus the measurements obtained reflect the relative supply of O_2 to the muscle versus its uptake (McCully & Hamaoka, 2000)

The Moxy was placed on the *vastus lateralis*, halfway between the greater trochanter and the lateral epicondyle of the femur and secured with a light shield and tape to block ambient near-infrared light from interfering with the detectors (see Figure 4.1). This muscle group was chosen as the location for the Moxy because it is part of the knee extensor group which is the primary contributor to force generation in the crank of the bicycle during down stroke of the pedal (Raasch, Zajac, Ma, & Levine, 1997). Data was recorded every two seconds throughout each trial.

Figure 4.1 Placement of the Moxy monitor. The device was positioned on the *vastus lateralis*, halfway between the greater trochanter and the lateral epicondyle.

4.3.10 Rating of Perceived Exertion scale

The Rating of Perceived Exertion (RPE) scale (Borg, 1998) (see Appendix A.6) was used to assess perceived effort during exercise. Participants ranked themselves on a numerical scale ranging from 6 (no exertion) to 20 (maximal exertion).

4.3.11 Health/side effects questionnaire

A health/side effects questionnaire was used to monitor for any adverse effects of supplementation (see Appendix A.7). This included questions pertaining to stomach and gut habits, abdominal discomfort and general health problems.

4.4 BLOOD ANALYSES

4.4.1 Full Blood Count

Whole blood samples were analysed by full blood count, using a fully-automated bench-top, five-part differential haematology analyser (LH 500, Beckman Coulter, Fullerton, CA, USA). The LH500 analyser has previously been validated against other such devices and with the haemoglobincyanide method, which is the reference method recommended by the International Committee for Standardization in Haematology (Kaplan et al., 2003; Zwart et al., 1996). The analyser was calibrated prior to use as per the manufacturer's recommendations and had shown acceptable performance on proficiency testing.

The full blood count analysis determines the number and volume of cells in whole blood by detecting changes in electrical resistance when a particle passes through a small aperture,

increasing the resistance of the electrical path between submerged electrodes and causing a measurable electronic pulse. The pulse count indicates the number of particles passing through, and the height of the electrical pulse indicates the cell volume. The specific erythrocyte parameters recorded were total erythrocyte count (RBC), haemoglobin (Hb) volume, haematocrit volume (Hct), mean cell volume (MCV) (the average volume of individual erythrocytes), mean cell haemoglobin concentration (MCH) and mean cell haemoglobin concentration relative to erythrocyte cell volume (MCHC). The total number of leukocytes (WBC) in whole blood was also measured.

4.4.2 Nitrate, nitrite assay

Plasma samples were analysed using a Nitric Oxide Colorimetric Assay Kit (BioVision Incorporated, Milpitas, California, USA) to measure NO_3^- and NO_2^-. This method is a two-step process, in which nitrate reductase is used to convert nitrate to nitrite, and then Greiss Reagents convert nitrite to a deep purple azo compound. Absorbance is read at 540 nm and plotted as a function of NO_3^- and NO_2^- concentration.

CHAPTER 5. CHRONIC CONSUMPTION OF A NOVEL KERATIN SUPPLEMENT

5.1 INTRODUCTION

Keratin proteins are tough, insoluble compounds, which are composed of a tight network of disulfide bonds courtesy of their high thiol content (Korniłłowicz-Kowalska & Bohacz, 2011). Poultry feathers contain ~90% of their total weight as keratin, which gives the material its characteristic light but rigid structure (Grazziotin, Pimentel, De Jong, & Brandelli, 2006). In New Zealand, chickens are widely bred for use in commercial meat production (~82,000,000 birds annually) (Fick, 2012), but their feathers, which represent ~10% of total chicken weight, are indigestible to humans and other monogastrics and are therefore considered a significant biological waste product. To reduce costs of waste disposal, feather meal is commonly used as a component of animal feed (El Boushy & van der Poel, 2013). Yet at dietary concentrations of >8%, its consumption is associated with significant impairments in growth, weight gain and food intake, because despite a favorable amino acid profile, its low digestibility renders much of the amino acids unavailable (Van Heugten & Van Kempen, 2002). However, if digestibility is improved, the high thiol content in keratin may set it up as a potential dietary antioxidant (McLeay et al., 2017). Cysteine (CYS) the predominant thiol in keratin is a precursor to the major endogenous antioxidants, glutathione (GSH) and taurine (TAU), which form a key part of the body's defense against oxidative stress (Brosnan & Brosnan, 2006; Shimada et al., 2015). As previously mentioned

(see 2.3.3), a thiol-induced reduction in oxidative stress has the potential to increase the efficiency of O_2 transport and uptake (VO_2).

Conventional methods to improve digestibility have included hydrolysis, chemical and enzymatic processing treatments (Korniłłowicz-Kowalska & Bohacz, 2011; Papadopoulos, 1985). However, during the cleaving of disulfide bonds, such methods also reduce the thiol content of keratin, causing the new product to be unsuitable as a dietary antioxidant (Papadopoulos, 1985). In addition, the oxidation processes involved in many previous methods of keratin extraction result in the production of peptides with strong off-tastes and odours, making the new product unpalatable (Kelly & Marsh, 2010).

However, recently a proprietary controlled hydrolysis process has been developed using poultry feathers, which produces a novel keratin product (KER) with a digestibility of ~83% *in vivo*, while maintaining a thiol component of ~87%. During this process, ~97% of the CYS in keratin is converted to cysteic acid, which cannot be converted to GSH, but is a direct precursor to the antioxidant, taurine (TAU) (Houlthham, 2014). The procedure yields a protein powder which can be mixed with liquid and ingested as a drink, or formulated into a protein bar (Kelly & Marsh, 2010). This product has been demonstrated to be safe and palatable when consumed in quantities of up to 40 g per day (Houlthham, 2014). Further, although the use of KER as a dietary antioxidant and ergogenic aid has not been previously investigated in humans, an initial rat study (Wolber et al., 2016) has indicated that KER may indeed enhance O_2 transport through its effects on blood parameters. Following the substitution of 50% of a casein protein diet with KER in male Sprague-Dawley rats over a four-week period, blood profiles exhibited significant increases (~5%) in haematocrit (Hct) and haemoglobin content (Hb). Further, the protein had a ~78% digestibility and increased the total CYS content absorbed by ~11% compared to a casein-only diet.

Considering the improvements in erythrocyte parameters in rats, the Wolber et al. (2016) study suggests that KER has the potential to also provide a small but significant improvement in the O_2 transport capacity of humans. The current study was designed to investigate the hypothesis that the chronic consumption of KER over a four-week period in highly-trained cyclists would increase the quantity of erythrocytes and their associated Hb proteins available for O_2 transport. Further, we investigated whether this would increase maximal O_2 uptake capacity (VO_{2max}) or improve VO_2 efficiency during submaximal cycling exercise.

5.2 METHODS

The current study formed part of a larger study, which investigated a range of potential benefits of KER supplementation. Information on the effects of KER on antioxidant defense and oxidative damage will be detailed in a doctoral thesis by McLeay.

5.2.1 Participant characteristics

Fifteen trained male cyclists, aged 18-55 years, were recruited from the regional cycle and triathlon communities. The criteria for inclusion was regular participation in endurance cycling exercise, at least three times per week in the three months prior to commencement of the study, absence of any contraindications to exercise or other procedures involved in the research, or allergy/objection to the consumption of dairy or animal products. The participants' baseline characteristics are presented in Table 5.1. Prior to participation in the study, participants were notified of all the potential risks and benefits associated with the study, and written and verbal consent were obtained, as described in 4.2.1. This study

was approved by the Massey University Human Ethics Committee (Southern A 14/49, in accordance with the Declaration of Helsinki.

Table 5.1 Baseline characteristics of participants in Study 1

Participant	Age (years)	Mass (kg)	Height (m)	% Body fat	VO_{2max} ($ml.min.kg^{-1}$)
1	39	103.2	1.92	23.4	56.2
2	42	84.5	1.85	18.9	65.7
3	47	100.0	1.80	27.1	53.4
4	41	75.7	1.80	23.8	47.0
4	18	68.2	1.67	17.9	63.4
5	37	119.2	1.86	33.1	53.4
6	18	71.2	1.76	13.6	64.5
7	28	70.9	1.75	18.7	52.4
8	40	93.6	1.68	24.6	52.8
9	55	80.0	1.82	21.6	52.8
10	41	86.0	1.78	26.7	60.0
11	22	82.2	1.81	18.1	81.3
12	18	68.2	1.67	17.9	67.0
13	39	81.5	1.76	20.3	61.3
14	27	78.6	1.87	16.1	71.9
Average ± SD	34 ± 11	84.2 ± 13.9	1.79 ± 0.7	21.5 ± 4.9	60.2 ± 8.7

5.2.2 Pre-study measurements

Anthropometric and body composition data was measured using the methods outlined in the general methodology (4.2.2, 4.2.3)

5.2.3 Study design

The study was a randomized, blinded, crossover design, with two four-week intervention periods (INT1: intervention 1; INT2: intervention 2) involving supplementation with either KER or a placebo ("gold standard" sodium caseinate, CAS), which were completed in a randomized order in a crossover study design. The interventions were separated by a minimum of eight weeks to allow wash-out of any remaining traces of the supplement from the participant. At the beginning of each intervention, participants completed two sessions of exercise testing on consecutive days. The first session was a four-stage submaximal exercise test, followed by a ramp protocol, to determine VO_{2max}. The second session was a two-hour steady state cycle with pre- and post-exercise blood collection. Supplementation began one-week after the exercise testing, and continued for four weeks. At the end of the supplementation protocol, the two days of exercise testing were repeated. A diagram showing the timeline for one intervention is shown in Figure 5.1

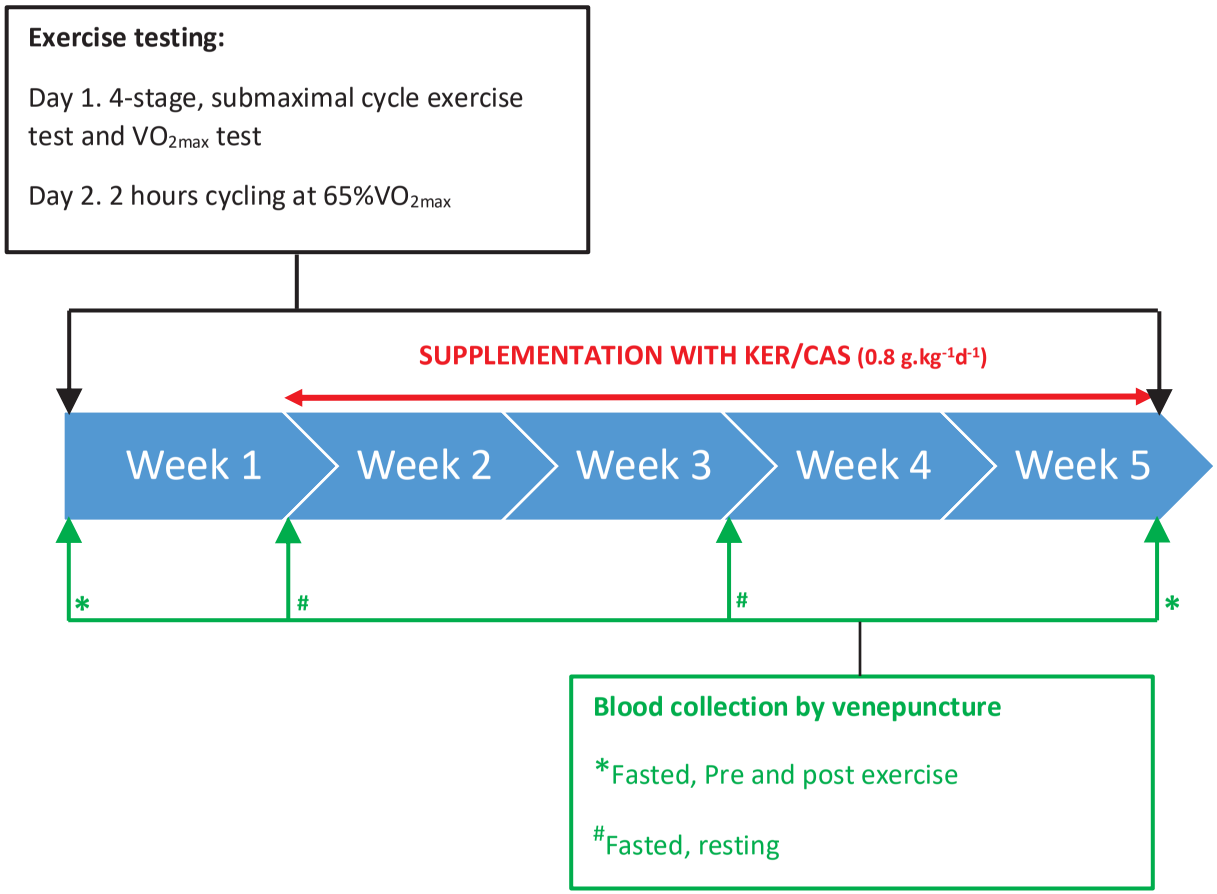

Figure 5.1 Timeline of study 1

5.2.4 Supplementation

During each intervention, participants consumed 0.8 g protein per kg bodyweight, per day ($g.kg^{-1}d^{-1}$), for six days per week, with one non-supplementation day each week. The supplement was consumed as two protein bars (of two different flavours), and the remainder of the protein requirement in powder form, which was mixed with water to form a drink. A breakdown of the macronutrient content of the supplements is provided in Table 5.2**.** A more detailed breakdown of supplement ingredients is provided in Appendix B. Participants received one week's worth of supplements at the beginning of each week during the supplementation period, and filled out a weekly health/side effects questionnaire (see 4.3.11) to monitor for any adverse effects of supplementation.

The novel KER supplement was supplied by Keraplast Research Ltd (Christchurch, New Zealand). KER was produced from the feathers of white leghorn chickens via a patented minimal hydrolysis process (patent application number US 13/381,766), which is described in detail by Kelly and Marsh (2010). Briefly, this involved treating keratin in an oxidizing solution at a low pH, and then heating the mixture to oxidize the CYS residues in the protein. Subsequently, the mixture was cooled and a base was added to raise the pH of the solution to form a protein salt. The solution was dried and milled to form a protein powder. The CAS supplement was purchased from Tatua (Morrinsville, New Zealand), and formed into a protein powder and bar of similar taste and protein content to KER by Keraplast Research Ltd.

During the three weeks preceding, and throughout the duration of each trial, participants were asked to refrain from taking any form of supplement, including other protein powders

or bars. Any prescribed supplements and/or medication were discussed with the researchers to deem whether they would have any potential effect on study measures.

Table 5.2 Macronutrient content of KER and CAS supplements.

Supplement	Energy (kJ/100 g)	Protein (g/100g)	Carbohydrate (g/100g)	Fat (g/100g)
KER powder	1536.9	69.0	17.7	1.7
CAS powder	1589.2	72.1	17.7	1.7
KER bar (banana)	1285.3	31.0	35.5	4.2
CAS bar (banana)	1306.1	32.2	35.5	4.2
KER bar (peanut)	1776.8	40.1	40.0	11.2
CAS bar (peanut)	1717.1	36.6	40.0	11.2

Supplements were consumed as two bars + protein powder totaling 0.8 g.kg^{-1} per day.

5.2.5 Exercise protocols

All exercise testing was completed in a fasted condition, on an electronically-braked ergometer (Lode Excalibur, Groningen, The Netherlands) as described in 4.1.1. A fasted condition was used to ensure consistency in blood glucose levels between trials. The four-stage, submaximal exercise test, followed by an incremental ramp to exhaustion were completed as described in the general methodology to calculate the volume of oxygen

consumed (VO_2) and carbon dioxide expired (VCO_2) during each submaximal stage and at exhaustion (VO_{2max}, VCO_{2max}) (see 4.3.1 and 4.3.2). This data was used to calculate the power output which would elicit ~65% of the participant's VO_{2max} (see 4.3.3).

On the day after the VO_{2max} test, participants completed a steady-state, constant-load exercise test involving two hours of cycling at 65%VO_{2max} with a cadence of 90–100 rpm. Perceived exertion (RPE) and heart rate (HR) data was recorded every 15 minutes throughout the exercise, as described in 4.3.5 and 4.3.10. Three participants completed one trial where a VO_{2max} value could not be obtained due to equipment error. For these participants, the equation for prediction of VO_{2max}, developed by the American College of Sports Medicine (Glass, Dwyer, & Medicine, 2007), was used

(1.8 * (PO * 6)/mass) + 7

where PO = power output in watts at exhaustion

5.2.6 Blood collection

Blood was collected by venepuncture as described in the general methodology (see 4.3.8) at the following time points: immediately before each constant-load exercise test, five minutes post each constant-load exercise test, and at the beginning and mid-point (two weeks) of each supplementation period. The post-intervention, pre-exercise blood collections doubled as the post-supplementation resting samples (see Figure 5.1). On every occasion throughout the study where a venepuncture was taken, participants were in

an overnight fasted state, and blood was collected in an EDTA vacutainer (~4 ml). Blood samples were refrigerated and analysed within 24 hours of collection, as described in 4.3.8.

5.2.7 Recording of diet and training

In each intervention, participants recorded their daily training schedule (see 4.2.4.1) and dietary intake (see 4.2.4.2) from one week prior to beginning pre-exercise testing, until the end of their post-exercise testing.

The participants attended two one-hour cycle training sessions per week at the laboratory. The power outputs prescribed for each protocol were determined by calculation of various percentages of the participant's VO_{2max}, as determined by their initial VO_{2max} test. The first session consisted of a 15-minute progressive warm-up, followed by two 15-minute steady state intervals at 80%VO_{2max}; these were separated by ten minutes at 60%VO_{2max} and were followed by a five-minute cool down. The second session involved a 5-minute warm-up followed by intervals of one minute at 85%, 90%, 100% VO_{2max}, 30 seconds at 130% VO_{2max}, then one minute at 100%, 90%, and 85% VO_{2max}. Light active recovery was given between intervals and a five-minute warm-down occurred at the end of the session. In additional to this prescribed exercise, participants completed their own training outside of the laboratory in their own time. To ensure training was consistent across trials, training recorded during INT1 was replicated in INT2.

The participants were asked to follow a similar diet in both interventions, although, it was acknowledged that it was not feasible for the diet to be replicated exactly.

5.2.8 Blood analyses

Blood samples were analysed by full blood count as described in 4.4.1 to determine total erythrocyte count (RBC), Hct, Hb, mean cell volume (MCV), mean cell haemoglobin volume (MCH), mean cell haemoglobin concentration relative to erythrocyte cell volume (MCHC) and total leukocyte count (WBC).

5.2.9 Statistical analyses

Statistical analyses to compare the values of all variables measured were performed using statistical computer software (SPSS Statistics, Version 23, IBM Corporation, New York). Statistical significance was accepted when $p \leq 0.05$, while a tendency was noted when $p \leq 0.1$. The expired gas variables taken during the four-stage submaximal protocol and VO_{2max} tests (VO_2, VCO_2) were analysed using three-way, repeated measures ANOVA to determine the main effects of, and interactions between, treatment (TR; KER vs. CAS), exercise stage (ST; 1-4) and time point during intervention (PREPOST; beginning vs. end). The performance measure from this test (power output at VO_{2max}; PO) was analysed using a two-way (TR x PREPOST), repeated measures ANOVA. For the analysis of HR during the steady-state constant-load exercise test, 30-minute time intervals were assessed using a three-way, repeated measures, ANOVA, which included TR, PREPOST and time point during the test (TIME; 30, 60, 90, 120 minutes).

Blood variables were analysed using a two-way repeated measures ANOVA (TR x PREPOST) for significant differences between variables pre, mid and post-chronic intervention. In addition, a three-way repeated measures ANOVA was used to determine whether any significant differences occurred between the pre- and post-exercise blood variables for the

constant load exercise test (EX; start vs. end of test), and whether this was affected by treatment or time point of intervention (TR x PREPOST x EX).

In all analyses, Mauchly's Test of Sphericity was used to validate the results of each ANOVA and in cases where aspherity was detected, the Greenhouse-Geiser correction was applied. Following each ANOVA, where significant main effects or interactions were observed, two-tailed paired t-tests with a Fisher's Least Significant Difference (Fisher's LSD) post-hoc analysis were used to identify the location of the significance. The effect size (ES) of significant interactions was calculated using Cohen's d. In accordance with Cohen (1988), effect sizes were classified as small (0.1), medium (0.5) or large (0.8). Data is presented as mean ± standard deviation (SD) or mean change and 95% confidence interval (CI) as appropriate. Graphs were produced using Prism 6.0 (GraphPad Software, CA, USA).

5.3 RESULTS

5.3.1 Side effects and blinding

No side effects of either condition were reported, aside from some anecdotal comments of occasional sulfur-smelling flatulence in both KER and CAS. Participants were blinded to the contents of the supplements and only one participant was able to confidently guess which condition they were on.

5.3.2 Diet and Training

Participants attended all compulsory training sessions at the laboratory, and reported that they followed the same training plan in both interventions. Five participants did not fully complete their food diary and were therefore excluded from the dietary analysis. The supplements (KER and CAS) provided to participants did not differ in energy, protein, fat or carbohydrate content ($p > 0.05$). When the supplements were not included, repeated measures ANOVA revealed that there were no significant main effects of treatment on total energy intake from food during both four-week interventions ($F_{1,9} = 0.56$, $p = 0.47$). There was a main effect of treatment on protein intake, with subjects consuming significantly less protein during all four weeks (pooled) of KER (-14.1 g, 95% CI, -26.7, -1.5, $F_{1,9} = 6.38$, $p < 0.05$, ES = 0.05).

5.3.3 Blood variables

The blood parameter values for pre, mid and post-supplementation with CAS and KER are presented in Figure 5.2, Figure 5.3 and Figure 5.4. The values for before and after the constant-load exercise test, pre and post supplementation with CAS and KER are presented in Figure 5.5, Figure 5.6 and Figure 5.7. The analysis of pre and post-exercise blood samples from one participant during one period of testing in the study did not measure Hct or MCHC, and that participant was consequently excluded from the analysis of pre/post exercise samples.

There were no significant main effects of treatment on RBC ($F_{1,14} = 0.616$, $p = 0.45$), Hb ($F_{1,14} = 0.645$, $p = 0.44$), Hct ($F_{1,14} < 0.0001$, $p = 0.99$), MCV ($F_{1,14} = 0.716$, $p = 0.41$), MCH ($F_{1,14} = 0.182$, $p = 0.68$), MCHC ($F_{1,14} = 1.02$, $p = 0.33$) or WBC ($F_{1,14} = 1.44$, $p = 0.25$) during the

supplementation period. There were also no main effects of PREPOST on RBC ($F_{1,14}$ = 2.37, p = 0.11), Hb ($F_{1,14}$ = 0.683, p = 0.51), Hct ($F_{1,14}$ = 1.86, p = 0.18), MCH ($F_{1,14}$ = 0.086, p = 0.92), MCHC ($F_{1,14}$ = 0.235, p = 0.79), MCV ($F_{1,14}$ = 0.547, p = 0.59) or WBC ($F_{1,14}$ = 0.680, p = 0.52).

The analysis of pre and post-exercise blood parameters (data not presented) did not indicate any changes in response to exercise due to TR, or any significant TR x PREPOST, TR x TIME or TR x EX interactions present for any of the variables (p > 0.05). There was a significant main effect of exercise, with post-exercise increases in RBC (+0.2*10^{12}/L, 95% CI, 0.1, 0.2, $F_{1,14}$ = 27.1, p < 0.0001, ES = 0.7), Hct (+0.1%, 95% CI, 0.1, 0.2, $F_{1,13}$ = 35.6, p < 0.0001, ES = 0.7) and WBC (+4.3*10^{9}/L, 95% CI, 2.8, 5.7, $F_{1,14}$, 39.6, p < 0.0001, ES = 1.8), and a decrease in Hb (-0.41 g.L^{-1}, 95% CI, -3.3, 2.5, $F_{1,14}$ = 40.6, p < 0.0001, ES = 0.8) compared to pre-exercise values. There were no significant effects of exercise on MCH ($F_{1,14}$ = 0.35, p = 0.11), MCHC ($F_{1,13}$ = 1.81, p = 0.20) or MCV ($F_{1,14}$ = 0.24, p = 0.63).

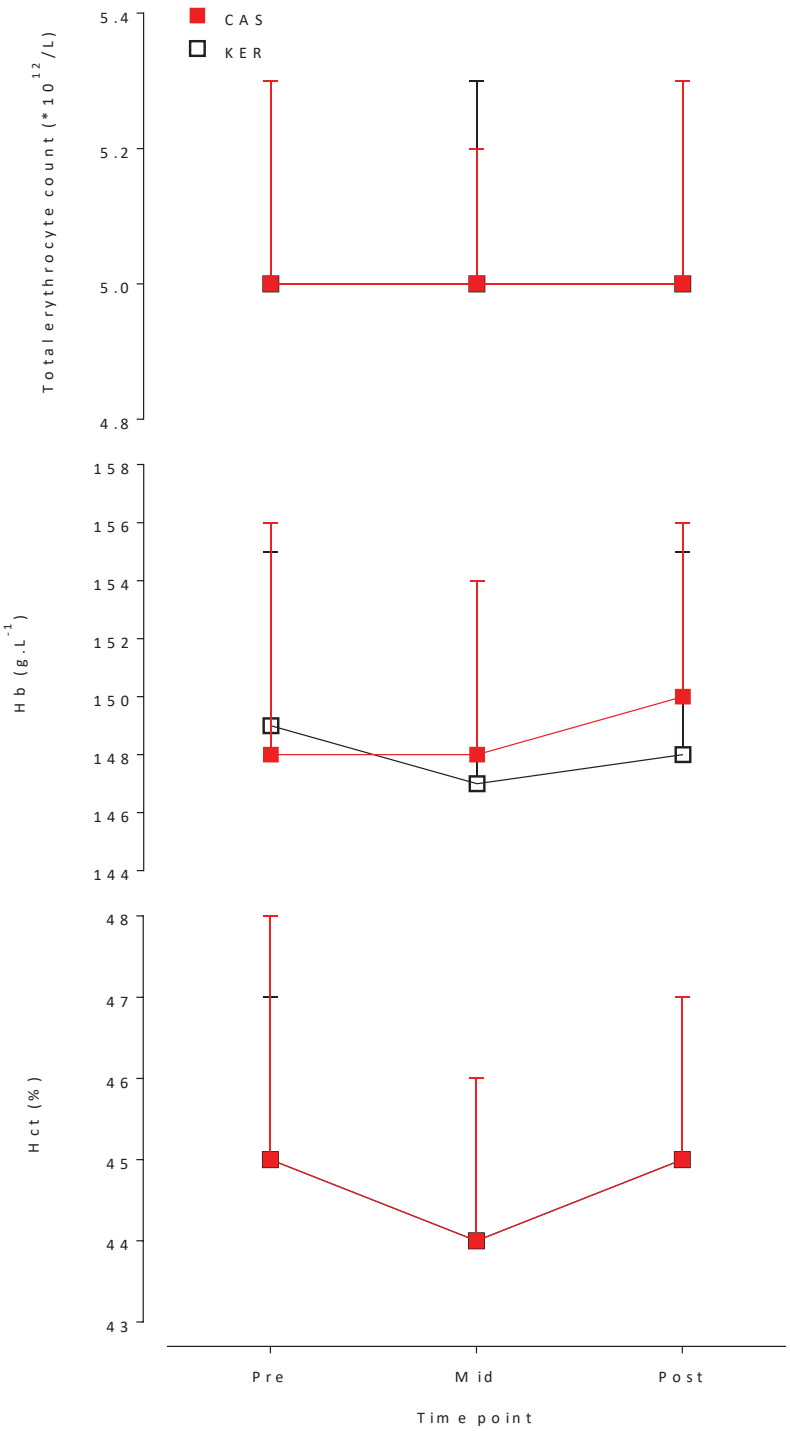

Figure 5.2 RBC, Hb and Hct pre-, mid- and post-supplementation with CAS and KER proteins for 4 weeks. Values are means ± SD, n = 14 (RBC, Hb) or 13 (Hct).

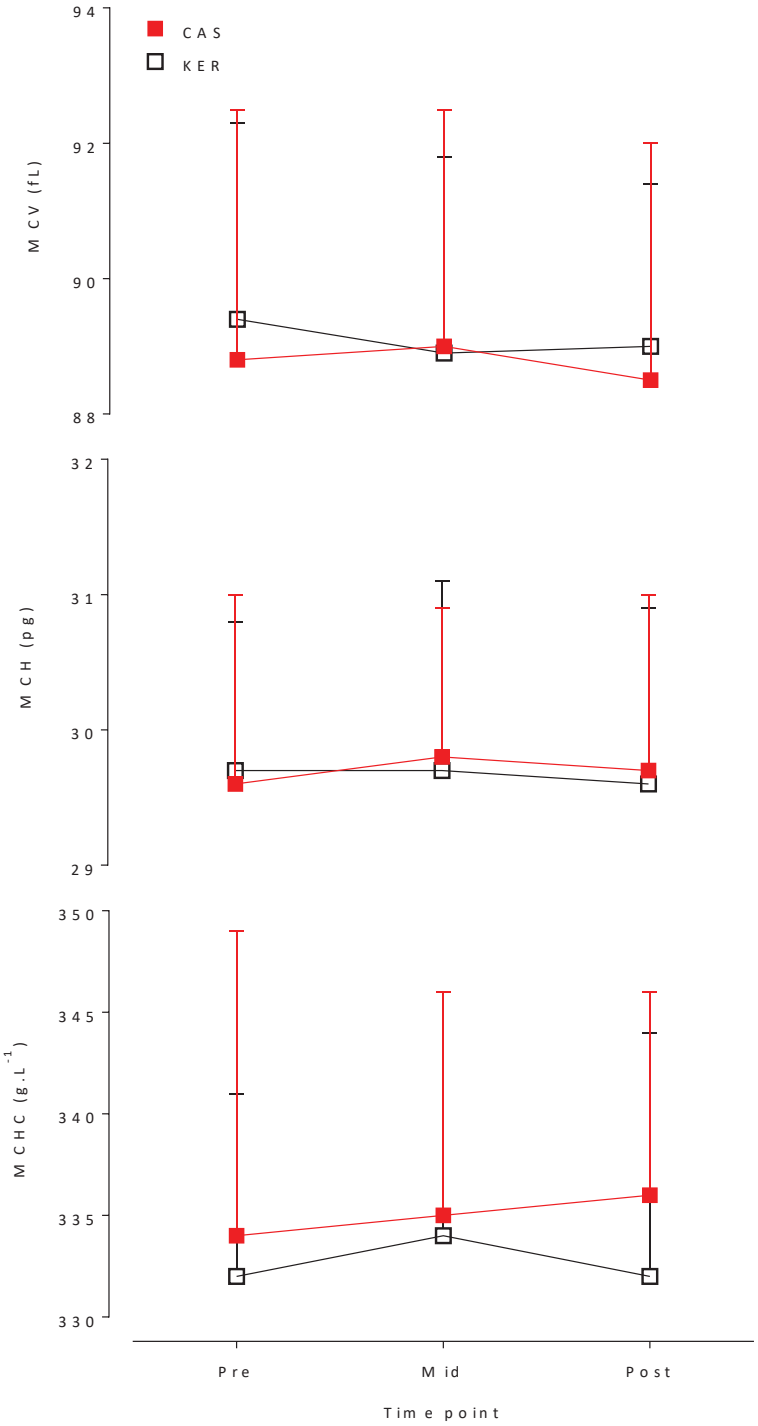

Figure 5.3 MCV, MCH and MCHC pre-, mid- and post-supplementation with CAS and KER proteins for 4 weeks. Values are means ± SD, n = 14 (MCV, MCH) or 13 (MCHC).

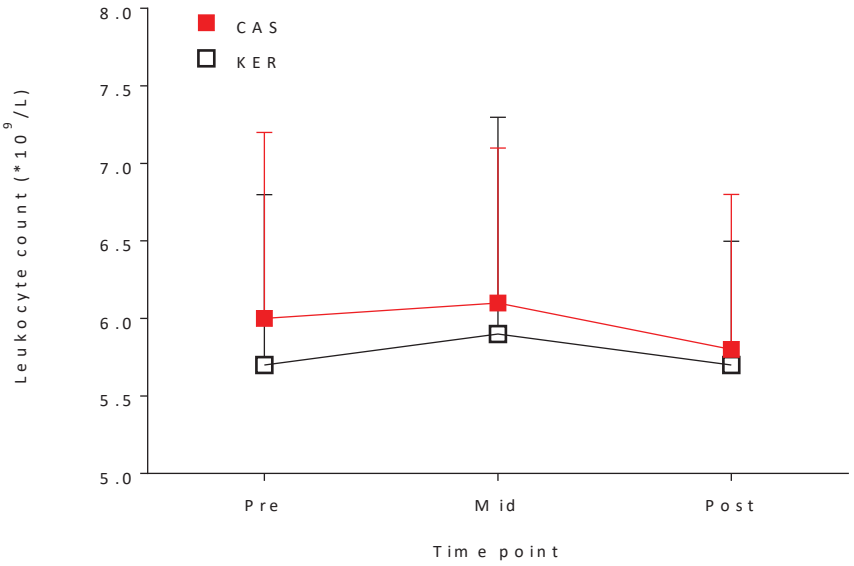

Figure 5.4 WBC pre-, mid- and post-supplementation with CAS and KER proteins for 4 weeks. Values are means ± SD, n = 14.

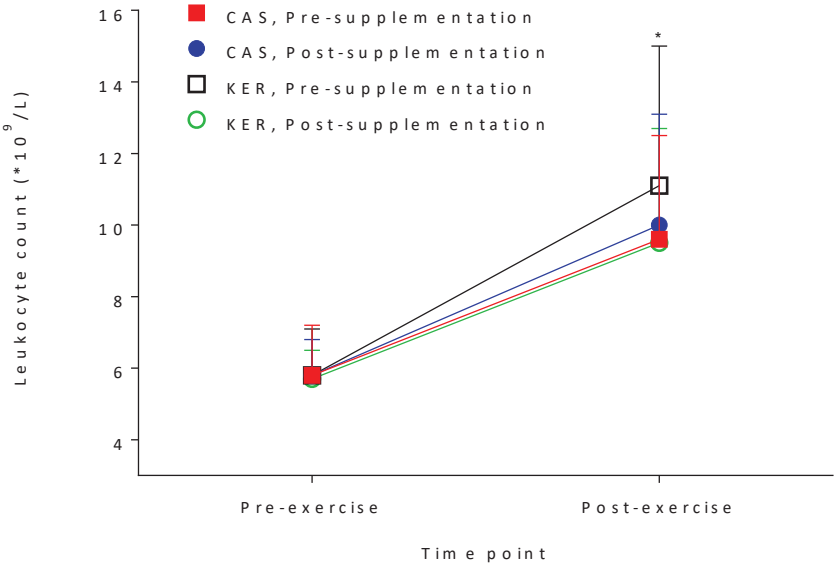

Figure 5.5 WBC pre and post the steady-state submaximal exercise test, pre and post supplementation with CAS and KER proteins for 4 weeks. Values are means ± SD, n = 14. * indicates $p \leq 0.05$ compared to pre-exercise for both treatments at both time points.

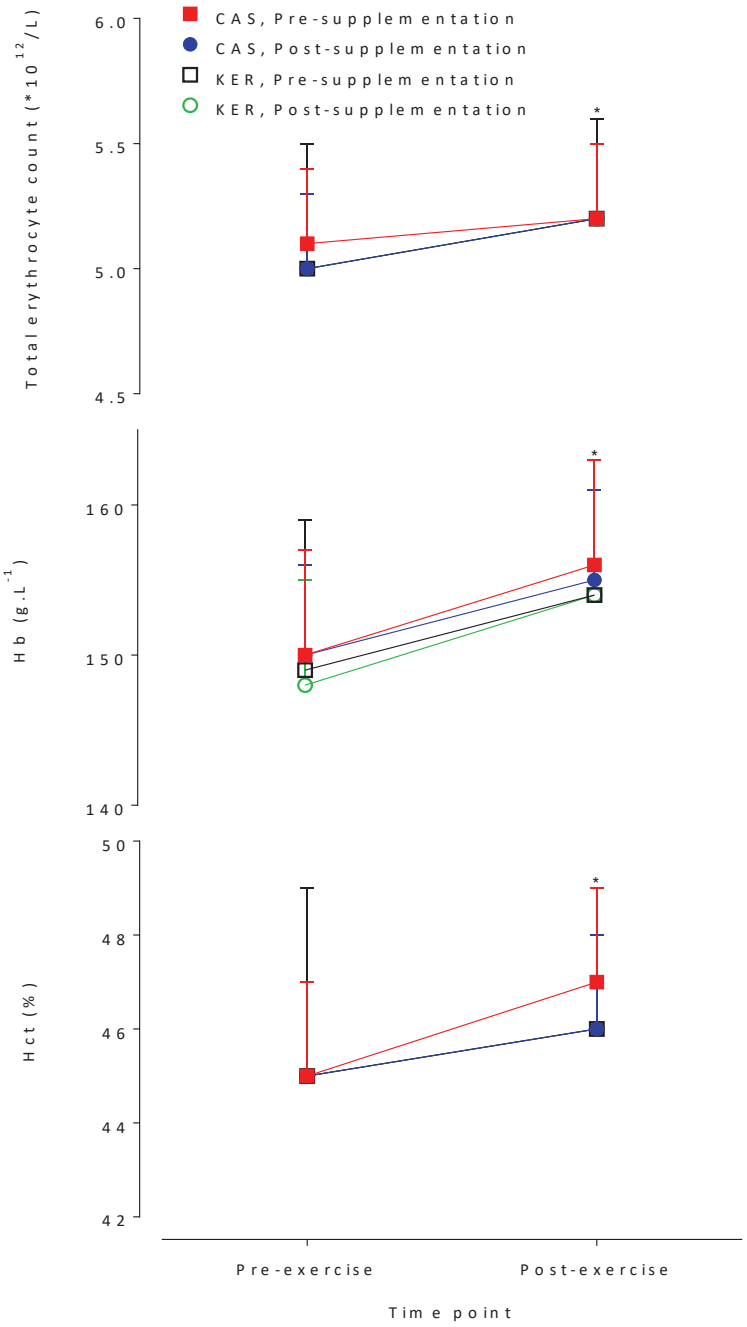

Figure 5.6 RBC, Hb and Hct pre and post the steady-state submaximal exercise test, pre and post supplementation with CAS and KER for 4 weeks. Values are means ± SD, n = 14 (RBC, Hb) or 13 (Hct). * indicates $p \leq 0.05$ compared to pre-exercise for both treatments at both time points.

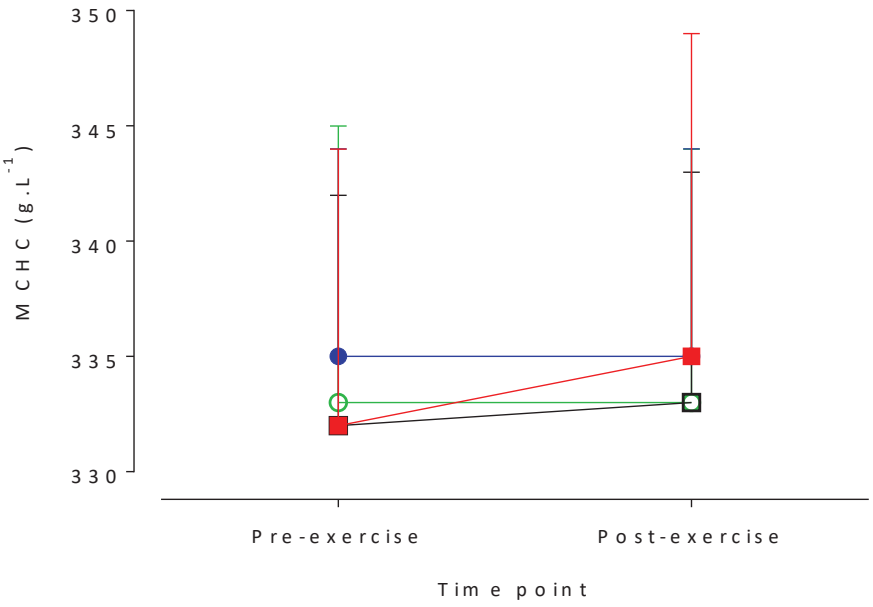

Figure 5.7 MCV, MCH and MCHC pre and post the steady-state submaximal exercise test, pre and post supplementation with CAS and KER for four weeks. Values are means ± SD, n = 14 (MCV, MCH) or 13 (MCHC).

5.3.4 Cardiorespiratory data

The cardiorespiratory data is presented in Figure 5.8 and Figure 5.9. Two participants had incomplete data for the submaximal part of the test, and three had incomplete data for the VO_{2max} test and were therefore excluded from the corresponding part of the analysis. The four stages during the steady-state exercise test corresponded to ~42, 54, 65 and 73% of the participants' initial VO_{2max}.

There were no main effects of TR on VO_2 ($F_{1,13}$ = 2.70, p = 0.13) or VCO_2 ($F_{1,13}$ = 0.12, p = 0.73) during the four-stage steady-state submaximal exercise or in VO_{2max} ($F_{1,12}$ = 1.48, p = 0.25) or VCO_{2max} ($F_{1,12}$ = 0.73, p = 0.411) during the VO_{2max} test (Figure 5.8). Further, there were no significant changes from pre to post-intervention in VO_2 ($F_{1,13}$ = 0.53, p = 0.48), VCO_2 ($F_{1.13}$ = 0.77, p= 0.52) or VCO_{2max} ($F_{1,12}$ = 0.006, p = 0.94) (Figure 5.9). There was a trend

towards an increase in VO_{2max} post-intervention compared to pre-intervention ($F_{1,12}$ = 3.67, p = 0.08)

There was a significant TR x PREPOST interaction for VCO_2 (p < 0.02), with post-hoc analysis showing greater values following KER compared to CAS, only in stage 1 of the exercise (+2.0 ± 0.7 ml.min.kg^{-1}, 95% CI, 0.2, 3.9, $F_{1,12}$ = 5.8, p < 0.04, ES = 0.1).

As expected, there was also a highly significant increase in all pulmonary gas exchange and ventilatory parameters as the intensity of exercise increased (F > 137, p < 0.0001).

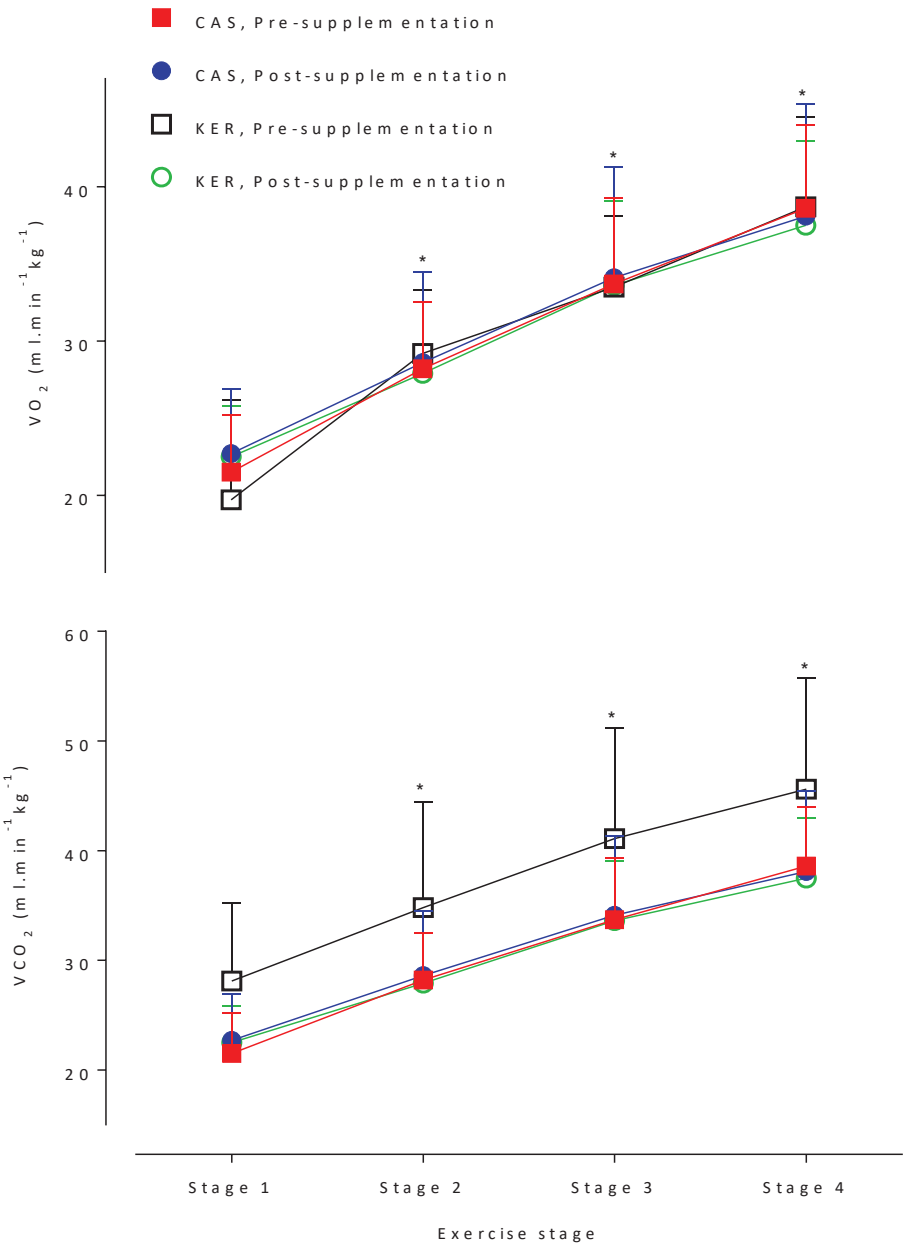

Figure 5.8 VO_2 and VCO_2 during the four-stage, submaximal exercise test, pre- and post-supplementation with CAS and KER for 4 weeks. Values are means ± SD, n = 12. * indicates $p \leq 0.05$ compared to previous intensity for both treatments.

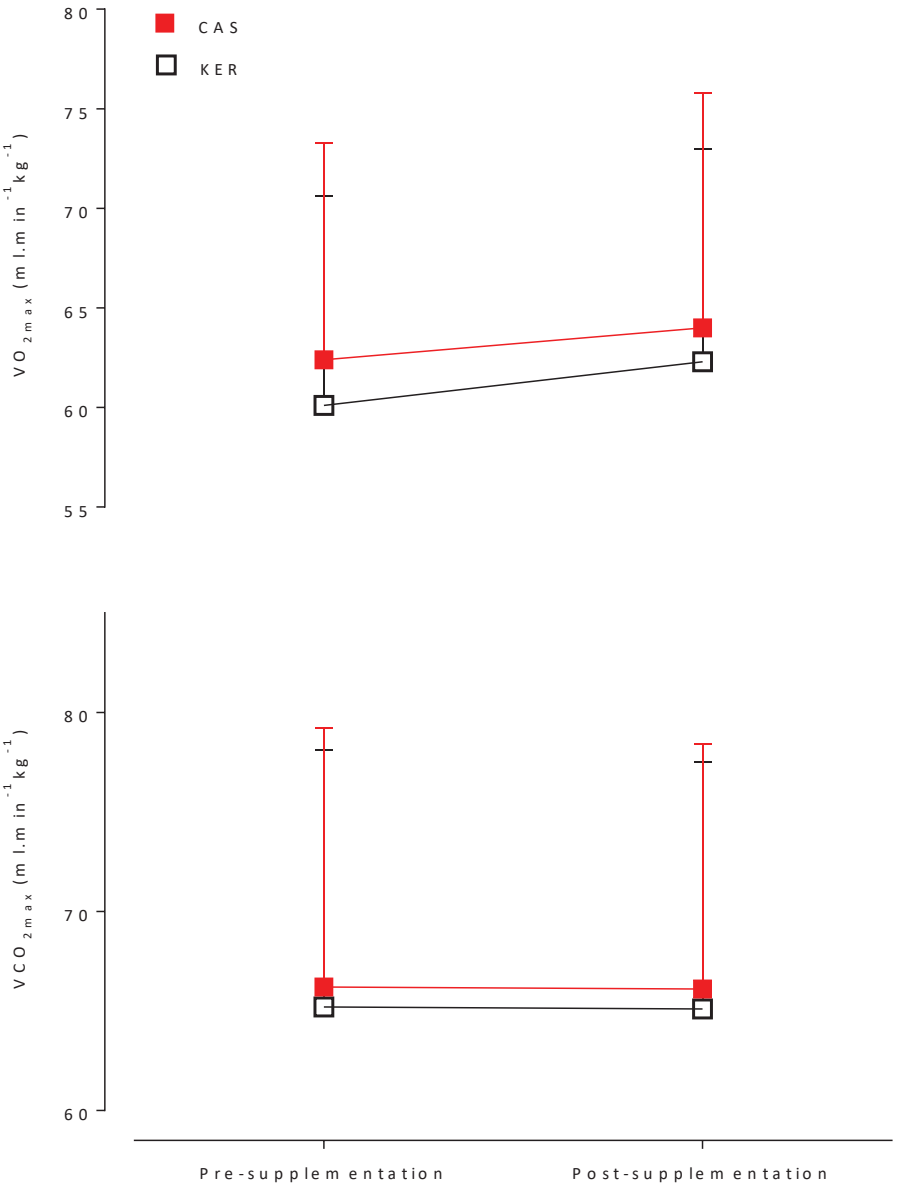

Figure 5.9 VO_{2max} and VCO_{2max} during the VO_{2max} test, pre- and post-supplementation with CAS and KER for 4 weeks. Values are means ± SD, n = 11.

5.3.5 Heart rate

There were no significant main effects of TR ($F_{1,14}$=0.14, p > 0.05) or PREPOST ($F_{1,12}$=2.6, p > 0.05), nor any significant interactions on HR. There was a highly significant increase in HR with TIME ($F_{1,14}$=24.4, p < 0.0001) (Figure 5.10).

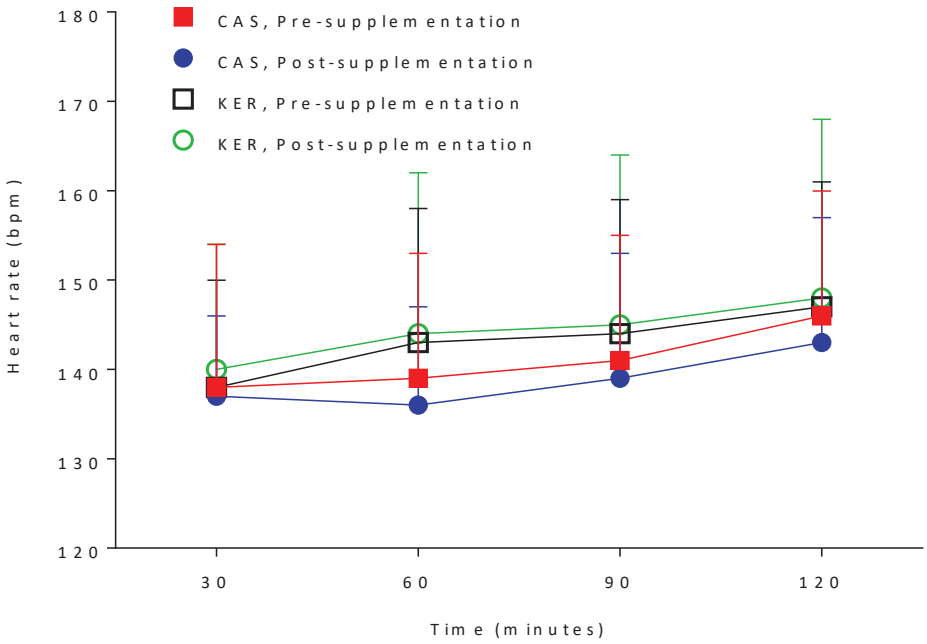

Figure 5.10 HR during the steady-state constant-load submaximal cycle test pre- and post-CAS and KER supplementation for 4 weeks. Values are mean ± SD, n = 14.

5.3.6 Performance variables

There was no significant main effect of TR on the maximal PO achieved during the VO_{2max} test ($F_{1,14}$ = 0.45, p = 0.51) and no significant TR x PREPOST interaction (p = 0.23). Regardless of treatment, there was a significant increase in maximal PO at post- compared to pre-intervention (+12 W, 95% CI, 2, 22, $F_{1,14}$ = 6.97, p = 0.02, ES = 0.2) (Figure 5.11). Accordingly, the PO at 65%VO_{2max} showed a strong trend towards being greater at post- compared to pre-exercise (+7 W, 95% CI, -0.1, 14, $F_{1,14}$ = 4.44, p = 0.054, ES = 0.2), although this was not affected by TR ($F_{1,14}$ = 0.065, p = 0.80).

There were also no significant effects of TR ($F_{1,14}$ = 0.59, p = 0.58) or PREPOST ($F_{1,14}$ = 0.60, p = 0.87) on perceived effort (RPE), but there was a highly significant increase in values as the test duration increased ($F_{1,14}$ = 44.2, p < 0.0001) (Figure 5.12).

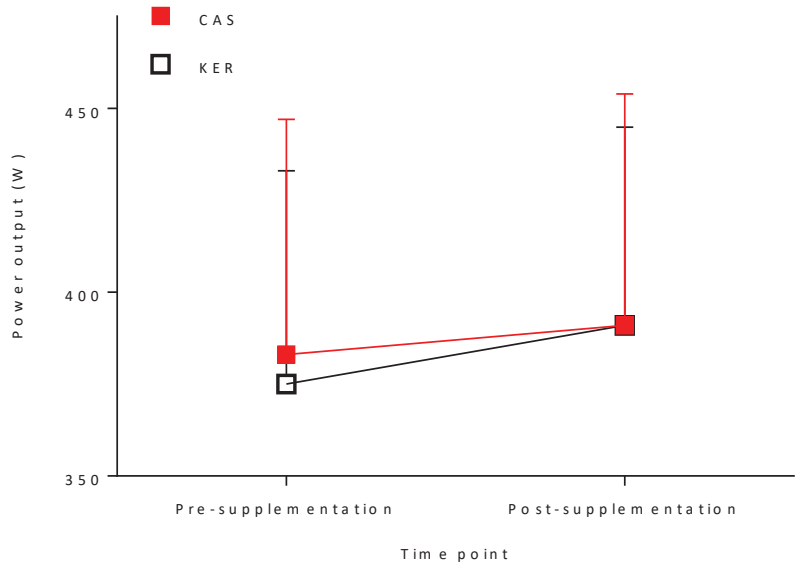

Figure 5.11 Power output at VO_{2max}, pre- and post-supplementation with CAS and KER for 4 weeks. Values are means ± SD, n = 14.

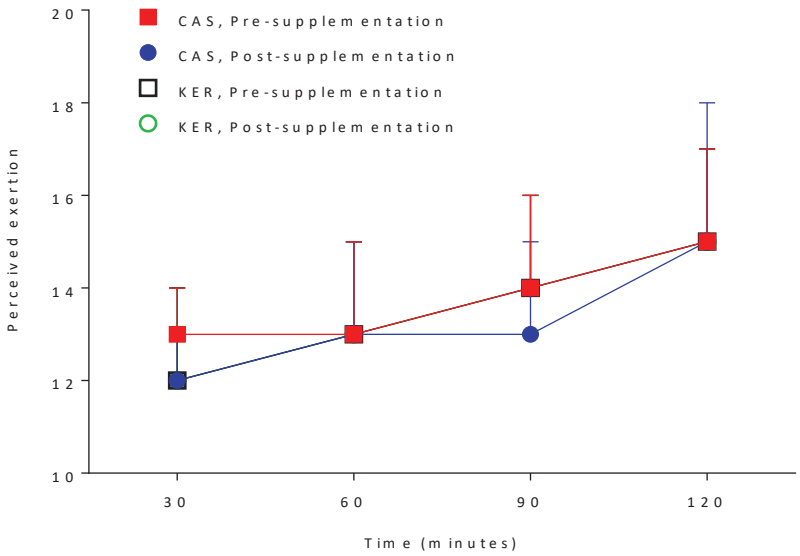

Figure 5.12 RPE during the steady-state constant-load submaximal cycle test pre- and post- supplementation with CAS and KER for 4 weeks. Values are means ± SD, n = 14.

5.4 DISCUSSION

This study was designed to test the hypothesis that a four-week period of KER supplementation would increase the O_2 transport capacity of the blood, by reducing the rate of erythrocyte breakdown. Our data do not support accepting this hypothesis as KER did not significantly affect any of the measured blood parameters, and was not associated with an increase in measured VO_{2max} or a decrease in the VO_2 required to sustain submaximal cycling intensities.

However, despite not inducing any significant changes in blood parameters, VO_2 or performance measures, KER was well-tolerated by participants, and has the potential to be used as a high-protein supplement.

5.4.1 Blood variables

The lack of change in blood parameters with KER in the current study is in contrast to the results from Wolber et al. (2016), who measured significant increases in Hct and Hb (both ~5%) in rats, following a four-week period of partial supplementation of a CAS diet with KER. Further, our results do not align with research involving the CYS donor, *N*-acetylcysteine (NAC), which has been associated with significant increases in plasma erythropoietin (EPO) (26%), Hct (10%), Hb (10%), MCV (12%) and MCH (3%) following eight days of supplementation (1200 $mg.d^{-1}$) in untrained individuals (Zembron-Lacny et al., 2010). Supplementation with NAC has also increased the EPO response to hypoxia when taken for five days prior to exposure to a normobaric hypoxia chamber (12% O_2) (Hildebrandt, Alexander, Bärtsch, & Dröge, 2002).

It is possible that the previously-described effects of thiol supplementation on blood parameters may not occur in already well-trained athletes, such as those who participated in the current study. Previous research has indicated that these individuals have an increased endogenous antioxidant capacity compared to untrained individuals as an adaptation to training. (Gomez-Cabrera et al., 2008). This adaptation has also been described in individuals exposed to hyperoxic (high O_2) gas, who show a significant increase in EPO production, despite an increase in oxidative species, a phenomenon known as the 'normobaric oxygen paradox'. This paradox is explained as an upregulation of antioxidant activity, stimulated by the sudden increase in reactive oxygen species (ROS), which continues to remove oxidative species at a high rate even after the hyperoxic stimulus is removed (Balestra, Germonpre, Lafere, Ciccarella, & Van der Linden, 2010). If the same concept is applied to exercise training, it follows that the increase in oxidative species induced by the greater flux of O_2 through metabolic pathways stimulates an upregulation in antioxidants. Thus, it is possible that the antioxidant levels in the current study's participants were already high enough to keep oxidative stress at a low level, thereby reducing any potential effects of KER on erythrocyte parameters. Alternatively, previous research has found KER to have a digestibility of 78% in rats (Wolber et al., 2016), but it is possible that the thiol content of the supplement was not digested sufficiently in our human participants to influence blood antioxidant status. However, this is speculative, because neither the digestibility, nor antioxidant status were measured in the current study.

5.4.2 Cardiorespiratory measures

Considering the lack of change in erythrocyte parameters, it is unsurprising that KER was not associated with any changes in VO_2. Our hypothesis was based on the major role of erythrocytes in transporting O_2 from the pulmonary system to the active muscles, and thus we assumed that any significant change in RBC, Hb or Hct would also lead to improved O_2 transport. Regardless, despite observing improvements in erythrocyte parameters following NAC supplementation, Zembron-Lacny et al. (2010) did not report any differences in VO_{2max} values obtained in a graded cycling test to exhaustion. Further, Kelly et al. (2009) proposed that NAC supplementation (1800 mg) could improve VO_2 through reducing respiratory muscle fatigue, thereby allowing faster O_2 transfer into the blood stream. However, despite inducing an increase in the mean maximal inspiratory pressure during cycling at 85% VO_{2max}, they showed no significant changes in VO_2. Thus, based on previous literature and the current study, it appears that the provision of exogenous CYS, either as cysteic acid in KER, or as NAC, is not associated with improvements in O_2 transport capacity.

Although VCO_2 was generally not affected by treatment, there was an increase in values in stage 1 of the submaximal test only, which despite having a small effect size (ES = 0.1) was statistically significant. However, because this finding did not correspond to any of other measures, we are unable to determine whether this change was a true effect of KER or due to a type I error.

5.4.3 Heart rate

KER supplementation was not associated with any changes in HR during the constant-load exercise test. This is in accordance with previous research involving NAC which showed no

effect of supplementation on HR during cycling time trial to exhaustion protocols (Kelly et al., 2009; Zembron-Lacny et al., 2010).

5.4.4 Performance

The absence of change in maximal PO in the VO_{2max} test with KER was also observed by Zembron-Lacny et al. (2010) following eight days supplementation with NAC in untrained individuals. Further, oral intake of thiol-containing supplements has not significantly improved performance in handgrip (Smith et al., 2016) or cycling time to exhaustion (TTE) protocols (Bailey et al., 2011). In addition, although Corn and Barstow (2011) measured an increase in TTE with oral NAC supplementation (6000-7800 mg) during cycling at 80% of maximal PO, performance in tests at 90%, 100% and 110% of maximal PO was not affected. Similarly Slattery et al. (2014) demonstrated improvements in repeated sprint performance, but no change in total work or mean PO during a simulated cycling race by highly-trained athletes, following nine days of NAC supplementation (1200 $mg.d^{-1}$). In contrast, Medved et al. (2004) measured a large (~26%) increase in TTE during cycling at 90% VO_{2max}, when a constant intravenous infusion of NAC was given during exercise to untrained but healthy individuals. Thus, it appears that thiol-containing supplements are most beneficial if taken in very large doses during exercise, a method which is impractical and disallowed in athletic competition.

Regardless of supplement, maximal PO was improved post-intervention compared to pre-intervention. Although the participants who participated in the current study were trained athletes, who were already participating in regular endurance cycling exercise, it is possible that the compulsory cycling sessions in the laboratory were at a higher intensity than their

regular training, and thus elicited an increase in post-intervention performance. However, the pre-intervention VO_{2max} values were not significantly different between treatment conditions, so it appears that the washout period was sufficient for fitness levels to return to baseline conditions.

5.4.5 Study limitations

Although significant effort was made to limit variation in exercise training and dietary intake between interventions, these factors could not be completely controlled, as demonstrated by the small, but significantly greater average protein intake by participants in CAS compared to KER. However, considering our primary measures were related to O_2 use and blood variables it is unlikely that this slight difference contributed to the lack of significant effects shown with KER.

In addition, because the current study did not measure blood antioxidant concentrations, it is unknown whether the lack of significant changes in the measured variables were due to the inability of KER to alter antioxidant status, or whether the lack of change occurred despite an improvement in antioxidant status.

Further, although this study observed no change in performance during a VO_{2max} test, it is possible that significant changes may have occurred with a different exercise protocol. However, the absence of significant changes in the measured blood and cardiorespiratory variables do not indicate that KER has potential as an ergogenic aid during endurance cycling.

Finally, this study compared KER versus intake of a CAS supplement, but did not include a non-supplemented condition. Therefore, we are unable to conclude whether supplementing with any type of protein is better than not supplementing at all.

5.4.6 Conclusions

Supplementation with KER does not appear to improve erythrocyte parameters, O_2 transport or exercise performance in highly-trained athletes. Therefore, while KER may be used as an alternative source of protein from an otherwise waste product, it should not be recommended to athletes as a method for improving VO_2 or exercise performance.

CHAPTER 6. ACUTE POMEGRANATE EXTRACT SUPPLEMENTATION

The published version of this chapter is presented in Appendix F.2

6.1 INTRODUCTION

Previously, this thesis has shown that chronic supplementation with a novel keratin supplement does not significantly increase oxygen (O_2) uptake (VO_2) or blood parameters, despite being rich in thiols. Thus, the current chapter will investigate whether polyphenols are a more effective class of antioxidants to improve markers of O_2 transport,

The source of the polyphenol-rich supplement, pomegranate (*Punica granatum*), is a seeded, red, fleshy fruit of Middle East origin which was used in traditional medicine to treat a variety of inflammatory conditions (Ismail, Sestili, & Akhtar, 2012). In modern-day research, consumption of pomegranate juice (POMJ) or extract (POMx) has been linked to a decline in cancer proliferation (Adams et al., 2010), the amelioration of cardiovascular disease markers (Aviram et al., 2004) and decreases in gut and joint inflammation (Sergent et al., 2010; Shukla et al., 2008). Recent research has indicated that pomegranate-based supplements may also enhance the matching of vascular O_2 provision to muscular requirements during exercise due to their high concentration of nitrates (NO_3^-) and polyphenol compounds (Roelofs et al., 2016; Trexler et al., 2014).

POMJ contains a greater concentration of polyphenols (~3.8 $mg.ml^{-1}$) than other polyphenol-rich beverages such as red wine (~3.5 $mg.ml^{-1}$), Concord grape juice (~2.6

$mg.ml^{-1}$) and cranberry juice (~1.7 $mg.ml^{-1}$) (Seeram et al., 2008). These are predominantly from the ellagitannin (ET) subclass (80–90%) with smaller amounts of anthocyanins (8–15%) (Seeram, Lee, & Heber, 2004). Consumption of ET or anthocyanin-rich foods is associated with a decrease in systolic blood pressure (SBP) and an increase in vessel diameter and blood flow (Barona et al., 2012; Labonté et al., 2013; Roelofs et al., 2016) suggesting a link between pomegranate consumption and O_2 delivery.

The NO_3^- content in POMx—12.93 ppm.(~0.2 $mmol.L^{-1}$) in POMJ and 109 $ppm.L^{-1}$ (~1.76 $mmol.L^{-1}$) in POMx, as reported by Roelofs et al. (2016)—is much lower than that in the well-known dietary NO_3^- source, beetroot juice (BRJ), which contains ~11 $mmol.L^{-1}$ NO_3^- (Bailey et al., 2009). However, polyphenols enhance the effects of dietary NO_3^- by promoting their conversion into Nitric Oxide (NO) (Gago et al., 2007; Peri et al., 2005), and protecting NO from damage caused by reactive oxygen species (ROS) (Ignarro et al., 2006). Thus, POMx may provide a source of dietary NO_3^- with greater bioavailability, despite the lower total NO_3^- content. Previously, supplementation with high-NO_3^- foods have been associated with a large increase (>92%) in plasma NO_2^-, a 3–5% reduction in VO_2 during submaximal exercise and a 15–25% improvement in performance during cycling, running and knee extension time to exhaustion (TTE) protocols in low to moderately-trained athletes (Bailey, Fulford, et al., 2010; Bailey et al., 2009; Breese et al., 2013; Lansley, Winyard, Fulford, et al., 2011; Wylie et al., 2013). In contrast, dietary NO_3^- has little or no benefit on these parameters in highly-trained athletes (VO_{2max} >60 $ml.min^{-1}kg^{-1}$), who may have greater NO synthase (NOS) activity, and consequently a lower reliance on NO production via NO_3^- (Arnold, Oliver, Lewis-Jones, Wylie, & Macdonald, 2015; Bescós et al., 2011; Cermak, Gibala, & van Loon, 2012; MacLeod et al., 2015; Peacock et al., 2012; Porcelli et al., 2015; Wilkerson et al., 2012). However, NO_3^- supplementation appears to benefit

these athletes during exercise at high altitude, where the lower atmospheric pressure of O_2 (PO_2) impairs O_2 transport and places greater reliance on the non-aerobic pathway to produce NO (Kelly et al., 2014a; Masschelein et al., 2012). This is significant because exercise at high altitude features in a number of major sporting competitions, such as the Tour de France.

Currently, only two studies have investigated the effect of pomegranate supplementation on endurance exercise performance at sea-level, with no previous research investigating its effects in low PO_2 conditions. In moderately-trained individuals, acute POMx intake (1000 mg) increased pre-exercise blood flow and TTE during treadmill running at 90–100% of peak velocity (Trexler et al., 2014). Conversely, following seven days of POMJ supplementation (1000 $ml.day^{-1}$) in a trained cohort, no change in performance was observed during a ten minute cycling time trial or TTE protocol in hot conditions (Trinity et al., 2014). Thus, further research is warranted to determine whether pomegranate improves O_2 transport and endurance exercise performance.

The outcome of the current study was primarily to determine the effect of acute POMx supplementation on O_2 transport parameters, and secondarily, to measure whether any observed effects are associated with improvements in endurance exercise performance. In addition, the study investigated whether any observed effects are greater during exercise in a moderate altitude environment. It was hypothesized that acute POMx supplementation will reduce the submaximal O_2 cost of exercise, and in doing so, improve performance in an environment (altitude) where O_2 availability may be limiting.

6.2 METHODS

6.2.1 Participant characteristics

All participants provided written consent after being informed about the study requirements and benefits and risks of participating and a health questionnaire was completed as detailed in 4.2.1. Eight highly-trained cyclists, aged 17–18 years, including seven males and one female, were recruited from the regional cycle community. This sample size was chosen based on previous studies which have successfully shown a decrease in VO_2 following NO_3^- supplementation with a sample size of eight participants (Bailey, Fulford, et al., 2010; Bailey et al., 2009). The participants' baseline characteristics are presented in Table 6.1. The study was approved by the Massey University Human Ethics Committee (Southern A 15/54) in accordance with the Declaration of Helsinki.

6.2.2 Pre-study measurements

Anthropometric and body composition data were measured using the methods outlined in the general methodology (4.2.2). A four-stage, submaximal exercise test, followed by an incremental ramp to exhaustion were completed as described in the general methodology to calculate VO_2 (see 4.3.1 and 4.3.2) on a cycle ergometer (see 4.1.1). This data was used to calculate the power output which would elicit ~50%, 65%, 80% and 100% of the participant's VO_{2max} (see 4.3.3).

Table 6.1 Baseline characteristics of participants in Study 2

Participant	Gender	Age (years)	Mass (kg)	Height (m)	VO_{2max} ($ml.min.kg^{-1}$)
1	Male	18	72.7	1.94	83.7
2	Male	17	70.0	1.79	76.1
3	Male	17	63.2	1.76	79.0
4	Male	18	68.0	1.77	70.6
5	Male	17	76.0	1.86	67.8
6	Female	18	52.9	1.64	64.7
7	Male	18	68.3	1.87	72.6
8	Male	17	69.5	1.76	81.0
Mean ± SD		18 ± 0.5	67.6 ± 7	1.80 ± 0.9	74.4 ± 6.2

6.2.3 Study design

The study protocol (Figure 6.1) was a randomized, double-blind, crossover design, which was completed on four occasions: twice in sea-level conditions (SEA) and twice in high-altitude conditions (ALT, 1657m, PO_2 equivalent to ~17% O_2). The testing location was the Turoa ski-field carpark, Ohakune, which was chosen due to being the highest road accessible to our mobile laboratory in the North Island of New Zealand. Participants ingested 1000 mg of either a POMx supplement (POM Wonderful LLC, California, USA) or a placebo (PLAC), in capsule form, before completing an exercise trial involving submaximal and maximal exercise on a cycle ergometer

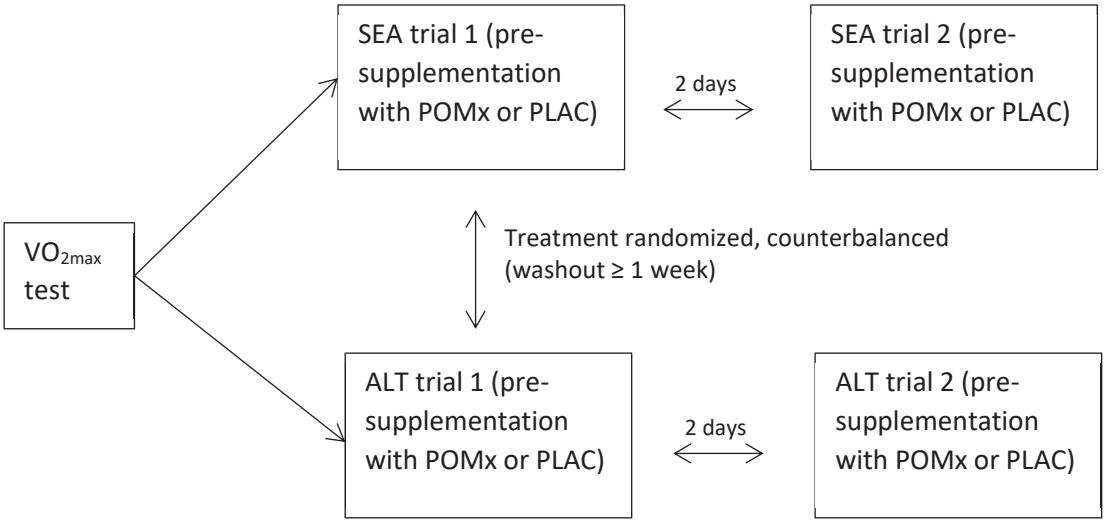

Figure 6.1 Design of Study 2

6.2.4 Supplementation

In accordance with previous POMx research, supplements were ingested 2.5 hours prior to each experimental trial to allow maximal absorption prior to exercise (Seeram et al., 2008). The quantity of POMx was based on that used by Trexler et al. (2014) who showed an association between acute POMx supplementation and improved running performance. The POMx supplement was produced by POM Wonderful LLC (California, USA, patent application number US 935007). The process by which POMx is produced is described in detail by Bates, Fritz, Henig, and Liker (2009). Briefly, a combination of the pericarp, inner membrane and seeds are heated and treated with enzymes to partially degrade the pomegranate solids, allowing the polyphenols to be collected. Previous analysis of the POM Wonderful LLC supplement have shown it to contain 95.5% ET, 3.5% ellagic acid and 1% anthocyanins (Trinity et al., 2014). The placebo supplement (brown sugar) was in a capsule of the same colour, size and shape as POMx.

Participants completed a POMx trial and a PLAC trial in each environmental condition in a randomized order; the supplement blinded to the participant and the researcher. The participant was instructed to swallow the capsule whole, without tasting it, to avoid an expectation bias. During the 48 hours prior to each session, participants were asked to limit consumption of NO_3^- and polyphenol-rich foods (detailed in Appendix A.8) and avoid strenuous exercise and antibacterial substances, such as mouthwash, which destroy NO_3^- - NO_2^- converting bacteria on the tongue (Govoni, Jansson, Weitzberg, & Lundberg, 2008). Participants consumed a standardized meal (524 calories, 8g protein, 11g fat, 51g carbohydrate) three hours prior to exercise. Participants were asked to arrive at the testing session in a hydrated state—this was confirmed verbally by the participant on arrival to the laboratory—and water was consumed ad libitum throughout the trial.

6.2.5 Exercise protocol

On arrival to the laboratory, body mass was obtained and a heart rate monitor (Garmin, Kansas, USA) was applied and recorded heart rate (HR) at a sampling rate of two seconds throughout the trial (see 4.3.5). A muscle O_2 monitor (Moxy, Fortiori Design LLC, Minnesota, USA) was positioned on the *vastus lateralis* muscle of each leg as detailed in the general methodology, and recorded local muscle O_2 saturation (SmO_2) and total haemoglobin (THb) throughout the trial as described previously (see 4.3.9). After sitting in a supine position for five minutes, systolic (SBP) and diastolic blood pressure (DBP) of the brachial artery was measured using an automated sphygmomanometer (see 4.3.4) Blood was collected via fingerprick sample and analysed for blood lactate concentration ($[La^-]_b$) (see 4.3.4). To determine haematocrit (Hct), additional blood (~35 µL) was collected from the fingertips in heparinized capillary microtubes and immediately spun in a microhaematocrit centrifuge

(see 4.3.7). In the SEA trials, a venous blood sample was also collected via venepuncture from the antecubital vein in heparinized vacutainers, immediately spun in a centrifuge and plasma was stored as described in the general methodology (see 4.3.8). The venous sample was not collected in ALT as samples could not be stored or analysed in the mobile laboratory.

Following the pre-exercise measures, the participant mounted the ergometer to begin cycle exercise. The exercise protocol is shown in Figure 6.2. The experimental protocol began with three x six-minute stages of stationary cycling exercise at power outputs corresponding to ~50%, 65% and 80% of their previously determined $VO_{2max.}$ In the last minute of each stage, VO_2 was measured as previously described and perceived exertion (RPE) was recorded (see 4.3.10). Then, following a five-minute rest, the load on the ergometer was increased to a workload calculated to elicit VO_{2max} and participants were instructed to ride at this intensity, at a cadence of ≥80rpm for as long as possible, with the trial being terminated once the participant could not maintain the required cadence (for the previous 10 seconds) or at volitional exhaustion. Time to fatigue at 100%VO_{2max} (TTE100%) was chosen in this study as a performance measure rather than a self-paced time trial to enable physiological data to be collected and compared at the five-minute point. Accordingly, five minutes into the TTE100% VO_2 was measured

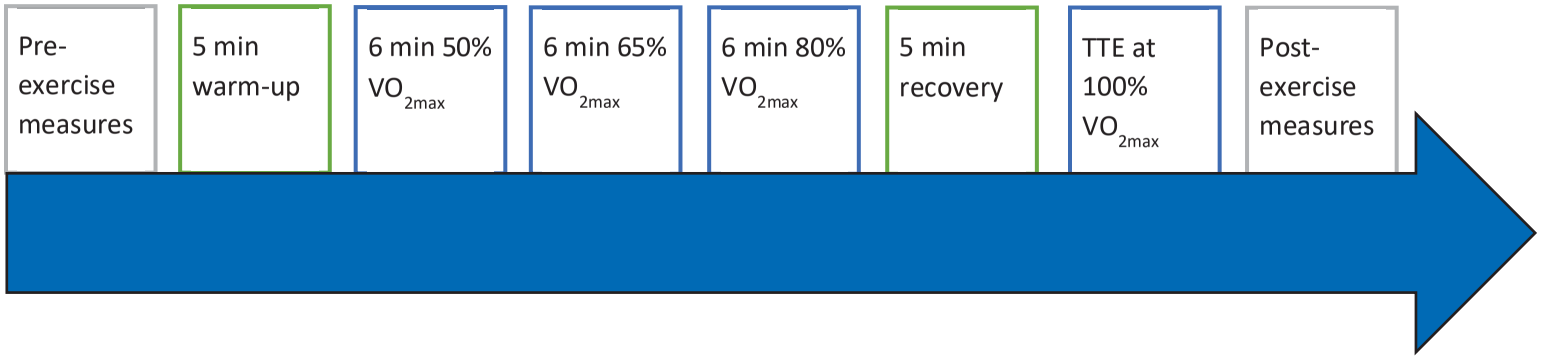

Figure 6.2. Trial protocol for Study 2

Figure 6.3 The mobile laboratory at the ALT testing location

6.2.6 Blood Analysis

Plasma samples were analysed using a Nitric Oxide Colorimetric Assay Kit as described in the general methodology (see 4.4.2).

6.2.7 Statistical Analyses

Statistical analyses to compare the values of all variables measured were performed using specialized statistical analysis software (SPSS Statistics, Version 23, IBM Corporation, New York). Variables measured during the submaximal exercise stages were analysed using three-way repeated measures ANOVA to test the significance level for main effects of, and interactions between environment (ENV; SEA or ALT), exercise intensity (INT; 50, 65 and 80%VO_{2max}) and treatment (TR; PLAC or POMx). Similarly, two-way repeated measures ANOVA were performed on all variables measured during the TTE100% (ENV x TR) and three-way repeated measures ANOVA were used to assess resting measures (ENV x TR x TIME).

In all analyses, Mauchly's Test of Sphericity was used to validate the results of each ANOVA and in cases where aspherity was detected, the Greenhouse-Geiser correction was applied. Statistical significance was accepted when $p \leq 0.05$, while a tendency was noted when $p \leq 0.1$. Where significant interactions were observed, two-tailed paired t-tests with a Fisher's LSD post-hoc analysis were used to identify the location of the significance. The effect sizes (ES) of significant interactions were calculated using Cohen's d (0.1 small; 0.5 medium; 0.8 large). The relationship between VO_2 and performance during the TTE100% was analysed using the Pearson correlation coefficient. Data is presented as mean ± standard deviation

(SD) or mean change and 95% confidence intervals (CI), as appropriate. Graphs were produced using Prism 6.0 (GraphPad Software, CA, USA).

6.3 RESULTS

Participants' self-reported adherence to supplement intake was 100% and no side effects of supplementation were reported.

6.3.1 Resting measures

Plasma NO_3^- was greater following POMx compared to PLAC (+10.3 µmol, 95% CI, 0.8, 19.7, $F_{1,5}$ = 7.83, p < 0.04, ES = 0.9) (Figure 6.4). However, the assay used was not sensitive enough to detect changes in NO_2^-. In addition, NO_3^- data was not obtained for two participants due to inability to obtain a blood sample or error during data analysis. Resting BP and Hct data is summarized in Table 6.2.

Resting SBP was not significantly affected by POMx or ALT (p > 0.05). However, there was a trend towards an increase in SBP with POMx vs. PLAC (+3.9 bpm, 95% CI, -0.6, 8.5, $F_{1,7}$ = 4.28, p=0.08, ES = 0.3). There was a strong trend towards an increase in DBP with ALT, which showed a moderate effect size (+5 mmHg, $F_{1,7}$ = 1.18, p = 0.054, ES = 0.6) and a significant TR x ENV x TIME interaction ($F_{1,7}$ = 7.64, p < 0.03). However, post-hoc analyses revealed no significant differences between pre- or post-exercise DBP with either treatment at either altitude (p > 0.05).

Hct was significantly increased in ALT (+1.4%, 95% CI, 0.2, 2.6, $F_{1,7}$ = 7.56, p < 0.03, ES = 0.4) and significantly decreased by POMx, although the ES was small (-0.76%, 95% CI, -1.3, -0.2, $F_{1,7}$ = 10.4, p < 0.02, ES = 0.2).

Regardless of TR or ENV, there were significant increases in post-exercise compared to pre-exercise values for SBP (+8.2 mmHg, 95% CI, -0.5, 16.9, $F_{1,7}$ = 4.93, p = 0.005, ES = 0.4), DBP (+5.7 mmHg, 95% CI, -0.9, 10.4, $F_{1,7}$ = 8.02, p < 0.03, ES = 0.6) and Hct (+1.2%, 95% CI, 0.6, 1.8, $F_{1,7}$ = 23.2, p = 0.002, ES = 0.4).

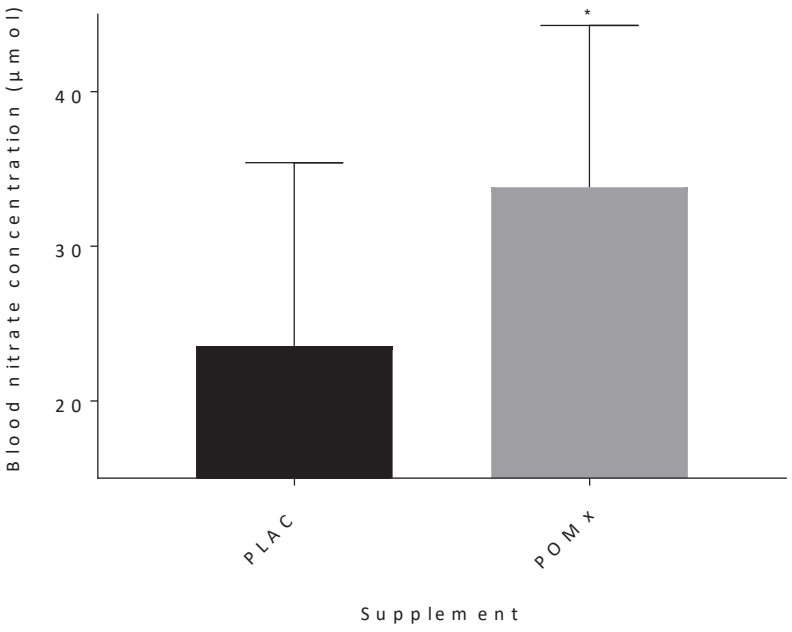

Figure 6.4 Resting plasma nitrate values at SEA following supplementation with PLAC and POMx. Values are means ± SD, n = 6. * indicates $p \leq 0.05$ compared to PLAC

Table 6.2 Resting BP and Hct data following POMx and PLAC in SEA and ALT conditions

Measure	SEAPLAC	SEAPOMx	ALTPLAC	ALTPOMx
SBP (mmHg)				
Pre-exercise	120 ± 13	125 ± 18	127 ± 12	130 ± 15
Post-exercise	125 ± 11[d]	129 ± 11[d]	135 ± 16[d]	139 ± 17[d]
DBP (mmHg)				
Pre-exercise	71 ± 9	73 ± 12	78 ± 6	78 ± 12
Post-exercise	78 ± 5[d]	78 ± 9[d]	80 ± 8[d]	86 ± 8[d]
Hct (%)				
Pre-exercise	45 ± 3	45 ± 3[a]	46 ± 4[a]	46 ± 3[bc]
Post-exercise	46 ± 3[d]	45 ± 3[ad]	48 ± 4[ad]	47 ± 3[bcd]

Data are presented as means ±SD, n = 8. POMx, Pomegranate extract, PLAC, Placebo; SBP, SEA, Sea-level; ALT, High-altitude; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; Hct, Haematocrit. [a] indicates $p \leq 0.05$ compared to SEAPLAC; [b] indicates $p \leq 0.05$ compared to SEAPOMx. [c] indicates $p \leq 0.05$ compared to ALTPLAC. [d] indicates $p \leq 0.05$ compared to pre-exercise value.

6.3.2 Cardiorespiratory, heart rate and blood lactate responses during submaximal exercise

6.3.2.1 Cardiorespiratory variables

The cardiorespiratory data are displayed in

Figure **6.5**. There was no significant main effect of POMx on VO_2 ($F_{1,7} = 0.193$, $p = 0.67$) or VCO_2 ($F_{1,7} = 1.34$, $p = 0.29$). Further there was no significant effect of ENV or any significant ENV x TR or INT interactions on VO_2 or VCO_2 ($F_{1,7} < 0.1$, $p > 0.05$). As expected, there was a

significant increase in VO_2 ($F_{1,7}$ = 182, p < 0.0001, ES = 1.7) and VCO_2 ($F_{1,7}$ = 152, p < 0.0001, ES = 1.7-1.8) as exercise intensity increased.

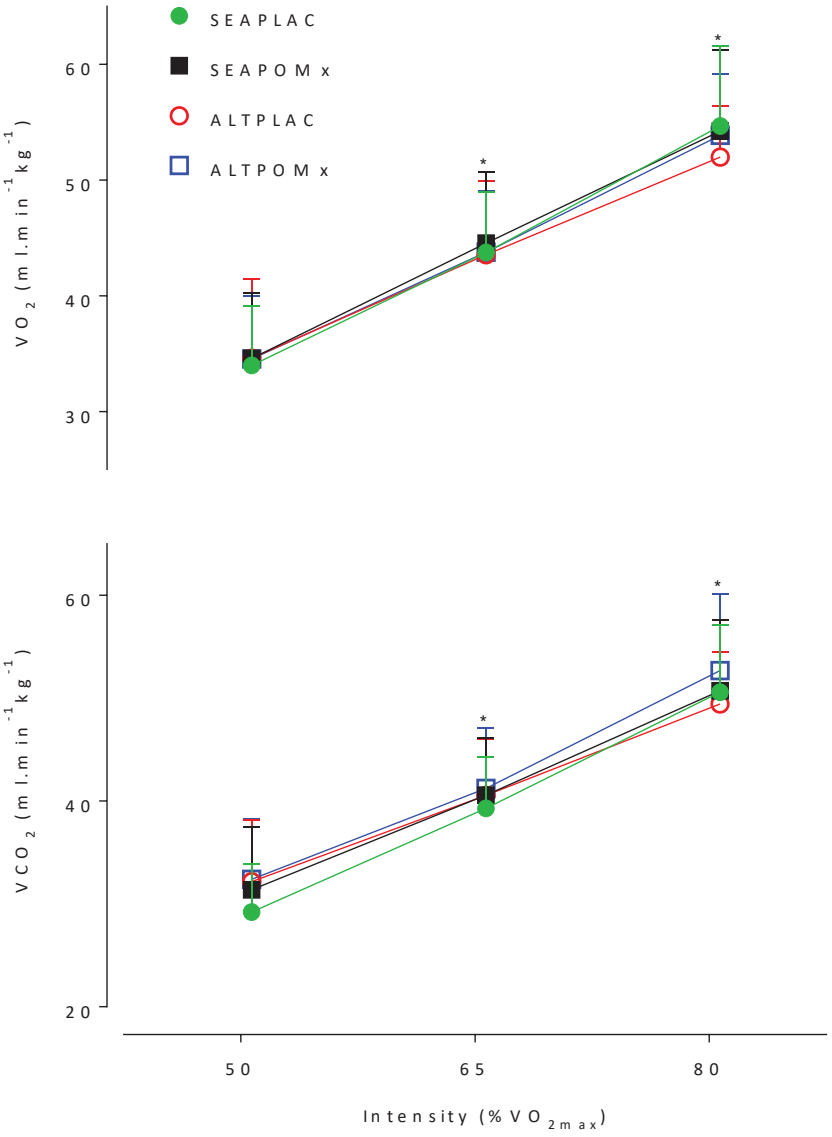

Figure 6.5. VO_2 and VCO_2 values during submaximal exercise following PLAC and POMx, in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

6.3.2.2 Muscle oxygenation

A full set of data for the Moxy measurements was not recorded for four participants in ALT, due to a battery error with the device during testing in the mobile laboratory. Therefore, two analyses were performed for the SmO_2 and THb data; one with the four full data sets, and one with data from all participants, at SEA only.

Analysis of the four full data sets showed no significant effect of POMx on either SmO_2 ($F_{1,3}$ = 0.001, p = 0.98) or THb ($F_{1,3}$ = 0.359, p = 0.59). There was a trend towards an increase in THb in ALT compared to SEA (+0.086, 95% CI, -0.018, 0.19, $F_{1,3}$ = 6.88, p < 0.08), but no effect of ENV on SmO_2 ($F_{1,3}$ = 2.99, p > 0.05). No significant effects of TR were observed for either parameter when just SEA data was considered (p > 0.05).

THb significantly decreased as intensity increased regardless of the type of analysis (p < 0.05). However, the expected decrease in SmO_2 was only significant in the 'SEA only' analysis ($F_{1,7}$ = 4.18, p < 0.04).

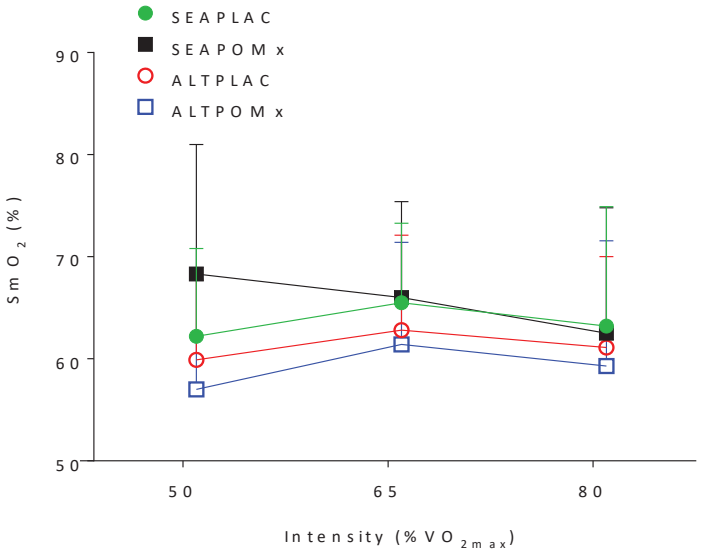

Figure 6.6 SmO_2 in the *vastus lateralis* during submaximal exercise, following PLAC and POMx, in SEA and ALT. Values are means ± SD, n = 4.

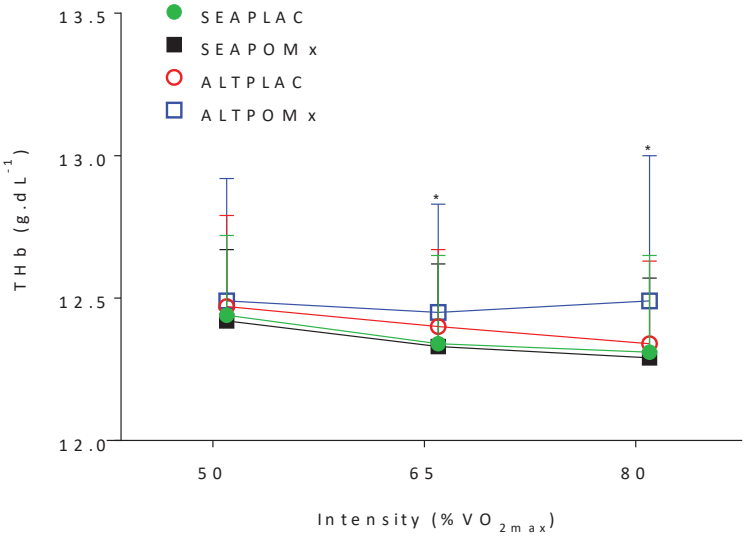

Figure 6.7 THb in the *vastus lateralis* during submaximal exercise, following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

6.3.2.3 Heart rate

There was also no effect of TR ($F_{1,7}$ = 3.80, p = 0.09) or ENV ($F_{1,7}$ = 2.95, p = 0.13) or any significant TR x ENV or INT interactions ($F_{1,7}$ < 0.1, p > 0.05) on HR. However, as expected there was a highly significant increase in values as exercise intensity increased ($F_{1,7}$ = 427, p < 0.0001, ES = 1.9-2.0) (Figure 6.8).

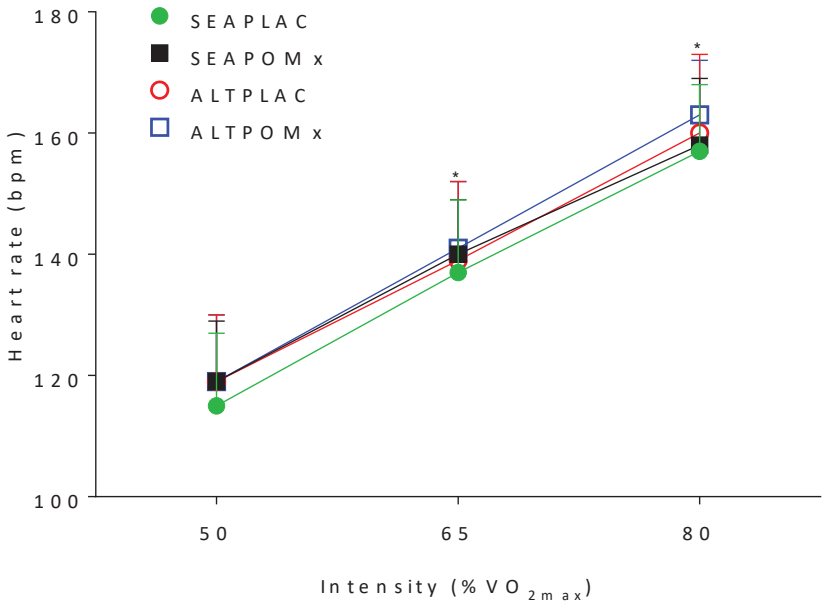

Figure 6.8 HR during submaximal exercise following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

6.3.2.4 Blood lactate

As with the other submaximal measurements, there was no significant effect of Tr on $[La^-]_b$ ($F_{1,7}$ = 1.22, p = 0.31) or any TR x ENV or INT interactions ($F_{1,7}$ < 0.1, p > 0.05). However, there was a significant increase in $[La^-]_b$ in ALT compared to SEA (-0.8 µmol,95% CI, 0.3, 1.3, $F_{1,7}$ = 2.95, p < 0.01, ES = 0.6) and a trend towards an increase in values with a greater exercise intensity ($F_{1,7}$ = 2.99, p < 0.09) (Figure 6.9).

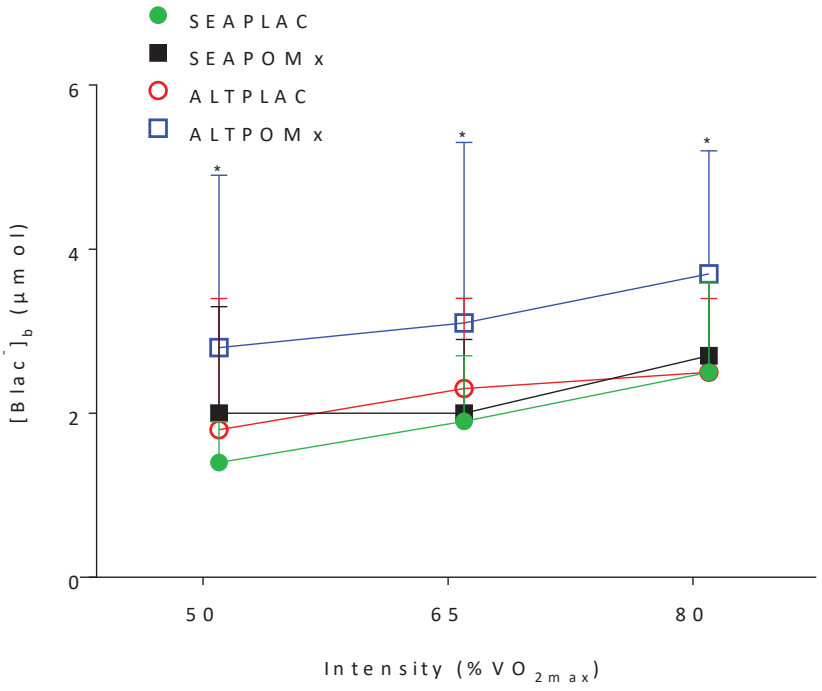

Figure 6.9 $[Blac^-]_b$ during submaximal exercise following PLAC and POMx, in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to SEA

6.3.3 Cardiorespiratory, heart rate and blood lactate responses to the time trial to exhaustion at 100%VO_{2max}

6.3.3.1 Cardiorespiratory variables

All data was recorded five minutes into the TTE100% and is displayed in Figure 6.11. There was no main effect of POMx on VO_2 ($F_{1,7}$ = 1.39, p = 0.28), but there was a significant decrease in VO_2 in ALT compared to SEA (-4.2 $ml.min^{-1}kg^{-1}$, 95% CI, 1.7, 0.04, $F_{1,7}$ = 6.37, p = 0.04, ES = 0.7). The two-way ANOVA showed a significant ENV x TR interaction for VO_2 ($F_{1,7}$=12.5, p=0.01). Post-hoc analysis identified that POMx significantly increased VO_2 at ALT (+3.8 $ml.min^{-1}kg^{-1}$, 95% CI, -5.7, 9.5, $F_{1,7}$=29.2, p=0.001 but not at SEA ($F_{1,7}$=0.95, p = 0.46, ES=0.2). Cohen's d showed a moderate effect size of 0.6. However, there was no correlation between VO_2 during the TTE100% and performance (r^2=0.0001) (Figure 6.10). For VCO_2, there were no main effects of Tr ($F_{1,7}$ = 0.516, p = 0.50) or ENV ($F_{1,7}$= 0.003, p = 0.96), but there was a significant ENV x TR interaction for VCO_2 ($F_{1,7}$=6.32, p= < 0.04). However, post-hoc analysis identified no significant differences between treatments at SEA ($F_{1,7}$=1.76, p > 0.5), and a trend towards an increase in VCO_2 with POMx at ALT with a moderate ES (+3.6, 95% CI, -0.665, 7.82, $F_{1,7}$=3.98, p=0.09, ES=0.5).

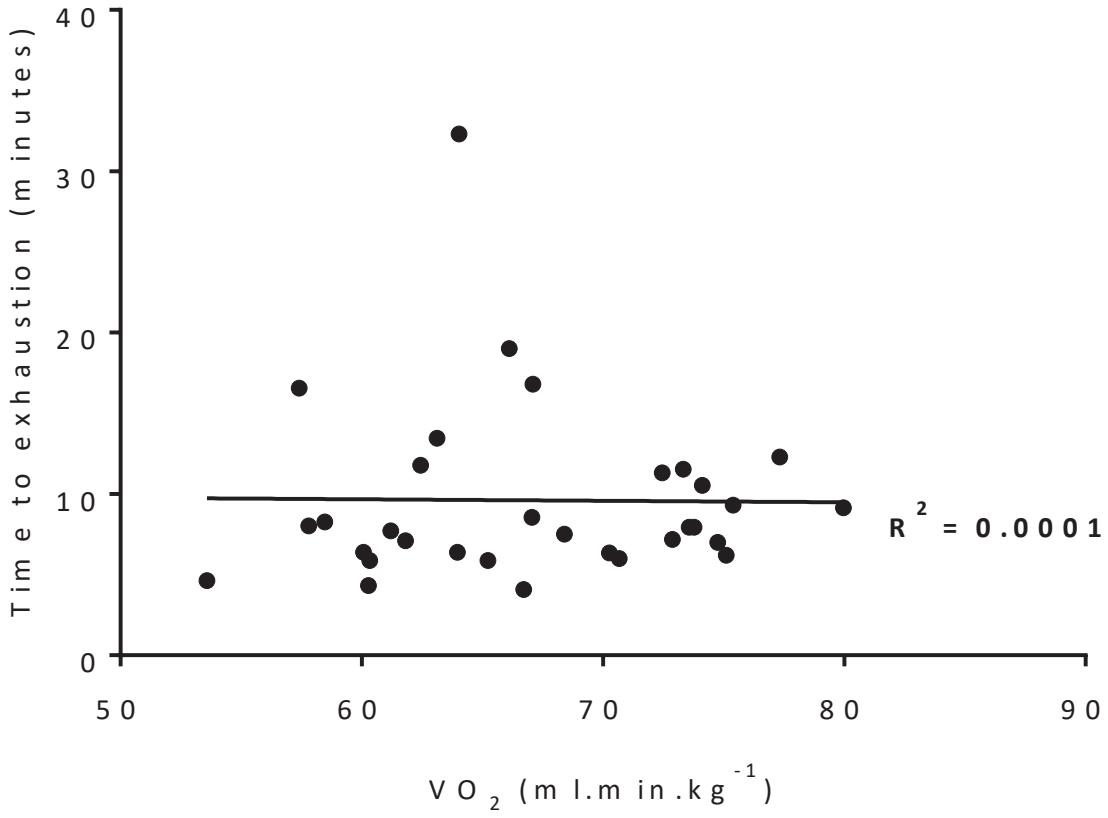

Figure 6.10 Correlation between VO_2 and TTE during the TTE100%

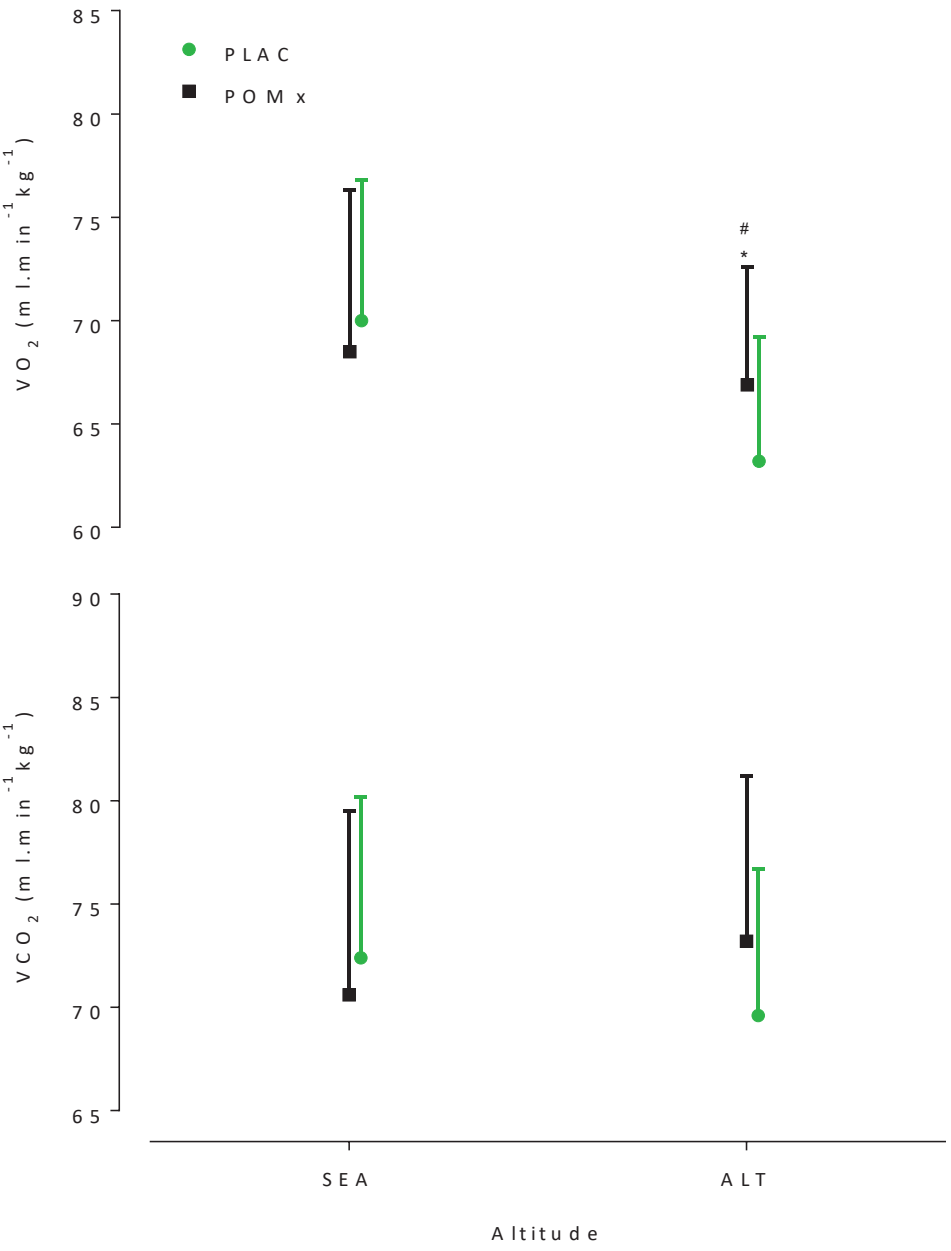

Figure 6.11 VO_2 and VCO_2 during the TTE100% following PLAC and POMx, in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to SEA. # indicates $p \leq 0.05$ for POMx compared to PLAC

6.3.3.2 Muscle oxygenation

There was no significant main effect of POMx ($F_{1,3}$ = 0.111, p =0.76) or ALT ($F_{1,3}$ = 2.65, p = 0.20) on SmO_2 with either of the two analyses described previously (see 6.3.2.2). There was a significant TR x ENV interaction for SmO_2 ($F_{1,3}$ = 38.1, p = 0.009), but post-hoc analysis showed no significant differences between conditions.

Similarly, regardless of analysis, THb was not affected by TR ($F_{1,3}$ = 5.42, p = 0.12) or Alt ($F_{1,3}$ = 1.72, p = 0.28) and there was no TR x ENV interaction ($F_{1,3}$ = 0.429, p = 0.56) (Figure 6.12)

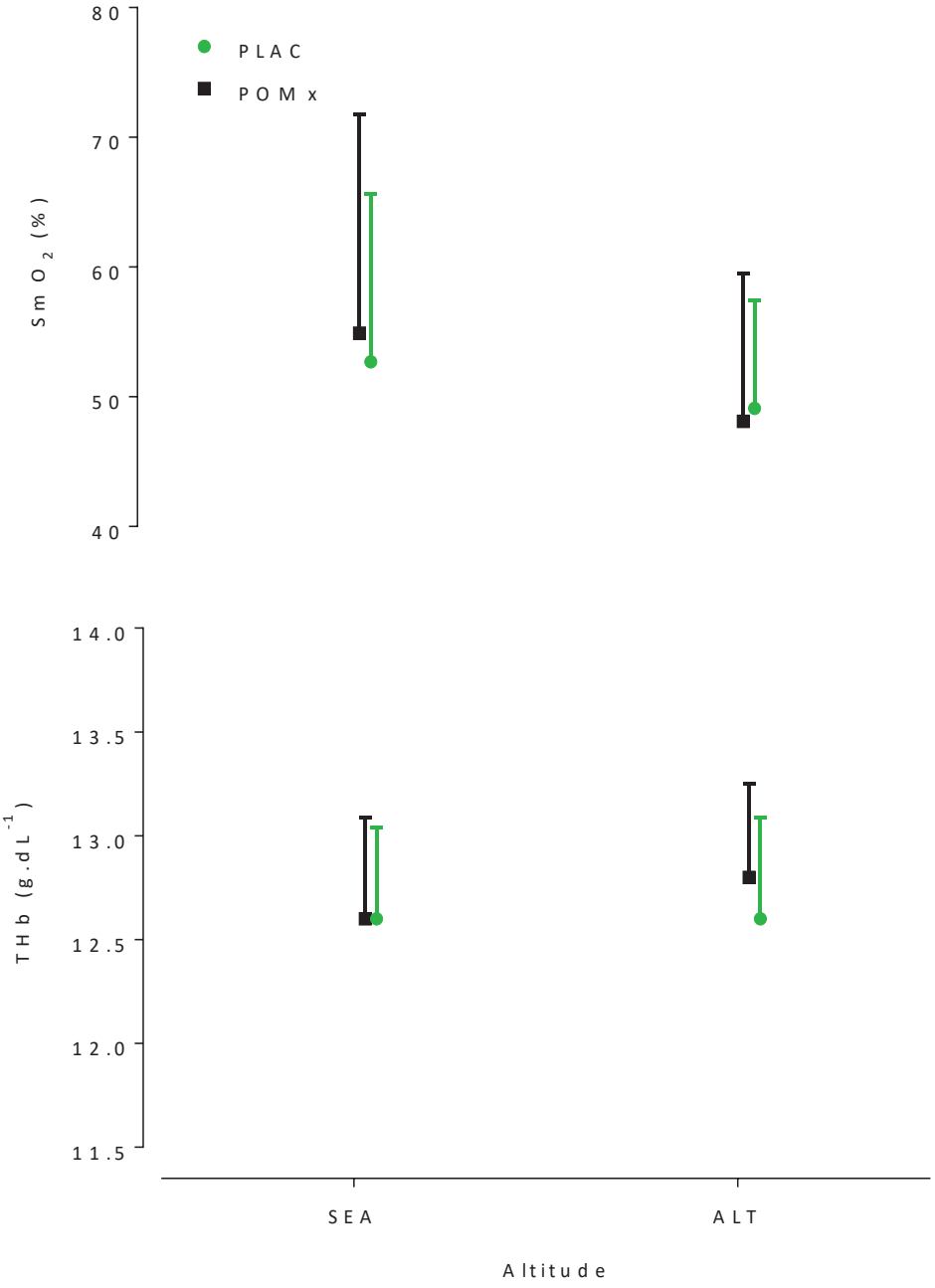

Figure 6.12 SmO_2 and THb in the *vastus lateralis* during the TTE100% following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 4.

6.3.3.3 Heart rate

There was no main effect of POMx ($F_{1,7}$ = 1.85, p = 0.22) on HR during the TTE100%, or any significant TR x ENV interaction ($F_{1,7}$ = 0.006, p = 0.94). However, HR was significantly greater in ALT compared to SEA (+3 bpm, 95% CI, 0.5, 6.3, $F_{1,7}$ = 7.6, p < 0.03) (Figure 6.13).

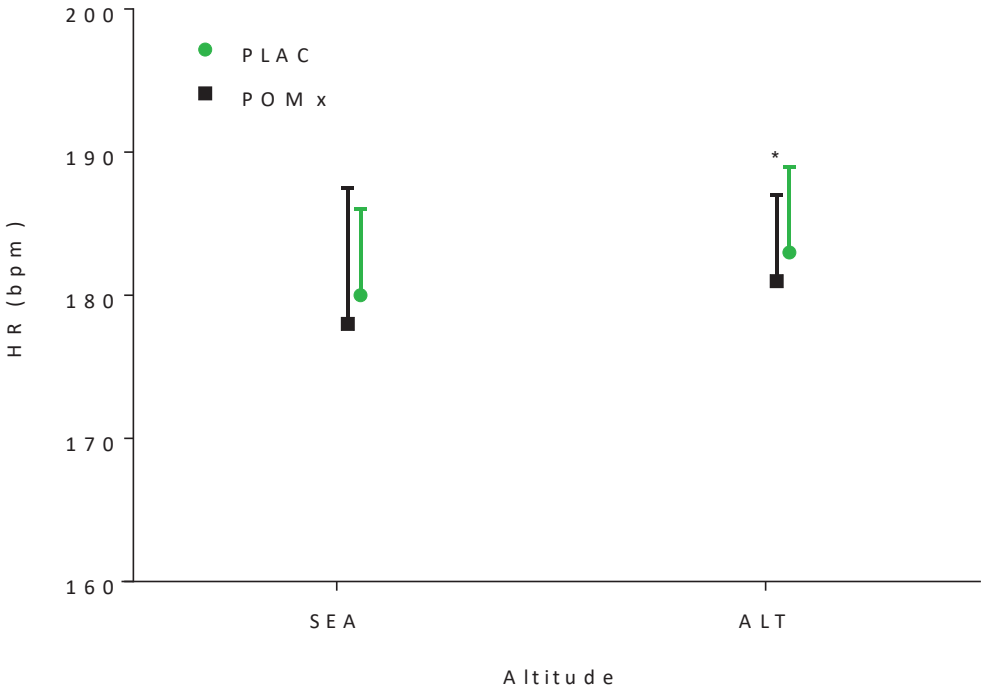

Figure 6.13 HR during the TTE100% following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to SEA.

6.3.3.4 Blood lactate

There were no significant main effects of POMx ($F_{1,7}$ = 0.615, p = 0.46) or ALT ($F_{1,7}$ = 3.25, p = 0.12) or any TR x ENV interaction ($F_{1,7}$ = 0.01, p = 0.92) on $[La^-]_b$ (Figure 6.14).

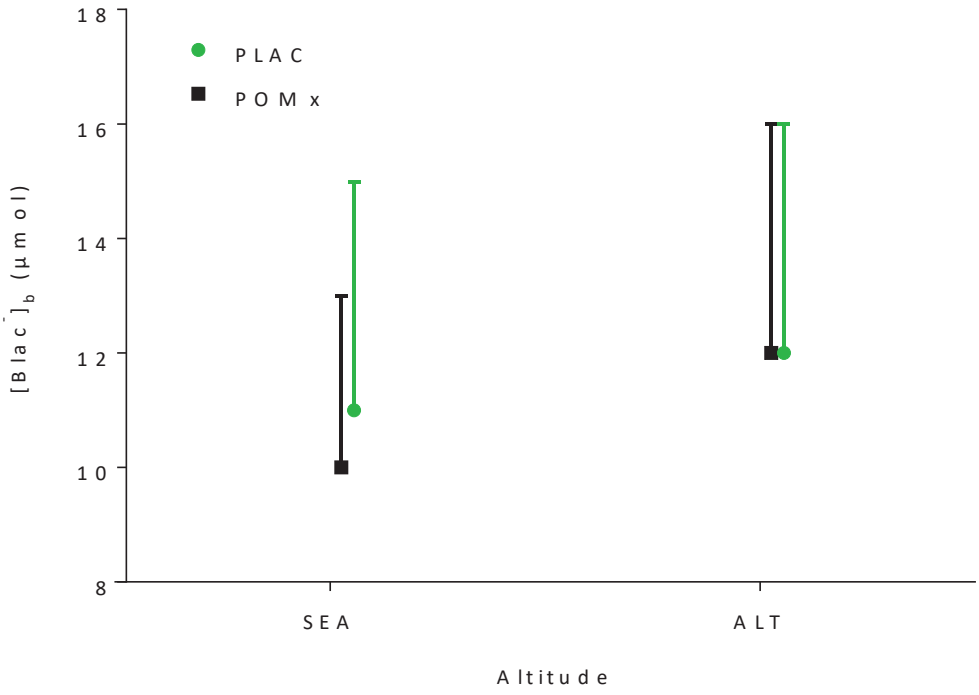

Figure 6.14 $[Blac^-]_b$ during the TTE100% following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 8.

6.3.4 Exercise performance

There was no main effect of POMx on performance during the TTE100% ($F_{1,7}$=0.776, p = 0.41). Performance was significantly decreased at ALT compared to SEA (-4.1 min, 95% CI, -6.9, -1.3, $F_{1,7}$ = 12.3, p < 0.02, ES=0.8) (Figure 6.15). The individual performance responses in the TTE100% to POMx in SEA and ALT are displayed in Figure 6.18. Despite no overall significant effect of POMx, there appear to be two participants (3 and 6) who increased their performance with POMx at both altitudes.

Perception of effort (RPE) during the submaximal stages was also unaffected by POMx ($F_{1,7}$ = 2.52, p = 0.16) and ALT ($F_{1,7}$ = 0.002, p = 0.97), but increased significantly with greater exercise intensity ($F_{1,7}$ = 156, p < 0.0001) (Figure 6.16).

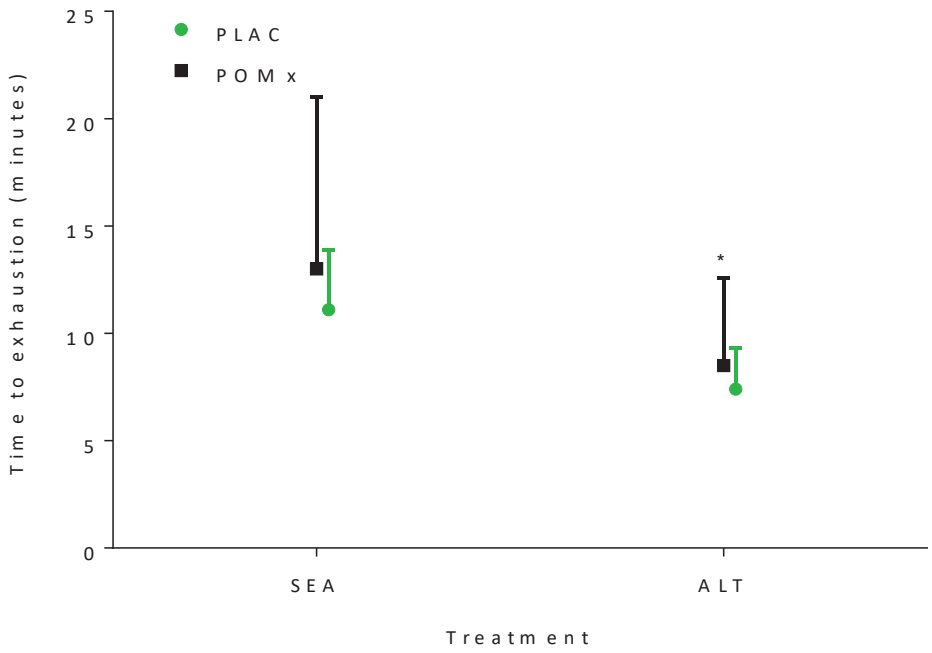

Figure 6.15 Performance in the TTE100% following PLAC and POMx, in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to SEA

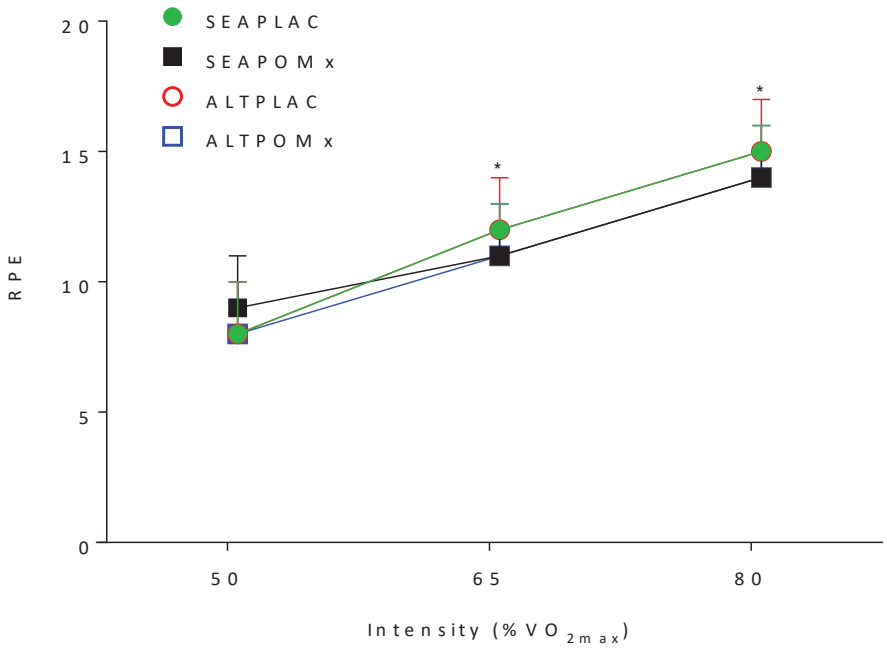

Figure 6.16 RPE during submaximal exercise following PLAC and POMx in SEA and ALT. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

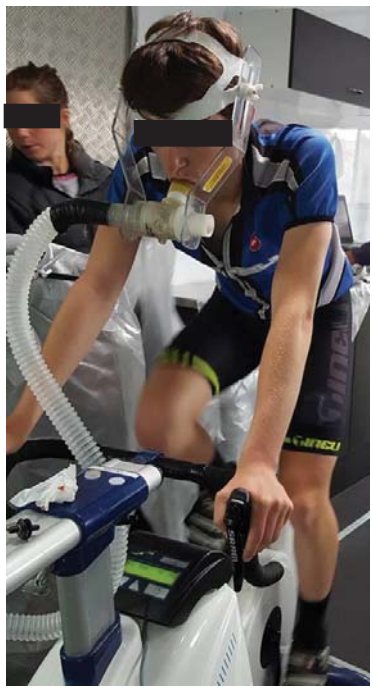

Figure 6.17 A participant completing the exercise protocol in the mobile laboratory

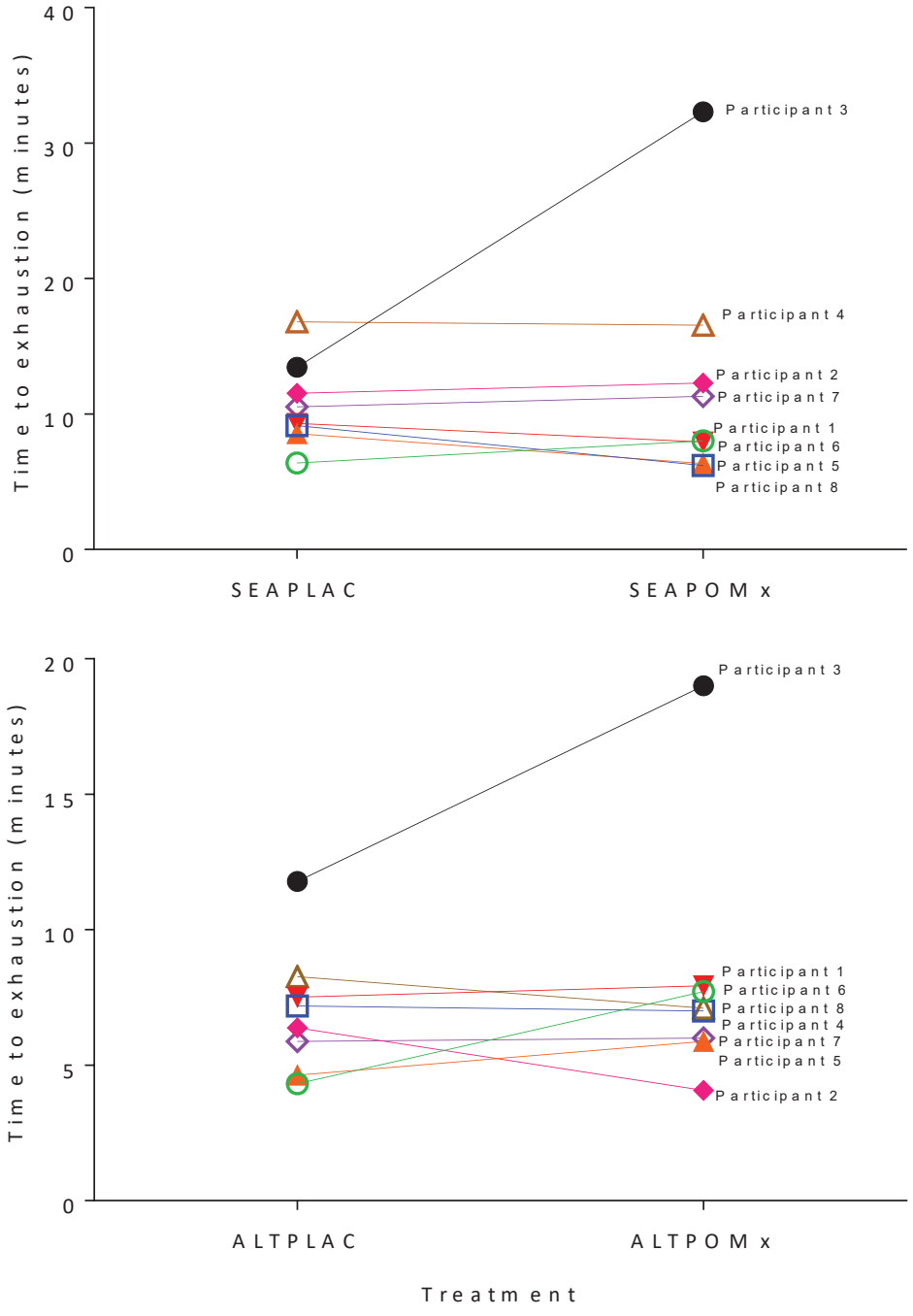

Figure 6.18 Individual performances in the TTE100% following PLAC and POMx in ALT and SEA

6.4 DISCUSSION

This study was designed to test the hypothesis that acute POMx supplementation would increase plasma NO concentration, reduce the submaximal O_2 cost of cycling exercise, and in doing so, improve strenuous aerobic performance in an environment (high altitude) where O_2 availability may be limiting. Although POMx was associated with a 10.3 ± 3.7 µmol increase in plasma NO_3^-, our data do not support the use of POMx as an ergogenic supplement as neither submaximal VO_2 nor performance were significantly altered by POMx ingestion. However, at a workload prescribed to elicit 100% VO_{2max} (at sea level), POMx enabled maintenance of VO_2 during exercise in low PO_2 conditions, indicating that the supplement may have some effect on O_2 transport or uptake under conditions of lowered O_2 availability.

6.4.1 Cardiorespiratory responses

To the author's knowledge, the current research is the first study to have measured VO_2 during cycling exercise following supplementation with POMx. The lack of effect of POMx on submaximal VO_2 or VCO_2 values in either environment despite an increase in plasma NO_3^- is in accordance with previous research involving acute supplementation with NO_3^--rich BRJ in highly-trained athletes (VO_{2max} > 66 ml $^{-1}min^{-1}kg^{-1}$) conducted at sea-level (Bescós et al., 2011; Nyakayiru et al., 2016; Peacock et al., 2012; Porcelli et al., 2015) or in low PO_2 conditions (13-15% O_2) (Arnold et al., 2015; MacLeod et al., 2015). In addition, the absence of change in VO_2 during the TTE100% at SEA supports the findings of Boorsma et al. (2014) who conducted a running protocol involving similar intensities to the current study, and found no significant difference in VO_2 values during a 1500m time trial. The restoration of

an ALT-induced lowering in VO_2 during intense exercise following POMx compared to SEA (+3.8 $L.min^{-1}$, 95% CI, -5.7, 9.5, p=0.001, ES=0.6) differed from previous NO_3^--based research involving highly-trained participants, although no other studies using hypoxia and VO_2 measurement have utilized a performance test protocol similar to the current study. Masschelein et al. (2012) found no change in VO_{2max} recorded during a graded exercise test to exhaustion in hypoxic conditions (11% O_2, ~5000m altitude) despite a 4% increase in the muscle tissue oxygenation index. However, in that study, exercise intensity was not stable, as it was in the current study and VO_2 was measured at exhaustion, rather than part-way into the exercise. In addition, MacLeod et al. (2015) recorded no change in VO_2 during a 10km cycling time trial completed in a normobaric hypoxic chamber (equivalent to ~2440m altitude). However, the duration of this test was significantly longer than ours, and was done under normobaric, rather than hypobaric hypoxia conditions, both of which may have influenced potential effects on VO_2.

The changes in VO_2 during the TTE100% in the current study were likely driven by the high polyphenol content of POMx, as POMx has a lower NO_3^- concentration than BRJ. While BRJ-based research in highly-trained athletes has recorded increases in plasma NO_3^- of 31-1907% (Boorsma et al., 2014; Cermak et al., 2012; Kelly et al., 2014a), the current study measured a 44% increase in NO_3^- with POMx. Although polyphenolic compounds are also present in BRJ, these are predominantly from the quercetin sub-class, in comparison to the high ET component in POMx (Kazimierczak et al., 2014). Previous research has generally found no link between quercetin intake, and changes in VO_2 or performance parameters (McAnulty et al., 2008; Quindry et al., 2008). However, an increase in pre or post-exercise blood flow and vessel diameter has been observed with consumption of ET or anthocyanin-containing fruit juices and extracts (Barona et al., 2012; Roelofs et al., 2016; Trexler et al.,

2014). Further, Ignarro et al. (2006) demonstrated that POMJ is more effective at protecting NO from breakdown than other polyphenol-containing juices and red wine, with significant antioxidant actions occurring at dilutions greater than 1000-fold. Thus, the increased VO_2 during the TTE100% following POMx supplementation may be due to a polyphenol-induced greater NO bioavailability.

Despite the large amount of research indicating a relationship between a supplement-induced increase in NO and changes in VO_2 during exercise, presumably through an increase in the efficiency of O_2 transport, the mechanisms behind these changes are currently irresolute. Although NO is known to mediate vasoactivity, an overall increase in vasodilation does not explain the reduction in VO_2 during submaximal exercise in normoxia, which has been observed in other studies (Bailey, Fulford, et al., 2010; Bailey et al., 2009; Breese et al., 2013; Wylie et al., 2013). Rather, the predominant mechanistic theories to explain the effects of NO on VO_2 involve an increase in either the efficiency of mitochondrial O_2 usage or in the muscular use of adenosine triphosphate (ATP), affording a lower VO_2 requirement to sustain a given work rate (Bailey, Fulford, et al., 2010; Larsen, Weitzberg, Lundberg, & Ekblom, 2010). Lansley, Winyard, Bailey, et al. (2011) provided indirect evidence for a mitochondrial-based effect with acute BRJ intake through an increase in the phosphate/VO_2 ratio, a measure of the ATP yield per O_2 molecule cycled through the electron transport chain, during two cycling time trials. On the other hand, Bailey, Fulford, et al. (2010) gave evidence for changes at the muscular level, via a reduction in post-exercise creatine phosphate degradation and estimated ATP turnover, and a trend towards a decrease in energy derived from oxidative phosphorylation during low and high-intensity knee extension. These results were indicative of a reduction in the energy required to develop a given amount of force. However, during exercise under hypoxic

conditions, a NO-induced augmentation in vasodilation has been shown to be important in maintaining blood flow to the active muscles (Casey et al., 2010). Thus, it is likely that under hypoxic conditions, the vasoactive role of NO contributes more greatly to VO_2.

6.4.2 Oxygen saturation responses

SmO_2 is a marker of local tissue oxygenation and gives a valid and reliable indication of O_2 uptake at the working muscles themselves (Crum, O'Connor, Van Loo, Valckx, & Stannard, 2017). Thus, given the lack of changes in whole-body VO_2 with POMx, it is not surprising that there were no significant effects of supplementation on SmO_2. Accordingly, Bond, Morton, and Braakhuis (2012) found no changes in SmO_2 following six days of BRJ supplementation (5.5 $mmol.d^{-1}$) despite an increase in performance during repeated rowing time trials. On the other hand, Masschelein et al. (2012) recorded a 4% increase in local oxygenation of the *vastus lateralis* during maximal cycling exercise with acute BRJ supplementation in very high altitude conditions. When the results of these studies are combined, they give further evidence to the potential mechanisms described in 6.4.1, which suggest that changes in VO_2 following an increase in NO are likely to be due to alterations in efficiency at the mitochondrial or muscular level, but the vasoactive role of NO becomes more important in hypoxic conditions. The disparity between the current study and that of Masschelein et al. (2012) may be due to the higher altitude, and thus greater PO_2 limitation, used in the latter (~5000 m). Alternatively, it is possible that the lack of change in our study was influenced by the small number of full data sets (n = 4) obtained for SmO_2 in ALT, which made it less likely that the power of the study would be high enough for a significant result to be obtained, and may have led to a type II error (Moher, Dulberg, & Wells, 1994).

Further, the SmO_2 data collected at SEA was in accordance with our previous observations (Crum et al., 2017), whereby values decrease as greater amounts of O_2 are taken up by the muscles. However, at ALT, SmO_2 increased from stage 1 to stage 2, before decreasing in stage 3. It is likely that the colder temperatures at ALT necessitated a longer warm-up period compared to SEA and therefore, the increase in SmO_2 in stage 2 reflects the greater duration taken for blood flow (and thus O_2 delivery) to the active muscles to increase following the onset of exercise. However, given the time restraints required to allow all participants to be tested on the same day, it was necessary to ensure each trial took less than 1 hour to complete.

As predicted, based on the acute nature of the current study, there were no significant changes in THb due to POMx or ALT. A change in this parameter requires alterations in either the production or degradation of Hb proteins, which is not likely to be seen over a period of several hours unless prolonged, intense exercise has been completed (Mairbäurl, 2013). In accordance with our previous validation study (Crum et al., 2017), THb was also not affected by exercise intensity, and showed little variation within each exercise period.

6.4.3 Performance during the TTE100%

The absence of changes in performance during the TTE100% is in agreement with Trinity et al. (2014) who demonstrated no effect of acute POMJ supplementation on a cycling time trial or TTE protocol in hot conditions in moderately-trained individuals. However, Trexler et al. (2014) found an increase in TTE during treadmill running at 90-100% of peak velocity, in moderately trained participants, following acute supplementation of POMx (1000mg, 30min before exercise) at sea-level. In addition, POMx has been shown to improve

performance and recovery from resistance and sprint cycling exercise (Machin et al., 2014; Trombold, Reinfeld, Casler, & Coyle, 2011). The current study is the first study to test POMx supplementation in highly-trained endurance athletes and is in accordance with previous research involving acute BRJ supplementation in this cohort, finding no change in performance in a running or cycling time trial in hypoxic conditions (Arnold et al., 2015; MacLeod et al., 2015). However, two studies involving multi-day periods of NO_3^- supplementation in low PO_2 conditions (11-13% O_2) showed changes in both O_2 parameters and exercise performance in moderately-trained individuals (VO_{2max} 58-61 $ml.min^{-1}kg^{-1}$). Kelly et al. (2014b) found that three days of BRJ supplementation (~8.4 $mmol.day^{-1}$) decreased steady-state VO_2 during a bout of moderate-intensity cycle exercise by 7.6% and this resulted in an 8% improvement in a subsequent high intensity TTE protocol, compared to exercise done following the intake of a placebo. Masschelein et al. (2012) found that six days of BRJ supplementation (~5$mmol.day^{-1}$) increased arterial O_2 saturation by 2.7% during submaximal cycling, resulting in a 5% increase in performance during a subsequent cycling graded exercise test. Thus, the small increase in VO_2 with acute supplementation of POMx may not be sufficient to produce an ergogenic effect during a 100%TTE in highly-trained athletes and a longer period of supplementation may be necessary. Alternatively, the absence of change in performance despite an increase in VO_2 may reflect the lack of correlation between VO_2 and performance times (r^2=0.0001), which indicates that VO_2 was not the determining factor in the TTE100%. Rather, it is likely that other factors, such as anaerobic capacity, or central fatigue, contribute more greatly to performance in a high-intensity TTE of a fixed-load nature.

Several reasons have been proposed for the lower efficacy of NO_3^- supplementation on submaximal VO_2 and performance in trained compared to untrained individuals. Firstly, as a

response to training, athletes tend to have elevated NOS activity (McConell et al., 2007), and higher resting NO_2^- and NO_3^- values (Vassalle, Lubrano, Domenici, & L'abbate, 2003). Consequently, they may have a lower requirement for NO_3^-–NO_2^-–NO pathway, and have sufficient NO_3^- present in the blood to use it when needed. Further, training adaptations which aid in O_2 transport and energy production, such as increased capillarization and mitochondrial density, may reduce the incidence of acidic and hypoxic muscular environments, which decrease NOS activity, and increase reliance on the NO_3^- pathway (Jensen, Bangsbo, & Hellsten, 2004; Wilkerson et al., 2012). Finally, research in rats has suggested that the NO_3^- pathway is predominantly used in type II muscle fibres, which work more frequently under acidic or hypoxic conditions (Ferguson et al., 2013). If this is the case, NO_3^- supplementation may be more effective in untrained individuals who tend to have a greater percentage of type II fibres (Holloszy & Coyle, 1984). However, currently, there is no direct evidence that type II fibres are preferentially affected in humans. Alternatively, the lack of overall performance response may be due to the presence of 'responders' and 'non-responders' to POMx. Despite no overall significant effect of POMx on performance in the current study, there were two participants who increased performance in both altitudes. Thus, a larger sample size of participants may be needed to determine the ratio of responders vs. non-responders in a highly-trained population.

6.4.4 Blood pressure and haematocrit responses

In addition to the results already presented in the discussion, the current study produced two results which are difficult to explain. Firstly, in contrast to previous research which has reported a lowering in SBP following acute BRJ or ET polyphenols from grapes in sedentary or moderately-trained individuals (Bailey et al., 2009; Barona et al., 2012; Vanhatalo et al.,

2011), and no effect on SBP following BRJ supplementation in highly-trained individuals (Cermak et al., 2012; MacLeod et al., 2015; Nyakayiru et al., 2016; Wilkerson et al., 2012), this study found a trend towards an increase in SBP with POMx (+3.9 bpm, 95% CI, -0.6, 8.5, $F_{1,7}$ = 4.28, p = 0.08). The only explanation we can give for this result is that the increase in SBP was a reactive response by the vasculature to ensure that the mean arterial pressure and thus, vessel perfusion and blood flow, were maintained, despite the NO-induced vasodilation.

Further, being an acute intervention, we did not anticipate changes in Hct with POMx, but surprisingly, there was a significant decrease in Hct following POMx compared to PLAC (-0.76%, 95% CI, -1.3, -0.2, $F_{1,7}$ = 10.5, p = 0.014), which indicated a small reduction in the O_2-carrying capacity of the blood. However, despite being significant, this change had a small effect size (ES = 0.2) and considering the increase in VO_2 with POMx at ALT, did not appear to affect VO_2 capacity. Hct varies from day to day by ~3% (Thirup, 2003) and it is possible that this result was due to differences in hydration between tests, as we did not measure pre-exercise hydration status, or standardize water intake during exercise. However, both these explanations are purely speculative and further research is required to determine whether POMx supplementation consistently results in similar effects on SBP and Hct.

6.4.5 Study limitations

A limitation to the current study is the relatively low altitude used in the hypoxic condition compared to the altitude generally associated with physiological changes (2500–3000 m) (Wilber, 2011) and that simulated in previous studies involving NO_3^- supplementation in hypoxia (~2500-5000m) (Arnold et al., 2015; Kelly et al., 2014a; Masschelein et al., 2012).

Due to our study being conducted in a mobile laboratory, we were restricted to areas with vehicle access in the North Island of New Zealand, and conducted the study at the highest altitude possible under these conditions. Further, the strength of conducting the study in a high-altitude environment, in comparison to the altitude chambers which have been used in previous research (Arnold et al., 2015; Kelly et al., 2014a; Masschelein et al., 2012), is the ability to test the supplement in hypobaric rather than normobaric conditions. Thus, the current study reflects the effect of POMx supplementation in true low PO_2 conditions, rather than just low O_2 conditions. However, future studies could investigate the potential ergogenic benefit of POMx at a higher altitude of >2500m.

In addition, the current study was limited by a relatively small sample size (n = 8), which was unavoidable due to the research taking place during a high-performance junior training camp. While previous research has demonstrated a reduction in VO_2 during submaximal cycling exercise (Bailey, Fulford, et al., 2010; Bailey et al., 2009), it is acknowledged that the lack of effect of POMx on submaximal VO_2 in the current study may have been due to an insufficient number of participants. Finally, the loss of battery life from the Moxy monitor in four participants on one of the ALT trials reduced the power of our statistical analyses for SmO_2 and THb. It is possible a full set of data may have shown significant changes in these parameters, although an analysis of the SEA data only did not show any effects of POMx.

6.4.6 Conclusions

In conclusion, acute POMx supplementation allowed a partial restoration of VO_2 during intense exercise in a hypoxic environment. However, no significant changes in VO_2 occurred during submaximal exercise and there was no effect of POMx on performance in either

environment. It is possible that acute POMx supplementation is not sufficient to influence VO_2 during normoxic conditions in highly-trained athletes, and a longer period of supplementation may be required. However, based on the results from the current study, the use of POMx as an ergogenic supplement is not supported.

CHAPTER 7. THE COMBINATION OF THIOL AND POLYPHENOL SUPPLEMENTATION

7.1 Introduction

In the previous chapter, acute POMx supplementation attenuated a hypoxia-induced decline in oxygen (O_2) uptake (VO_2) during a cycling time trial to exhaustion at a fixed workload calculated to elicit 100% of maximal O_2 consumption (VO_{2max}) in highly-trained cyclists. However, no changes in VO_2 were recorded during submaximal cycling or in normoxic conditions. It was concluded that, in our highly-trained participants, acute supplementation was insufficient to influence O_2 transport parameters, and a multi-day protocol may be required for this cohort.

As previously described, athletes have greater basal NO_2^- and NO_3^- concentrations and levels of NOS activity as a result of training adaptations. Thus, it has been suggested that in order to produce a meaningful increase in plasma NO, greater levels of dietary NO-stimulating supplements may be required (Jonvik, Nyakayiru, van Loon, & Verdijk, 2015). Although prior to this thesis, no previous research has measured the effect of POMx supplements in trained individuals, multi-day supplementation protocols using products containing its two components of interest—nitrates (NO_3^-) and polyphenols—have been used with success in this population. For example, acute supplementation with NO_3^--rich beetroot juice (BRJ) does not alter VO_2 during a running or cycling time trial at altitude (Arnold et al., 2015; MacLeod et al., 2015) but multiple days of BRJ supplementation has been shown to decrease VO_2 during moderate-intensity cycling exercise (Kelly et al., 2014a),

and improve O_2 saturation in the *vastus lateralis* during submaximal and maximal cycling exercise (Masschelein et al., 2012). Similarly, the majority of research which has shown ergogenic effects of polyphenol supplementation in trained athletes has used protocols of three to six weeks (Eichenberger, Colombani, & Mettler, 2009; Lafay et al., 2009; Skarpańska-Stejnborn, Basta, & Pilaczyńska-Szcześniak, 2006), further suggesting that prolonged supplementation is required.

In addition, the previous chapters in the current thesis have investigated the effects of thiol and polyphenol supplementation separately, but there is some evidence that the two may have a greater influence on O_2 transport if ingested together; an increased thiol concentration may reduce the degradation of nitric oxide (NO) by superoxide, thereby augmenting the effect of a polyphenol-induced increase in the compound, while also preventing an increase in peroxynitrite generation (Arteel et al., 1999). Further, the combination of thiols with NO and O_2 may increase the formation of *S*-nitrosothiols (SNO), thus improving the stability and half-life of NO (McMahon et al., 2016). Accordingly, McKinley-Barnard et al. (2015) found that when glutathione (GSH) was co-supplemented with L-citrulline (CIT), a component of the endogenous NO production pathway, the combination of the two was more effective at increasing markers of NO concentration and activity, than with CIT alone. However, no previous studies have measured the effects of a supplementation protocol of this nature in athletes or on markers of O_2 transport or endurance exercise performance.

Therefore, the current study had two major hypotheses: Firstly, that eight days of POMx supplementation would decrease the VO_2 required to perform submaximal cycling exercise through an increase in plasma NO concentration (as indicated by an improvement in plasma

NO_3^-), and increase performance in a subsequent time trial. Secondly, it was predicted that these improvements would be augmented by co-supplementation with a thiol supplement.

7.2 METHODS

7.2.1 Participants

Nine moderately-trained cyclists (three females) were recruited from the regional cycle and triathlon communities. The criteria for inclusion were regular participation in endurance cycling exercise, at least three times per week, in the three months prior to commencement of the study. The participants' baseline characteristics are presented in Table 7.1. One participant (female) did not complete the study due to a non-study related injury, and is not included in any of the data presented. Prior to participation in the study, the participants were notified of all the potential risks and benefits associated with the study, as described in 4.2.1. This study was approved by the Massey University Human Ethics Committee (Southern A 16/21) in accordance with the Declaration of Helsinki.

Table 7.1 Baseline characteristics of participants in Study 3

Participant	Gender	Age (years)	Mass (kg)	Height (m)	VO_{2max} ($ml.min.kg^{-1}$)
1	Male	47	90.0	1.82	55.1
2	Male	40	82.5	1.82	54.8
3	Female	27	54.3	1.60	62.7
4	Female	20	54.5	1.67	52.9
5	Male	30	72.0	1.75	54.9
6	Male	50	77.8	1.73	46.0
7	Male	35	80.6	1.80	43.9
8	Male	49	72.0	1.72	67.3
Mean ± SD		37 ± 10	73.0 ± 12.0	1.74 ± 0.7	54.7 ± 7.2

7.2.2 Pre-study measures

In order to determine workloads for the supplemented trials, the participants completed a four-stage, submaximal exercise test, followed by an incremental ramp to exhaustion, on an electronically-braked cycle ergometer (see 4.1.1) as described in the general methodology (see 4.3.1 and 4.3.2). Subsequently, the power outputs to elicit 50%, 65%, 80% and 90% VO_{2max} were calculated (see 4.3.3).

7.2.3 Study Design

The study was a cross-over design, with four eight-day periods of supplementation, each separated by a one-week washout period, based on a previous NAC supplementation protocol used by Zembron-Lacny *et al.* (2010). On the eighth day of supplementation, an

exercise protocol, involving submaximal and intense cycling exercise, was performed. A diagram showing the timeline for the study is shown in Figure 7.1

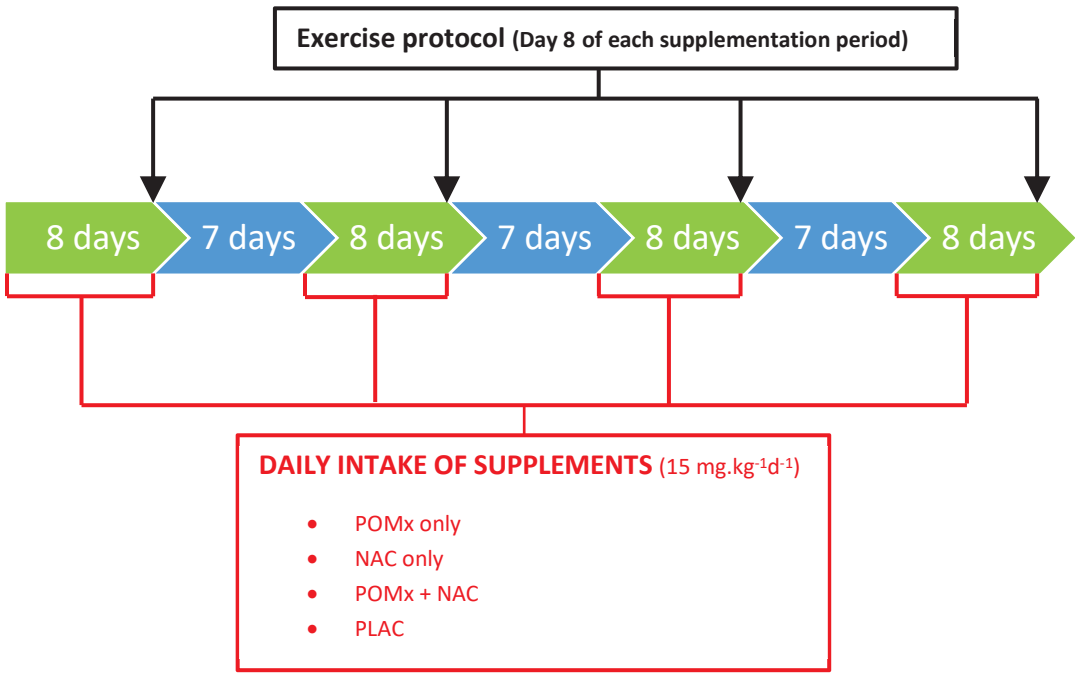

Figure 7.1 Design of Study 3

7.2.4 Supplementation

The four supplementation protocols were completed in a randomized order, with the supplement blinded to the participant and the researcher. The participant was instructed to swallow the capsule whole, without tasting it, to avoid an expectation bias. Each supplement was ingested in capsule form, in a quantity of 15 $mg^{-1}kg^{-1}day^{-1}$ (815–1350 $mg^{-1}day^{-1}$), based on similar quantities used in successful POMx and thiol supplementation protocols employed by Trexler et al. (2014) and Zembron-Lacny et al. (2010). The protocols were as follows: POMx only (POM Wonderful LLC, USA), thiol only (*N*-acetylcysteine (NAC),

Solgar, UK), POMx and thiol combined (BOTH) and placebo (PLAC; Stevia, NuNaturals, Oregon). In the BOTH condition, a total of 30 $mg^{-1}kg^{-1}day^{-1}$ of supplements were ingested (15 $mg^{-1}kg^{-1}day^{-1}$ POMx + 15 $mg^{-1}kg^{-1}day^{-1}$ NAC). Thus, in BOTH, the total amount of supplementation was twice as much as in the other conditions. However, this was necessary to ensure that any effects seen with BOTH could be attributed to the interaction between the two compounds, rather than being due to having a different quantity of a particular compound. As described in the previous chapter (see 6.2.4), previous analyses of the POM Wonderful LLC supplement have shown it to contain substantial amounts of polyphenols (Trinity et al., 2014). NAC was chosen as the thiol supplement because it has previously been shown to improve VO_2 and exercise performance, and unlike the previously-used keratin supplement, can be taken in capsule form (Reid et al., 1994; Slattery et al., 2014; Zembron-Lacny et al., 2010). In accordance with previous research, the final supplement was ingested 2.5 hours prior to each experimental trial to allow maximal absorption prior to exercise (Seeram et al., 2004). Participants were provided with a list of NO_3^- and polyphenol-rich foods (Appendix A.8) and asked to limit intake of these during the supplementation periods, as well as avoiding antibacterial substances, such as mouthwash, which destroy NO_3^-–nitrite converting bacteria on the tongue (Govoni et al., 2008). Participants received one week's worth of supplements at the beginning of each week during the supplementation period.

7.2.5 Exercise protocol

The exercise protocol was completed on five occasions, firstly as a familiarization session, and then following each of the four supplementation protocols. Prior to the first supplemented trial, participants completed a 48-hour training diary (see 4.2.4.1) which was

replicated for all following trials. They also consumed a standardized meal, 2.5 hours prior to exercise, containing approximately 0.8 g carbohydrate, 0.25 g protein and 0.2 g fat, per kg of bodyweight.

On arrival to the laboratory, body mass was obtained and a heart rate (HR) monitor (Garmin, Kansas, USA) was applied, which recorded HR every two seconds throughout the trial (see 4.3.5). A muscle O_2 monitor (Moxy, Fortiori Design LLC, Minnesota, USA) was positioned on the *vastus lateralis* muscle halfway between the greater trochanter and the lateral epicondyle of the femur as detailed in the general methodology, and recorded local muscle O_2 saturation (SmO_2) and total Hb (THb) throughout the trial, as described previously (see 4.3.9). In contrast to the previous chapter, a Moxy monitor was used on each leg, and the average value between legs was calculated for each data point, to reduce the chance of data points being lost.

After sitting in a supine position for five minutes, systolic (SBP) and diastolic (DBP) blood pressure of the brachial artery was measured using an automated sphygmomanometer (see 4.3.4). To determine haematocrit (Hct), blood was collected via fingerprick sample (~35 µL) in heparanized capillary microtubes and immediately spun in a microhaematocrit centrifuge (see 4.3.7. A venous blood sample was also collected via venepuncture from the antecubital vein in heparinized vacutainers and immediately spun in a centrifuge and plasma was stored at -80^{o}C as described in the general methodology (see 4.3.8).

Following the pre-exercise measures, the participant mounted the ergometer to begin cycle exercise. The experimental protocol began with three six-minute stages of stationary cycling exercise at power outputs corresponding to 50%, 65% and 80% of their previously

determined VO_{2max}. In the last minute of each stage, VO_2 was measured as previously described and perceived exertion (RPE) was recorded (see 4.3.10). Then, following a five-minute rest, a linear factor was applied to the ergometer (see 4.1.1) which was based on a workload corresponding to 90% of the participant's previously measured VO_{2max} at 90 rpm and participants completed a five-minute 'best effort' time trial. This workload was selected based on previous research in our laboratory involving time trial efforts of this length. SmO_2, THb and HR were recorded throughout the time trial, with the data collected being analysed for average values across the time trial as a whole, and across the last minute of the time trial only. Post-exercise measures of BP, HR, SmO_2, THb and Hct were recorded after resting in a supine position for five minutes after the time trial.

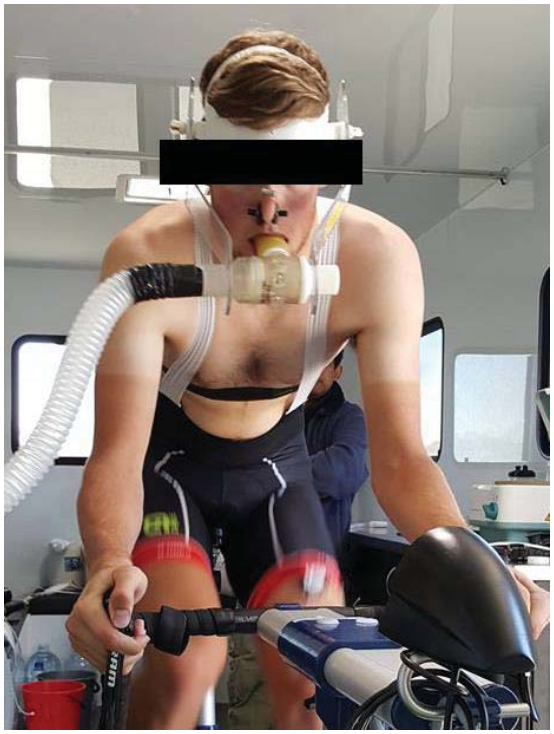

Figure 7.2 A participant completing the submaximal part of the exercise protocol

7.2.6 Blood Analysis

Plasma samples were analysed for NO_3^- content using a Nitric Oxide Colorimetric Assay Kit, as described in the general methodology (see 4.4.2)

7.2.7 Statistical Analyses

Statistical analyses to compare the values of all variables measured were done using statistical computer software (SPSS Statistics, Version 23, IBM Corporation). Three-way repeated measures ANOVA were used to test the significance level for main effects of treatments containing POMx (POMx, i.e. POMx, BOTH vs. no POMx, i.e. NAC, PLAC), treatments containing NAC (NAC vs. no NAC), and intensity (INT; 50, 65 and 80%VO_{2max}) for all submaximal exercise variables. Similarly, pre- and post-exercise measures were analysed using three-way repeated measures ANOVA (POMx x NAC x TIME). This method of analysis allowed identification of the effects of POMx and NAC regardless of treatment condition, as opposed to a design which just analysed the four treatment conditions individually. Time trial measures were analysed for main effects and interactions between POMx and NAC using two-way repeated measures ANOVA.

In all analyses, Mauchly's Test of Sphericity was used to validate the results of each ANOVA and in cases where aspherity was detected, the Greenhouse-Geiser correction was applied. Statistical significance was accepted when $p \leq 0.05$, while a tendency was noted when $p \leq 0.1$. Following each ANOVA, where significant main effects or interactions were observed, two-tailed paired t-tests with a Fisher's Least Significant Difference (Fisher's LSD) post-hoc analysis were used to identify the location of the significance. The effect size of significant interactions was calculated using Cohen's d. In accordance with Cohen (1988), effect sizes

(ES) were classified as small (0.1), medium (0.5) or large (0.8). Data is presented as mean ± standard deviation (SD) or mean change and 95% confidence interval (CI), as appropriate. Graphs were produced using Prism 6.0 (GraphPad Software, CA, USA).

7.3 RESULTS

Participants' self-reported adherence to supplement intake was 100% and no major harmful side effects of supplementation were reported, apart from minor gastrointestinal issues (bloating) with NAC and BOTH in three participants. Participants were unable to determine which treatment they were on during each intervention period.

7.3.1 Resting measures

Plasma NO_3^- values are presented in Figure 7.3. The blood analysis was unsuccessful for one participant, so this figure represents average values from seven participants only. Plasma NO_3^- were significantly greater following treatment conditions containing POMx compared to no POMx (+12.9 µM, 95% CI, 3.7, 22.1, $F_{1,6}$ = 13.1, p < 0.02, ES = 0.9) but were not affected by NAC ($F_{1,6}$ = 0.281, p = 0.62). Post-hoc analysis revealed that although there was a significant increase in NO_3^- with POMx compared to PLAC (+17 µM, 95% CI, 6.7, 27.2, $F_{1,6}$ = 18.0, p = 0.008, ES = 1.0), there was no significant difference between POMx and BOTH ($F_{1,6}$ = 2.12, p = 0.21).

Pre- and post-exercise responses to treatment are presented in Table 7.2. Resting BP was not significantly affected by POMx (SBP, $F_{1,7}$ = 1.88, p = 0.21; DBP, $F_{1,7}$ = 0.535, p = 0.49) or NAC (SBP, $F_{1,7}$ = 1.13, p = 0.32; DBP, $F_{1,7}$ = 0.753, p = 0.41). In addition, neither resting Hct nor resting HR were affected by POMx (Hct, $F_{1,7}$ = 1.37, p = 0.28; HR, $F_{1,7}$ = 0.218, p = 0.66) or

NAC (Hct, $F_{1,7}$ = 0.710, p = 0.43; HR, $F_{1,7}$ = 0.004, p = 0.95). Similarly, muscle oxygenation was not affected by POMx (SmO_2, $F_{1,7}$ = 0.006, p = 0.94; THb, $F_{1,7}$ = 0.001, p = 0.98) or NAC (SmO_2, $F_{1,7}$ = 0.325, p = 0.59; THb, $F_{1,7}$ = 0.300, p = 0.60). None of the resting variables showed a significant POMx x NAC interaction (p > 0.05).

As expected, SBP (+7 mmHg, 95% CI, 3, 10, $F_{1,7}$ = 22.5, p = 0.002, ES = 0.5), SmO_2 (+21%, 95% CI, 15, 27, $F_{1,7}$ = 64.9, p < 0.0001, ES = 9.1) HR (+35 bpm, 95% CI, 27, 43, $F_{1,7}$ = 99.2, p < 0.0001, ES = 7.5) and Hct (+2%, 95% CI, 0.1, 4, $F_{1,5}$ = 7.35, p < 0.05, ES = 0.7) were increased post-exercise compared to pre-exercise.

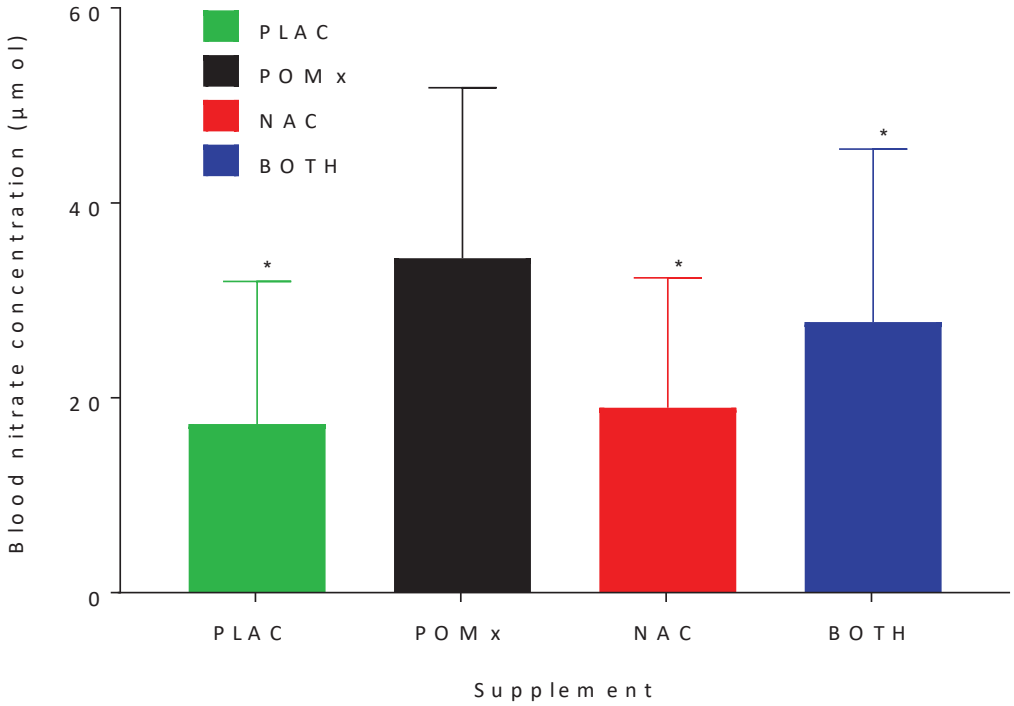

Figure 7.3 Resting plasma nitrate values following supplementation with PLAC, POMx, NAC and BOTH. Values are means ± SD, n = 7. * indicates $p \leq 0.05$ compared to POMx.

Table 7.2 Pre- and post-exercise variables following supplementation with PLAC, POMx, NAC and BOTH

Measure	PLAC	POMx	NAC	BOTH
SBP (mmHg)				
Pre-exercise	120 ± 14	120 ± 17	122 ± 12	119 ± 14
Post-exercise	132 ± 18[a]	128 ± 18[a]	125 ± 14[a]	123 ± 14[a]
DBP (mmHg)				
Pre-exercise	78 ± 11	79 ± 17	77 ± 12	77 ± 8
Post-exercise	74 ± 9	75 ± 9	72 ± 9	75 ± 6
SmO_2 (%)				
Pre-exercise	60 ± 9	60 ± 7	60 ± 6	60 ± 11
Post-exercise	82 ± 6[a]	82 ± 6[a]	80 ± 8[a]	81 ± 8[a]
THb ($g.dL^{-1}$)				
Pre-exercise	12.5 ± 0.4	12.5 ± 0.4	12.5 ± 0.4	12.5 ± 0.4
Post-exercise	12.5 ± 0.4	12.6 ± 0.4	12.6 ± 0.4	12.5 ± 0.4
Heart rate (bpm)				
Pre-exercise	57 ± 9	59 ± 10	59 ± 12	59 ± 12
Post-exercise	92 ± 13[a]	95 ± 14[a]	94 ± 18[a]	93 ± 12[a]
Hct (%)				
Pre-exercise	45 ± 3	44 ± 3	44 ± 4	43 ± 3[a]
Post-exercise	45 ± 3[a]	46 ± 5[a]	47 ± 5[a]	45 ± 5[ab]

Data are presented as means ± SD, n = 8. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; SmO_2, muscle oxygen saturation; THb, total haemoglobin; HR, heart rate; Hct, haematocrit; PLAC, placebo; POMx, pomegranate extract; NAC, N-acetylcysteine; BOTH, POMx + NAC. [a] $p < 0.05$ compared to pre-exercise. [b] $p < 0.05$ compared to PLAC.

7.3.2 Cardiorespiratory and heart rate responses during submaximal exercise

7.3.2.1 Cardiorespiratory variables

Data detailing the responses of VO_2 and VCO_2 during submaximal exercise are presented in Figure 7.4. POMx significantly decreased VO_2 (-2.1 ml.min^{-1}kg^{-1}, 95% CI, -2.8, -0.23, $F_{1,7}$ = 7.10, $p < 0.04$, ES = 0.2) and VCO_2 (-1.54 ml.min.kg^{-1}, 95% CI, -2.6, -0.45, $F_{1,7}$ = 11.1, $p < 0.02$) compared to no POMx. In contrast, there was a significant increase in VO_2 with NAC compared to no NAC (+1.9, 95% CI, 0.26, 3.6, $F_{1,7}$ = 7.48, $p < 0.03$, ES = 0.2) but no main effect of NAC on VCO_2 ($F_{1,7}$ = 3.37, p = 0.11). Post-hoc analysis revealed that VO_2 was significantly lower with POMx compared to PLAC (-2.6 ml.min^{-1}kg^{-1}, 95% CI, -4.4, -0.9, p = 0.009, ES = 0.3), NAC (-4.0 ml.min^{-1}kg^{-1}, 95% CI, -6.8, -1.1, $p < 0.02$, ES = 0.4) and BOTH (-2.5 ml.min $^{-1}$kg^{-1}, 95% CI, -5.0, -0.05, $p < 0.05$, ES = 0.2). However, there were no significant differences in VO_2 between PLAC and either BOTH or NAC, or between BOTH and NAC ($p >$ 0.5).

Post-hoc analysis for VCO_2 showed that following POMx, values were significantly lower than following NAC (-2.5 ml.min^{-1}kg^{-1}, 95% CI, -4.4, -0.5, $p < 0.03$, ES = 0.2), and there was a strong trend towards lower values with POMx compared to PLAC (-1.1 ml.min^{-1}kg^{-1}, 95% CI, -2.2, 0.05, p = 0.059, ES = 0.1). There was no difference in VCO_2 in POMx vs. BOTH, PLAC vs. BOTH or NAC, or NAC vs. BOTH ($p > 0.05$). There was a significant increase in VO_2 ($F_{1,7}$ = 103, $p < 0.0001$) and VCO_2 ($F_{1,7}$ = 76.3, $p < 0.0001$) with increasing intensity.

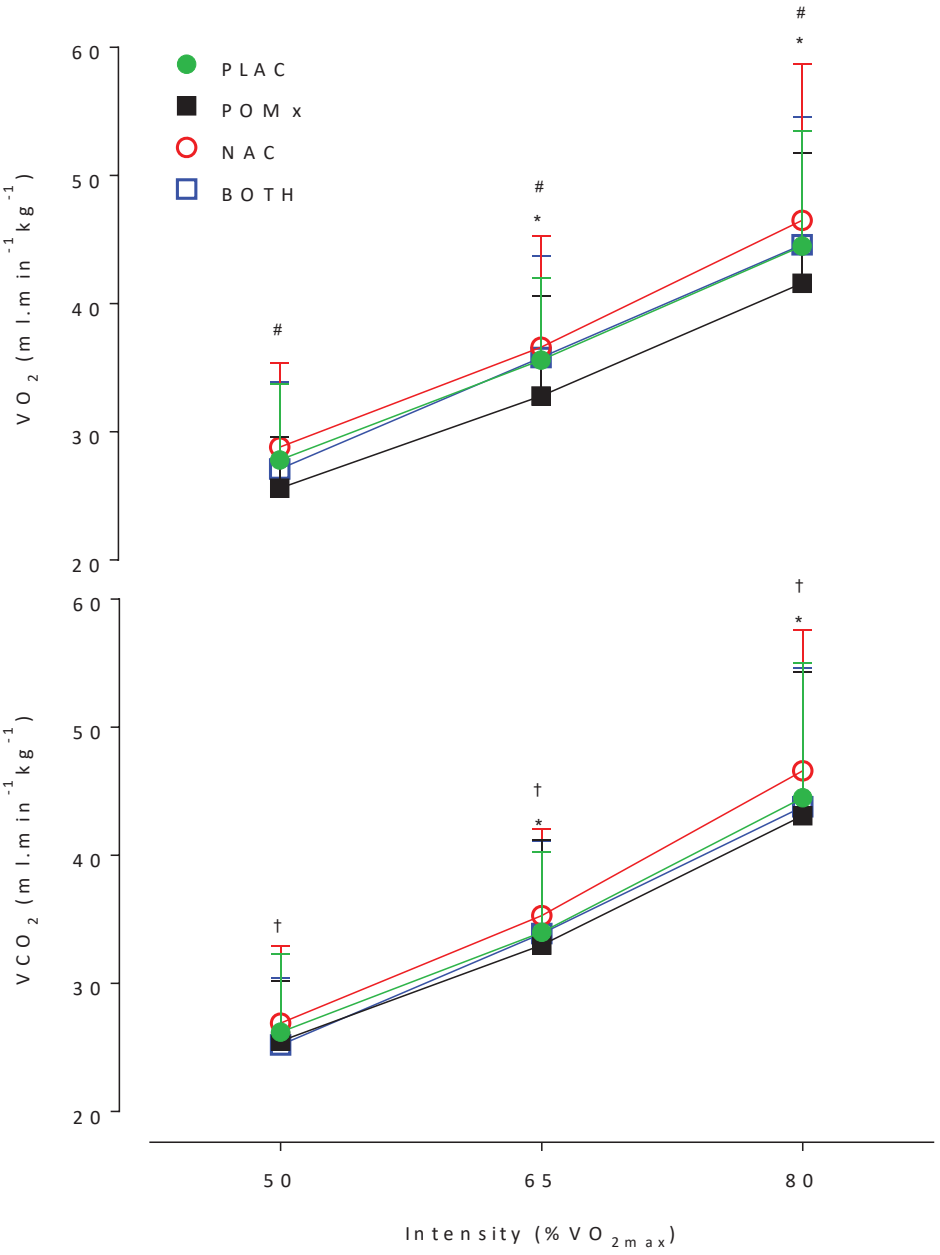

Figure 7.4 VO_2 and VCO_2 during the submaximal exercise following PLAC, POMx, NAC and BOTH. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to the previous intensity for all treatment conditions. # indicates $p \leq 0.05$ for POMx compared to all other treatments. † indicates $p \leq 0.05$ for POMx compared to NAC only.

7.3.2.2 Muscle oxygenation variables

There were no significant changes in SmO_2 or THb due to POMx (SmO_2, $F_{1,7} = 0.054$, $p = 0.82$, THb, $F_{1,7} = 0.001$, $p = 0.97$) or NAC (SmO_2, $F_{1,7} = 0.072$, $p = 0.80$, THb, $F_{1,7} = 0.141$, $p = 0.72$), or any significant interactions between POMx, NAC and INT ($F < 1.0$, $p > 0.05$). However, there was a significant decrease in SmO_2 ($F_{1,7} = 6.76$, $p = 0.001$) and increase in THb ($F_{1,7} = 8.11$, $p = 0.005$) as exercise intensity increased (Figure 7.5)

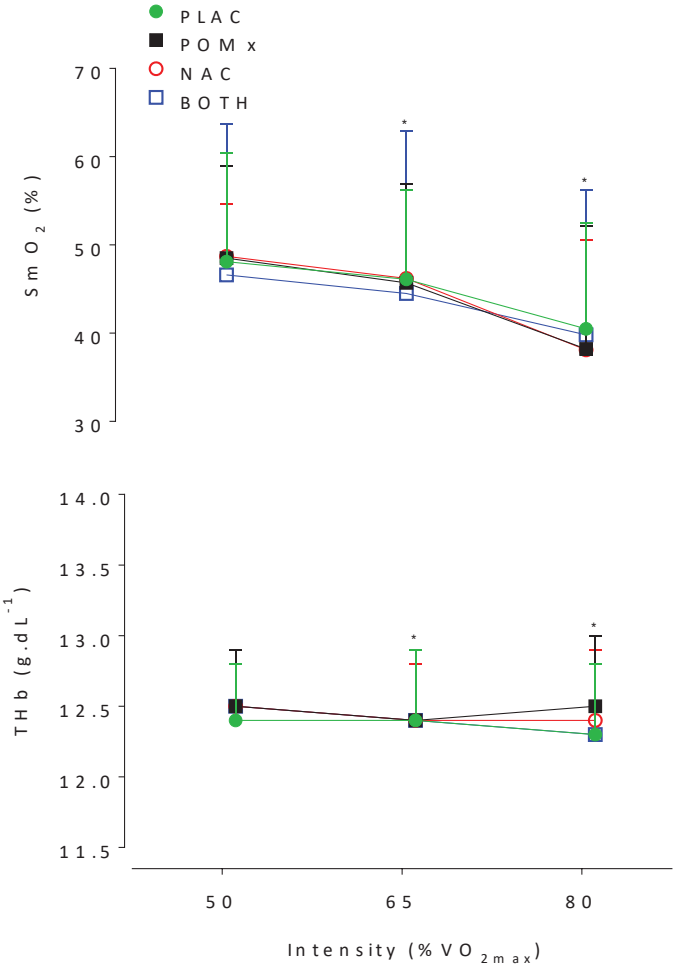

Figure 7.5 SmO_2 and THb in the *vastus lateralis* during submaximal exercise, following PLAC, POMx, NAC and BOTH. Values are means ± SD, n = 8. * indicates $p \leq 0.05$ compared to the previous intensity for all treatment conditions.

7.3.2.3 Heart rate

There was no effect of POMx on HR ($F_{1,7}$ = 0.737, p = 0.42), but there was a trend towards an increase in HR with NAC (+3.9 bpm, 95% CI, -0.26, 8.1, $F_{1,7}$ = 4.93, p = 0.06, ES = 0.2). However, post-hoc analysis revealed no significant differences between treatments. As expected, there was a significant increase in HR with increasing exercise intensity ($F_{1,7}$ = 152, p < 0.0001) (Figure 7.6)

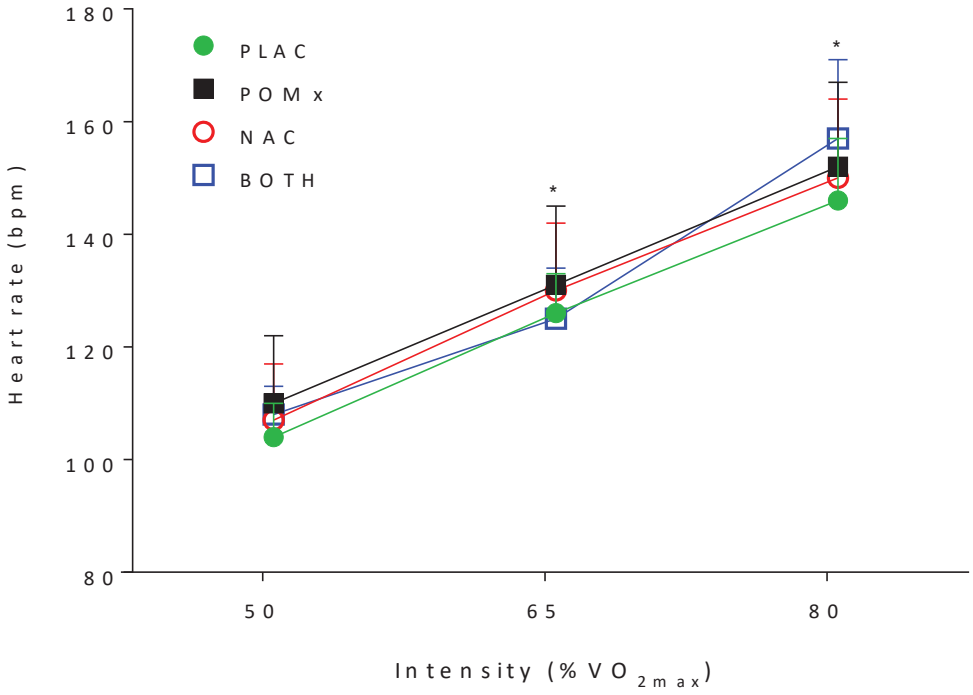

Figure 7.6 HR during submaximal exercise following PLAC, POMx, NAC and BOTH. Values are means ± SD. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

7.3.3 Muscle oxygenation and heart rate responses to the time trial

7.3.3.1 Muscle oxygenation responses

Examples of typical THb and SmO_2 responses to the five-minute time trial are shown in Figure 7.7. While THb values stayed relatively stable throughout the exercise, there was a large decrease in SmO_2 in the first minute of exercise. However, there was no significant effect of POMx or NAC on SmO_2 during the time trial as a whole (POMx, $F_{1,7} = 0.094$, $p = 0.77$; NAC, $F_{1,7} = 0.085$, $p = 0.78$) , or during the last minute only (POMx, $F_{1,7} = 0.077$, $p = 0.79$; NAC, $F_{1,7} < 0.0001$, $p > 0.99$). Similarly, THb was not affected by POMx or NAC in either analysis of the time trial ($F < 1.0$, $p > 0.05$).

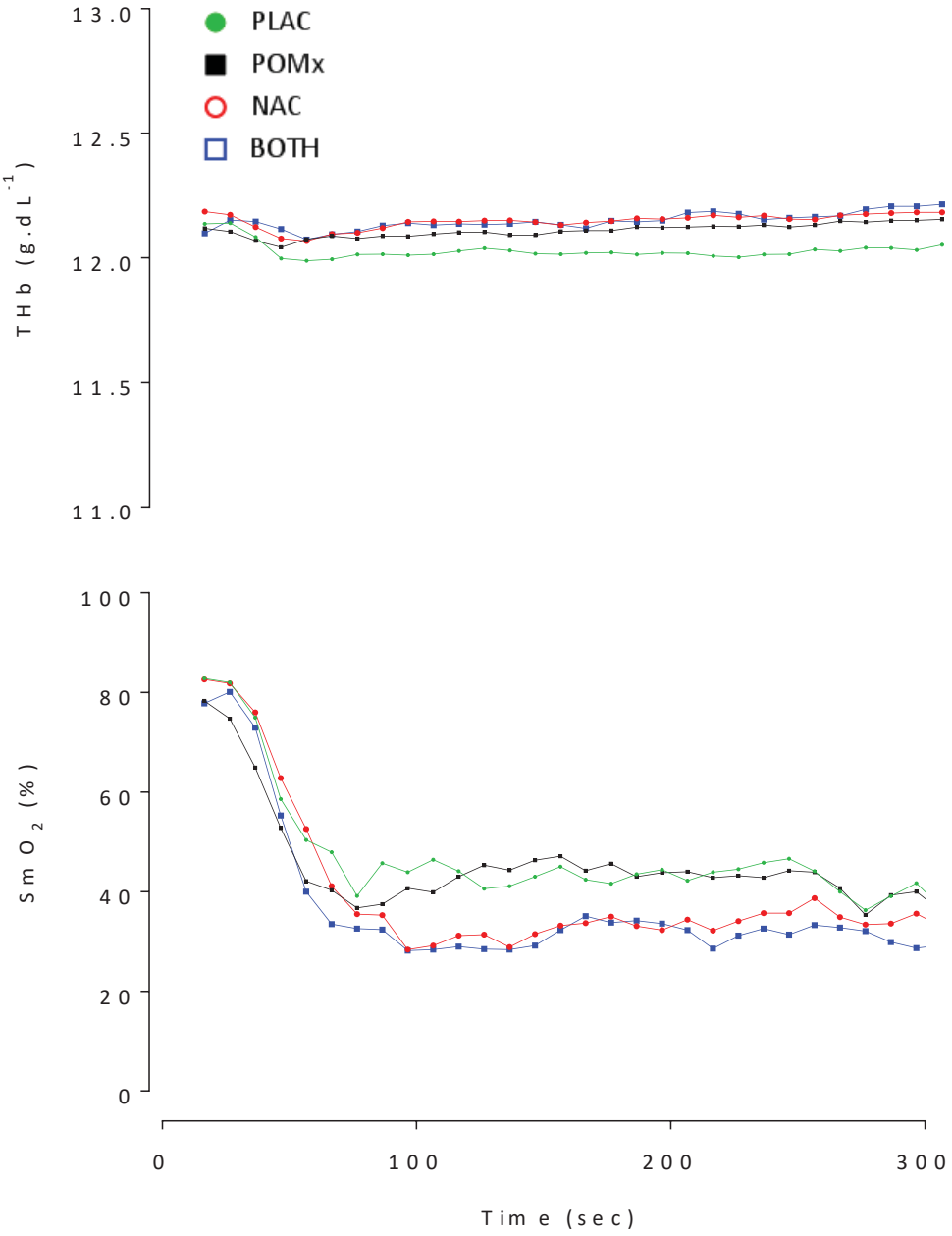

Figure 7.7 Typical muscle oxygenation responses to the five-minute time trial (participant EC)

7.3.3.2 Heart rate

A typical HR response during the time trial is shown in Figure 7.8. There was no significant effect of POMx or NAC on HR either during the time trial as a whole (POMx, $F_{1,7}$ = 0.514, p = 0.50; NAC, $F_{1,7}$ = 0.269, p = 0.62) or in the last minute of the exercise (POMx, $F_{1,7}$ = 0.159, p = 0.70; NAC, $F_{1,7}$ = 0.259, p = 0.63).

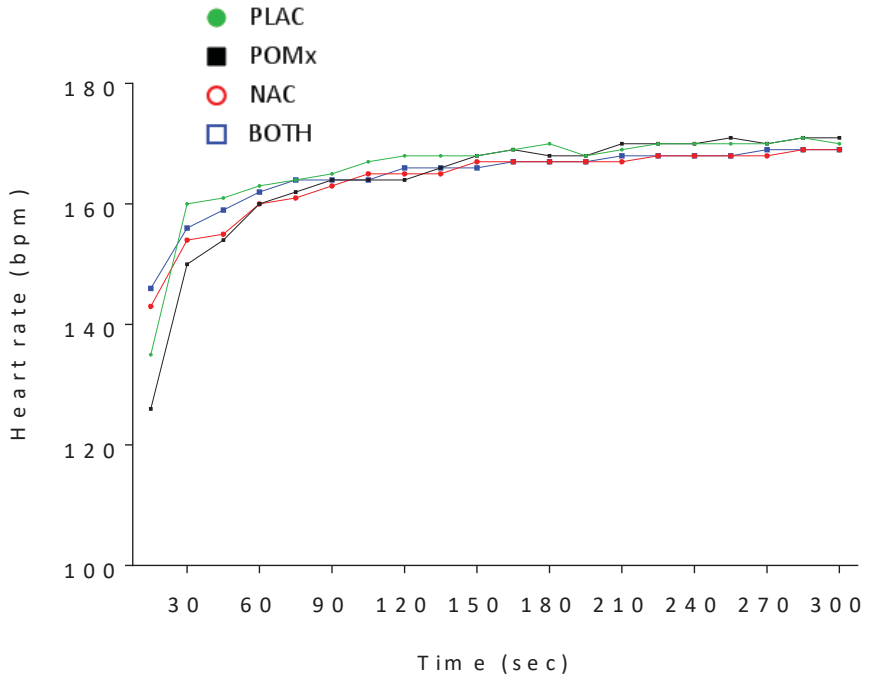

Figure 7.8 A typical heart rate response to the five-minute time trial (participant 3)

7.3.4 Exercise performance

Performance during the time trial, as measured by energy expenditure (kJ) was not affected by POMx ($F_{1,7}$ = 0.192, p= 0.68) or NAC ($F_{1,7}$ = 1.12, p = 0.33) (Figure 7.9). In addition,

perception of effort (RPE) during the submaximal exercise stages was not altered by POMx ($F_{1,7}$ = 0.191, p = 0.68) or NAC ($F_{1,7}$ = 2.23, p = 0.18).

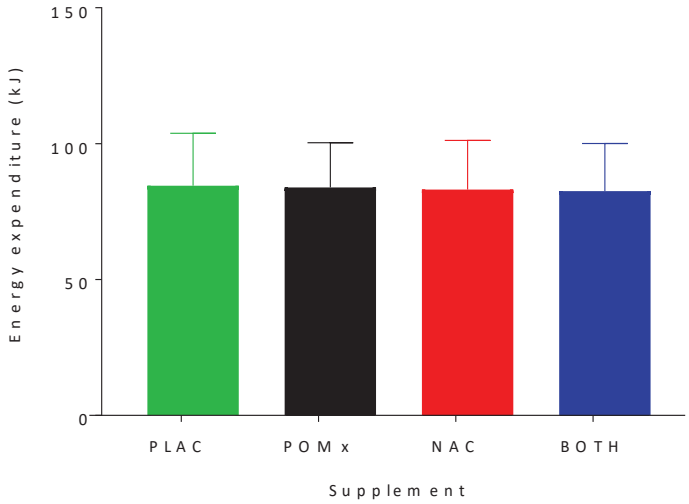

Figure 7.9 Total energy expended during the five-minute time trial following PLAC, POMx, NAC and BOTH. Values are presented as means ± SD.

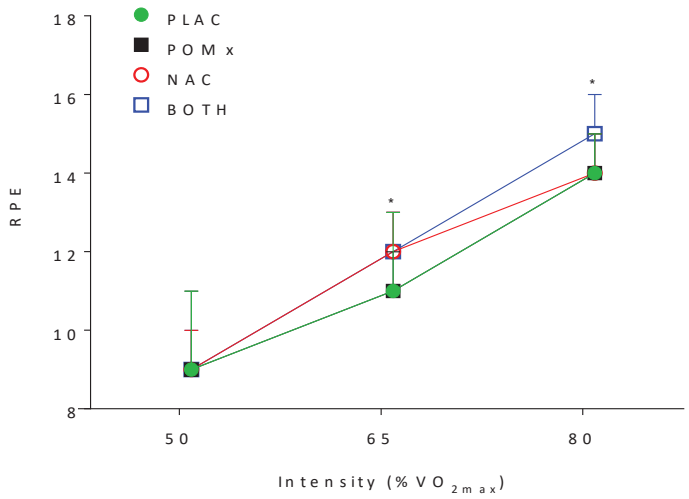

Figure 7.10 RPE during submaximal exercise following PLAC, POM, NAC and BOTH. Values are means ± SD. * indicates $p \leq 0.05$ compared to previous intensity for all treatment conditions.

7.4 DISCUSSION

This study was designed to test the hypothesis that short-term POMx supplementation would reduce the submaximal O_2 cost of cycling exercise, and in doing so, improve performance during a subsequent cycling time trial. It was further proposed that co-supplementation with NAC would enhance the effects of POMx through a reduction in NO degradation. That VO_2 was reduced during submaximal exercise following POMx compared to NAC and PLAC, partially supported our hypotheses. However, co-supplementation with NAC reversed the POMx-induced improvements in the efficiency of O_2 usage, despite no significant difference in plasma NO_3^- between POMx and BOTH. In addition, the changed efficiency in POM and NAC did not translate into difference in time trial performance, nor any changes in the other dependent variables measured.

7.4.1 Cardiorespiratory responses

In contrast to the acute POMx supplementation protocol in the previous chapter (Chapter 6), eight days of POMx supplementation was associated with a decrease in submaximal VO_2, compared to non POMx trials. This is in accordance with a number of previous studies, which have reported a decrease in VO_2 in trained athletes during submaximal exercise following multi-day (Cermak et al., 2012; Kelly et al., 2014a) but not acute (Arnold et al., 2015; Bescós et al., 2011; MacLeod et al., 2015; Peacock et al., 2012; Wilkerson et al., 2012) BRJ supplementation. Thus, although POMx provides a much lower dose of dietary NO_3^- compared to BRJ, it appears that the protective effect of polyphenols is sufficient to increase the bioavailability of NO. As described in the previous chapter (see 6.4.1), an

increased plasma NO availability is likely to reduce the VO_2 required to exercise at a given submaximal intensity through increasing either the efficiency by which mitochondria use O_2 to produce energy (adenosine triphosphate, ATP), or the amount of ATP required to yield a given amount of muscular contraction. At the mitochondrial level, NO is a known inhibitor of O_2 binding to cytochrome c, the terminal electron acceptor of the electron transport chain, and therefore impedes its use in energy production (Brown & Cooper, 1994). Consequently, it has been suggested that NO reduces O_2 consumption at proximal muscle fibres, which are richly perfused, and allows greater O_2 movement to more distal fibres. This may increase O_2 distribution throughout the muscle, improve the matching O_2 delivery to requirements and reduce the amount of O_2 required to produce a given amount of energy (Lansley, Winyard, Fulford, et al., 2011). At the muscular level, there is some evidence that NO may enhance calcium ion channel regulation, through activating thiols in the ryanodine receptor components of these channels, in low PO_2 conditions, but reducing their activation when PO_2 is high. Again, this is suggested to allow more precise matching of energy requirements to muscular contraction, thereby reducing the O_2 that is required to produce a given amount of muscular work (Bailey, Fulford, et al., 2010; Jones, Chiesa, Chaturvedi, & Hughes, 2016).

Although the multi-day protocol in the current chapter provided the stimulus needed to reduce VO_2 during submaximal exercise, the increase in NO_3^- induced from eight days of POMx supplementation was only slightly greater (17 µmol) than in the previous chapter's acute protocol (10 µmol). However, since NO_3^- was only used as an indicator of NO production, this marker may not reflect the changes in NO availability, particularly because polyphenols have been shown to enhance the conversion of NO_3^- to NO, thereby reducing

its presence in the plasma. On the other hand, Vanhatalo et al. (2010), demonstrated that when BRJ was ingested over a period of 15 days, the initial rise in plasma NO_2^- at 2.5 hours post-supplementation gradually declined until day eight, after which levels began to increase again, reaching maximal values at day 12 (Figure 7.11A). Thus, it is possible that greater changes in NO_3^- may have been seen with a slightly longer supplementation protocol. However, it is important to note that despite the large increase in NO_2^- after day eight in their study, no significant lowering in VO_2 values or the O_2 cost of exercise (VO_2/work rate) during moderate cycling exercise after day five was seen by the authors (Figure 7.11B). It is possible that once a certain threshold of plasma NO_3^- or NO_2^- is reached, the rate of NO_3^- to NO conversion is maximized and any further NO_3^- accumulates in the plasma.

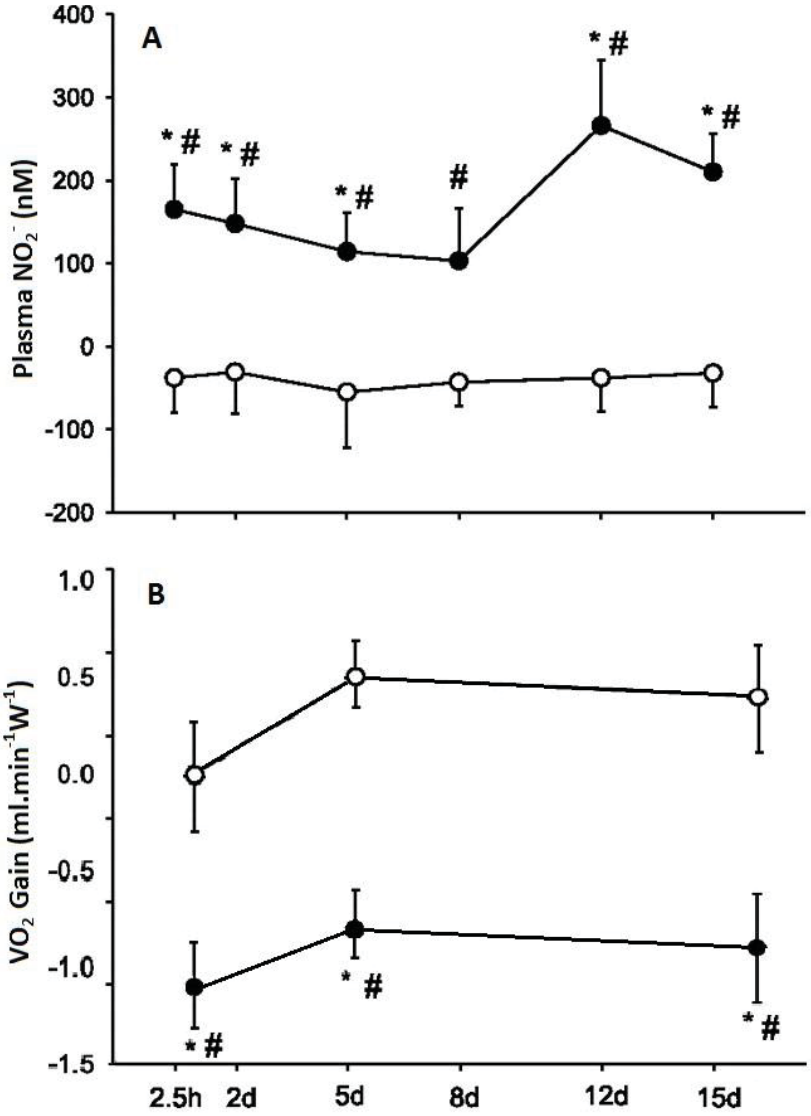

Figure 7.11 Change in (A) plasma nitrites and (B) the oxygen cost of exercise (VO_2/work rate) relative to pre-supplementation baseline during a 15-day supplementation period with beetroot juice (●) and placebo (○). *indicates $p \leq 0.05$ compared to pre-supplementation baseline. # indicates $p \leq 0.05$ compared to placebo. Adapted from Vanhatalo et al. (2010)

Further evidence for the lack of correlation between plasma NO_3^- and VO_2 was shown by the results from the BOTH trials. Despite no significant difference in plasma NO_3^- between POMx and BOTH, the VO_2-lowering effects of POMx were reversed when co-supplemented

with NAC. This appears to be the result of an NAC-induced increase in VO_2, which negated the VO_2-lowering effect of POMx. This is in contrast to previous research which has shown an increased antioxidant status and decreased oxidative damage with NAC (Slattery et al., 2014; Zembron-Lacny et al., 2010) and led us to hypothesize a protective effect on NO and augmentation of the effects of POMx. This result also differed from research by McKinley-Barnard et al. (2015) who found an increase in markers of NO concentration and activity with the addition of a thiol (GSH) to a component of the endogenous NO pathway (CIT), compared to supplementation with CIT alone. Thus, it appears that the interaction of NAC with NO or POMx reduces the POMx-induced actions of NO. One possible mechanism for this effect is the formation of SNO compounds from NO, thiols and O_2, which is driven by an increased ratio of NO to superoxide. Although previous research has described SNOs as forms of NO with greater stability and longer half-lives (Pacher, Beckman, & Liaudet, 2007), the extent to which SNOs carry out the functions of NO is not fully determined. There is evidence that SNOs formed from cysteine in the erythrocytes, lungs and muscles are essential signalling molecules in processes such as blood flow (Stamler et al., 1997), matched alveolar perfusion (Lipton et al., 2001) and skeletal muscle function (Sun, Xu, Eu, Stamler, & Meissner, 2003). However, little data exists on SNOs formed from thiols which don't exist as components of target proteins, and the activity of SNOs during exercise performance has not previously been investigated (Reid, 2015). It is possible that in its SNO form, the undetermined mechanism by which NO influences VO_2 (see 6.4.1) is less efficient, thus reducing the effects of POMx. However, this is purely speculative, as the current study did not measure the level of SNO formation, so it is unknown whether there was indeed an increase in SNO with NAC.

7.4.2 Oxygen saturation responses

In accordance with the previous chapter (see 6.4.2), multi-day supplementation with POMx and/or NAC had no effect on SmO_2 or THb at rest, or during submaximal or intense exercise, despite POMx being associated with a decrease in VO_2. Since we have shown that the Moxy monitor is a reliable device for measuring such parameters, particularly during resting or low to moderate exercise (Crum et al., 2017), this gives further evidence that the VO_2-lowering effects of POMx supplementation are not due to changes in O_2 delivery, at least in normoxic conditions.

7.4.3 Performance during the five-minute time trial

Despite the decrease in VO_2 required to complete the submaximal part of the exercise test, subsequent performance in the time trial was unchanged. This is in accordance with the previous chapter, where acute POMx supplementation did not improve performance in a TTE at 100%VO_{2max} in hypoxia, despite an improved VO_2 capacity. Similarly, Trinity et al. (2014) found no change in performance during a cycling TTE at 100%VO_{2max} in hot conditions following seven days of POMJ (1000ml.day^{-1}). We predicted that the time trial in the current study would be more sensitive to changes in exercise capability because the definite end-point of the time trial gives participants a clear goal to aim for, as opposed to the open-ended nature of a TTE (Currell & Jeukendrup, 2008). In addition, we did not measure VO_2 during the time trial, to allow participants to fully concentrate on the exercise. However, we suggest that the high-intensity of the short time trial may have resulted in a relatively large anaerobic energy production contribution, thereby reducing the potential effect of POMx on performance. This is comparable to research by Peeling, Cox, Bullock,

and Burke (2015), who found that although VO_2 was reduced during a four minute kayaking time trial following BRJ supplementation, there was no change in performance. On the other hand, several authors of previous BRJ studies have suggested that a dietary NO_3^--induced increase in NO preferentially affects the performance of type II fibres, which are more likely recruited during high-intensity exercise (Bailey et al., 2015; Breese et al., 2013). In addition, performances during longer running or cycling time trials following POMx or BRJ supplementation have shown mixed results. For example, BRJ was associated with a 2.9% increase in a 16.1 km cycling time trial performance in one study (Muggeridge et al., 2014), but did not improve performance in a 10km time trial (~48 minutes) in seven out of ten runners in another (Arnold et al., 2015). Thus, although it seems realistic that POMx could have the potential to improve longer bouts of endurance exercise with a greater aerobic component, without knowing the mechanism by which NO affects VO_2, this is difficult to predict. It is suggested that future research involving POMx should employ a longer exercise test to test this theory.

7.4.4 Blood pressure and haematocrit responses

In contrast to the results from the acute supplementation protocol (see 6.4.4), POMx supplementation was not associated with any changes in BP or Hct. This provides some evidence that the unexpected increase in SBP and decrease in Hct in the previous chapter may have been due to day-to-day variation, rather than an effect of supplementation.

As previously described, although dietary NO_3^- intake in untrained individuals has been associated with significant decreases in SBP (4–7%), presumably due to greater NO-induced vasodilation (Bailey, Fulford, et al., 2010; Bailey et al., 2009; Larsen et al., 2007; Vanhatalo et al., 2010), trained individuals generally do not show the same changes (Bond et al., 2012;

Cermak et al., 2012; Nyakayiru et al., 2016; Wilkerson et al., 2012). Similarly, while SBP has not been measured in trained endurance athletes following intake of POMx, acute supplementation did not alter its magnitude during sprint cycling or resistance exercise in 'active individuals' (Roelofs et al., 2016). As detailed in 6.4.3, there are a number of training-induced adaptations which may explain the lack of change in SBP in such individuals, despite an increase in NO. In essence, such adaptations have already increased the vasodilatory capacity in these individuals sufficiently, causing further increases in NO to have minimal effect on this parameter.

On the other hand, NAC supplementation has not been associated with changes in BP, but has increased Hct in untrained individuals when ingested for eight days in similar quantities to that used in the current study (Zembron-Lacny et al., 2010). In that study, it was proposed that a NAC-induced reduction in oxidative stress had increased Hct by reducing erythrocyte degradation. However, it appears that the same effect does not occur in trained individuals, either when supplemented alone, or with POMx. This reinforces the lack of changes in blood parameters measured in Chapter 1 with chronic thiol supplementation (see 5.4.1). Thus, based on these two studies, thiol supplementation is not supported as a method for inducing changes in O_2 transport.

7.4.5 Study limitations

As in the previous chapter, this study was limited by a relatively small number of participants (n = 8) due to the training status required for participation. Further, one additional participant pulled out of the study before completion, due to a non-study related injury. However, all participants who finished the study completed the supplementation

protocol and exercise protocol with full commitment, giving the researcher confidence that the results presented are a good representation of the effects of POMx and NAC supplementation on trained athletes.

7.4.6 Conclusions

In conclusion, eight days of POMx supplementation decreased the VO_2 required to perform submaximal cycling at 50-80% VO_{2max}. However, co-supplementation with NAC negated this effect, due to the VO_2-elevating effect of the latter. Despite the decrease in submaximal VO_2 with POMx, performance during a subsequent time trial was unaffected. Therefore, while POMx may improve the efficiency of O_2 use, its use as an ergogenic aid for short-duration endurance exercise is not supported by the current study, although it is possible that benefits may be seen during a longer time trial.

CHAPTER 8. DISCUSSION AND CONCLUSIONS

This thesis proposed to determine whether supplementing the diet with antioxidants could improve the efficiency of oxygen (O_2) transport during exercise in trained athletes.

Chapters 5–7 describe three distinct studies in which acute, short-term and chronic supplementation of thiol and/or polyphenol-containing products are compared with a placebo to ascertain their impact on various parameters in the O_2 carrying and utilization processes during exercise. Importantly, the study participants were well-trained cyclists and triathletes. Thus, the outcomes of these studies can be extrapolated to the competitive endurance athletic population where nutritional supplementation is widely practiced, often where scientific knowledge of the efficacy of the supplements is lacking (Maughan, Depiesse, & Geyer, 2007).

Based on the information presented in Chapter 1, it was predicted that the intake of such antioxidants would: 1) reduce the breakdown of erythrocytes and their associated O_2-carrying protein, haemoglobin (Hb), thus improving maximal O_2 utilization (VO_{2max}) capacity; 2) reduce the breakdown of the major signaling hormone and vasodilatory compound, Nitric Oxide (NO), thereby enhancing the efficiency of blood delivery and oxidative energy production; and 3) further augment the effects of NO, by enhancing its formation from dietary nitrates (NO_3^-). The combination of these effects, if shown, had the potential to improve aerobic power output and thus performance.

In contrast to previous research conducted in rodents (Wolber et al., 2016), four weeks of supplementation with a novel, thiol-rich keratin product (KER) did not improve any of the blood parameters associated with O_2 transport. Accordingly, post-supplementation exercise

testing also showed no change in the O_2 cost of submaximal cycling exercise, or in VO_{2max}. Therefore, although providing a rich source of protein, KER did not show any potential to improve the efficiency of O_2 transport or uptake. Considering this finding, it is unsurprising that KER was also ineffective at improving cycling performance, as measured by maximal power output in a VO_{2max} test. It was nevertheless well tolerated for the four-week supplementation period, providing additional evidence to previous research (Houlthham, 2014) which indicated that the hydrolyzed and soluble keratin is a safe form of protein supplement. In addition, because the current study did not measure blood antioxidant concentrations, it is unknown whether the lack of significant changes in the measured variables were due to the inability of KER to alter antioxidant status, or whether the lack of change occurred despite an improvement in antioxidant status.

Conversely, as described in Chapter 6, supplementation with a high-polyphenol dietary supplement (pomegranate extract; POMx) appears to influence O_2 transport in certain situations. That is, when ingested acutely, POMx regained some of the reduction in VO_2 which is typical during intense exercise in a hypoxic environment. This effect was likely driven by the high content of flavonoid polyphenols in POMx. These compounds are associated with protective effects on Nitric Oxide (NO), a compound which has previously increased the efficiency of O_2 usage following the intake of dietary supplements containing large concentrations of its precursor, NO_3^- (Bailey, Fulford, et al., 2010; Bailey et al., 2009; Lansley, Winyard, Fulford, et al., 2011)

In contrast, acute POMx supplementation was ineffective at altering VO_2 during submaximal exercise and in exercise conducted in a normoxic environment. Further, despite improved VO_2 values during the cycling time trial to exhaustion at 100%VO_{2max} in hypoxia, POMx was not associated with an improvement in performance, although analysis

of individual responses identified two participants for whom the supplement appeared to be ergogenic. It was proposed that the lack of changes in VO_2 (in normoxia) and performance following POMx in this initial study were partly influenced by the highly-trained nature of the study participants. Previous research has demonstrated that due to training adaptations, such individuals have greater basal levels of nitric oxide synthases (NOS), plasma NO_3^- and nitrites (NO_2^-) (McConell et al., 2007; Vassalle et al., 2003), and therefore may receive less benefit from the effects of polyphenols on NO production, bioavailability and protection. On the other hand, an acute dose of POMx may have been insufficient to alter the efficiency of O_2 transport, and a longer supplementation period may be required to induce changes in trained individuals.

In agreement with the above, Chapter 7 demonstrated that eight days of POMx supplementation was indeed sufficient to reduce the O_2 cost of submaximal cycling exercise in a normoxic environment, as measured by a reduction in the VO_2 required to sustain a given intensity of exercise. However, this did not alter performance in a subsequent five-minute time trial. It is possible that the performance tests used in this thesis were too short in duration for the benefit of a decreased O_2 requirement to be observed. The protocols chosen for this thesis were limited by time restraints, due to the hypoxic setting used in Chapter 6. It is suggested that further studies involving POMx and exercise performance should employ a time trial protocol of sufficient length to allow a predominantly aerobic contribution to energy production (≥ 1 hour). Until such research has been conducted, POMx cannot be recommended as an ergogenic aid, because despite a decrease in submaximal exercise O_2 consumption, the absence of a performance enhancement renders use of the supplement ineffective for athletes. The evidence presented nevertheless warrants further scientific exploration to investigate the effect of POMx on VO_2 in different

conditions of O_2 availability as it may prove ergogenic in specific circumstances for some athletes.

In theory, thiol and polyphenol antioxidants have erythrocyte and NO-protecting properties which could combine to enhance O_2 transport to a greater extent than supplementation with either antioxidant alone. However, the co-supplementation of a thiol-rich supplement (*N*-acetylcysteine, NAC) with POMx for eight days (Chapter 7) reversed the reduction in VO_2 values during submaximal cycling exercise which were observed with POMx intake only. To our knowledge, this is the first study to observe this effect, and contrasts with previous research (McKinley-Barnard et al., 2015) in which the co-supplementation of the thiol, glutathione, with the NO precursor, L-citrulline (CIT) was associated with an increase in markers of NO concentration and activity compared to supplementation with CIT alone. Further, other studies have shown ergogenic benefits of NAC supplementation, albeit using constant infusion protocols or untrained participants (Medved et al., 2004; Zembron-Lacny et al., 2010) . The results of those studies have seen this supplement gain popularity amongst supplement manufacturers and it is now widely available to the consumer. Our findings do not support the recommendation for NAC supplementation for endurance exercise, certainly in situations such as high-intensity aerobic exercise, which require efficiency of O_2 use. Further, the therapeutic use of NAC is that of paracetamol overdose, and knowledge of this efficiency-reducing effect, as shown by an increase in VO_2 and a trend towards an increase in heart rate during submaximal exercise, may be useful in a clinical setting in that regard. Despite also having a high thiol content, we did not observe any negative effects on indicators of O_2 transport or usage efficiency with KER. However, it would be worthwhile to investigate the effect of other thiol-rich supplements on such parameters. Our findings add to growing evidence which demonstrates that excessive

intake of certain antioxidant-containing compounds (e.g. vitamin C) reduce, rather than enhance, exercise performance.

Although Chapters 6 and 7 demonstrated some evidence of a change in whole-body VO_2 with POMx, local muscle O_2 saturation (SmO_2) and total Hb content was unaltered by polyphenol supplementation at any exercise intensity. Given that the predominant mechanistic theories for the effect of NO on VO_2 involve improvements in efficiency at the mitochondrial or muscular level, this may not be surprising. However, it gives further evidence that any changes in VO_2 are not simply the result of improved vasodilation and blood flow to the muscles.

In summary, based on the lack of evidence for enhancement of performance, this thesis does not support the recommendation of the selected dietary antioxidant supplements for athletes, for performance-enhancing effects at least. However, because the primary outcome measure of this thesis, VO_2, appears to be altered by POMx, the intake of polyphenol-rich products warrants further investigation. Based on the results of Chapters 6 and 7, it appears that the benefits of POMx become more significant as demands on O_2 transport and utilization processes increase. Therefore, the suggested areas of future research would involve exercise of greater duration, and environmental conditions where O_2 availability and/or limitations to the various transport parameters differ.

CHAPTER 9. REFERENCES

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CHAPTER 10. APPENDICES

TABLE OF APPENDICES

APPENDIX A FORMS, QUESTIONNAIRES AND SCALES

A.1. Example of a participant information sheet

Participant Information Sheet

The effect of acute pomegranate extract supplementation on blood parameters and exercise performance in normoxic and hypoxic conditions in highly-trained cyclists.

Introduction

Pomegranate extract

Pomegranate extract (POMx) is produced from pomegranate fruit skins and arils in a process which removes the sugar content of the fruit and concentrates its other nutrients. The resulting supplement has a high nitrate concentration and is rich in polyphenols (a type of antioxidant). The potential benefits of POMx to exercise performance are related to these compounds.

Nitrates

Following ingestion of nitrate-containing foods, a portion of the nitrates absorbed are secreted in salivary glands in the mouth. These react with anaerobic bacteria on the tongue and are converted to nitrites, before being re-swallowed to enter the digestive system and are converted to nitric oxide in either the stomach or the bloodstream. An increased blood concentration of nitric oxide has the potential to improve exercise performance through its role as a blood vessel dilator (vasodilator). Previously, increased levels of nitric oxide have been associated with a reduction in blood pressure and an increase in blood flow during rest and exercise. These findings have prompted a large amount of research investigating the effect of pre-exercise consumption of nitrate-rich beetroot juice (BRJ. In sedentary and active individuals, the increased blood flow caused by BRJ supplementation has improved the efficiency of oxygen delivery to the active muscles, thereby reducing the effort required to sustain a given level of exercise. Consequently, this has improved exercise performance in a range of endurance sports.

Polyphenols

In addition to nitrates, the large number of polyphenols present in POMx may help to increase nitric oxide concentration in the blood. Polyphenols are antioxidant compounds which protect nitric oxide from breakdown via neutralization of the damaging reactive

oxygen species produced during exercise. In addition, polyphenols help to stimulate the conversion of nitrites to nitric acid, allowing faster production following nitrate intake.

Altitude

The effects of nitrate supplementation may be enhanced during exercise at altitude. In conditions of reduced oxygen pressure (PO_2), nitric oxide production via other endogenous pathways is limited, leading to stimulation of the nitrate-nitrite-nitric oxide pathway. This may be important for endurance athletes who commonly use periods of high altitude training as a method of naturally stimulating an increase in red blood cell content. However, the ability to train with intensity at altitude is limited by the low PO_2. By reducing the oxygen required to sustain a given intensity, nitrate supplementation could improve the capacity to train under these conditions.

This study aims to determine the effects of pre-exercise POMx intake (1000mg) on blood parameters and exercise performance in normoxic (sea level) and hypoxic (altitude) conditions.

Participation

We are recruiting ten healthy cyclists between the ages of 18 and 50 years to participate in this study. Participation is voluntary and you may withdraw from participating in the study at any time. The study involves three exercise testing sessions of approximately one hour at the Massey University Practical Teaching Complex, HPL lab and two exercise testing sessions of approximately one hour in high-altitude conditions in the Ruapehu area.

Protocol

After initial interest and contact, you will be asked to visit Massey University Practical Teaching Complex, HPL lab at a time that suits you, to fill out a health screening questionnaire to determine suitability to the research project. If deemed suitable, you will be invited to come to the laboratory prior to the first performance testing for familiarization of the protocol and equipment, and to ensure you are comfortable in the laboratory setting.

The study involves testing in both normoxic (sea-level) and hypoxic conditions. The tests under normoxic conditions will be carried out in the HPL. Firstly, you will complete a maximal aerobic capacity (VO_{2max}) test which will involve cycling at four six minute progressively-increasing intensities on a stationary bike, while ventilatory gases are collected via a portable gas mask and heart rate is monitored via a heart rate monitor. After a short rest, this will be followed by a 'ramp' protocol in which the intensity of exercise is increased gradually until exhaustion. This will allow us to measure the maximum amount of oxygen that you can take in and use to produce energy. This test will take approximately one hour.

Over the next two weeks, you will visit the HPL twice to complete a cycling trial on the stationary bike. You will arrive at the laboratory 2.5 hours prior to exercise and be given a standardized meal as well as a supplement in capsule form. This will be either pomegranate extract or a placebo (brown sugar). You will not be told which supplement you are receiving and supplementation will occur in a random order between the two trials. Just before the

exercise test, a blood sample will be taken via venepuncture to determine blood nitrate concentration.

Approximately one week after the normoxic trials, you will complete two trials in high-altitude conditions. These trials will follow the same protocol as the laboratory tests, with pre-exercise intake of either PE or a placebo. However, you will not be required to complete a VO_{2max} test at altitude. The trials in high altitude conditions will be conducted in the Ohakune-Ruapehu area over three days. Transport and accommodation (2 nights) will be provided for all participants free of charge. Participants will however need to provide their own food. You will also need to bring clothes suitable for cycling in (summer and winter as weather can be variable), your cycling shoes and your bike.

Experimental measures

VO2 max test
During this protocol your expired respiratory gases will be collected in a bad and analysed for O_2 and CO_2. This will provide a measure of your VO_{2max}. Due to the nature of the test (maximal effort) there will be some discomfort associated with it. However it will be temporary and if you feel you need to pull out before the test is complete, you may do so.

Normoxic and hypoxic cycling protocol
This protocol will involve cycling at three progressive submaximal intensities at 50, 65 and 80% of your previously measured VO_{2max}. After a short rest, the intensity will be increased to 100% of your VO_{2max} and you will cycle at this intensity for as long as possible. Again, there will be some discomfort associated with the intensity of this test. However, as with the VO_{2max} test this will be temporary, and you are welcome to pull out at any time.

Oxygen saturation
During the cycling tests you will be fitted with a pulse oximetry meter. This is a small sensor device which is placed on your quadriceps, and determines the oxygen saturation of your blood via the passing of light wavelengths through a body part to a photodetector. This process should not present any discomfort.

Blood pressure
You will have your blood pressure taken using a blood pressure cuff. You will feel a slight tightness as the cuff is inflated but this will only occur for a minimal period of time.

Blood samples
Blood samples will be taken by venepuncture (during the normoxic trials) and capillary blood sample (during the normoxic and hypoxic trials).

Venepuncture
Blood will be taken in 10ml samples from the antecubital vein of either arm. Blood samples will be used to analyse your blood for levels of nitrates. Because needle insertion into a vein is required for blood samples to be taken, you may experience minor to moderate

discomfort. Slight bruising may occur at the site of needle insertion; however this can be minimized by keeping direct pressure on the sampling site immediately after sample is taken.

Capillary blood sampling
Blood will be collected via a small fingerprick sample prior to each exercise test, after each submaximal state, and at the conclusion of each test (total sample = 5 per test). These samples will be used to analyse blood lactate production during exercise.

Blood samples are disposed of in their vacutainers (blood collection tubes) or storage tubes into biohazard bags, which are autoclaved at 121 degrees C and disposed of by Nuplex Medical Waste. Alternatively, you can request to have any/all portions of your samples returned to you.

If analysis of your blood reveals pathological levels of any of the markers measured you will be informed of the results and advised to see your GP.

Supplement consumption
Prior to two of the trials you will consume 1000mg of a POMx supplement. This supplement is available commercially and has been previously been shown to have no adverse health effects when consumed for 28 days in quantities of 1420 mg/day (Heber *et al.* 2007). Thus, the intake in the current study should not present any adverse effects. However, if you experience any side effect (e.g. nausea, vomiting) you are advised to inform the researchers and the trial will be stopped.

Reference:

Heber D, Seeram NP, Wyatt H, Henning SM, Zhand Y, Ogden LG, Dreher M, Hill JO (2007). Safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol dietary supplement in overweight individuals with increased waist size. *Journal of Agriculture and Food Chemistry* 55: 10050-10054.

Exercise in high altitude conditions
High altitude environments have a reduced partial pressure of oxygen, which diminishes the binding and subsequent transport of oxygen in the blood. Therefore, exercise at a given intensity is more difficult under these conditions, than at sea level, and you may feel greater discomfort exercise at altitude than you would when cycling at sea level. In addition, at high altitude environments, there is a small risk of developing altitude sickness. While this is normally associated with higher altitudes than those at Ruapehu (>2500m), it is important to be aware of the signs and symptoms of the condition (dizziness, nausea, headache) and to inform the researchers if you experience any of these symptoms. If altitude sickness does occur, you will be taken to a lower altitude to recover.

What to bring and do prior to the tests.

Normoxic trials and VO_{2max} test

- Consume a meal 2.5 hours prior to the testing and record what you eat. You will then replicate this meal for all the exercise tests.
- Bring clothes suitable for cycling in, including cycling shoes.

Hypoxic trials

- Transport and accommodation in Ohakune will be provided. Further details will be provided once we have determined who is taking part in the study
- Bring:
    - Clothes suitable for cycling in (summer and winter), including cycling shoes and a helmet.
    - Money to purchase food during the trip and/or food to eat during the trip
    - Other clothing and items that you require for an overnight stay
- Prior to testing you will consume the same meal that you selected for the previous testing in normoxic conditions.

Foods to avoid

We ask you to avoid the nitrate and polyphenol-rich products detailed on the sheet provided for one week prior to each test.

We also ask that you avoid using mouthwash or chewing gum for 24 hours prior to each test, as these products reduce the ability of salivary bacteria to react with nitrates.

Participants Rights

- You can ask questions on any aspect of the project at any time, and we will do our best to answer them to your satisfaction.
- As a participant in the study you will provide information on the understanding that your name will not be used unless you give permission to the researcher.
- You have the right to view your own data at any stage and have it explained to you.
- You have the right to have any blood samples returned to you after they have been analysed.
- You will also be given access to a summary of the project findings when it is concluded.
- You can withdraw from the project at any time, without giving any reason and without penalty.
- If you are studying with the School of Sport & Exercise, your participation/non-participation will not affect your grades in any way.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident

Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Exclusion Criteria
If any of the following apply:

- *You have a known heart or cardiovascular condition or if a member of your family died below the age of fifty (50) as a result of a heart condition.*
- *If you feel pain in your chest when you do physical activity or have chest pain at rest*
- *In the last six months you have suffered from any painful injury or condition that lasted more than one week.*
- *You have had an injury or medical condition that you think may affect your ability to sense pain or discomfort.*
- *You are taking prescribed medication.*
- *You have cultural or religious sensitivities about human body measurements.*
- *You have an objection to having blood samples taken*
- *You have any other reason to consider that you are not in good health and of average, or better than average, fitness.*
- *If you have been hospitalized recently.*
- *If you have any infectious diseases that may be transmitted via blood.*
- *If you have any disorder of bleeding or clotting of the blood.*
- *If you have any family history of any bleeding disorder or disorder of blood clotting.*
- *If you currently take non-prescribed medication and/or other drugs.*
- *If you suffer from anemia/iron deficiency*
- *If you have an allergy to pomegranate*
- *If you have a history of altitude sickness*
- *If you are currently participating in another research study*

…you should <u>NOT</u> participate in this project.

However, if you are interested in participating please contact Emma Crum, using the contact details above, anytime in the next 3 weeks.

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 15/54. If you have any concerns about the conduct of this research, please contact Mr Jeremy Hubbard, Chair, Massey University Human Ethics

Committee: Southern A, telephone 04 801 5799 x 63487, email humanethicsoutha@massey.ac.nz.

A.2. Example of a consent form

The effect of acute pomegranate extract supplementation on blood parameters and exercise performance in normoxic and hypoxic conditions.

PARTICIPANT CONSENT FORM

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature:		**Date:**	
Full Name - printed			

A.3. Example of a health screening questionnaire

Name:_____

Address: _____

Phone: _____

Age: _____

Please read the following questions carefully. If you have any difficulty, please advise the exercise specialist who is conducting the exercise test.

Please answer all of the following questions by circling only one answer for each question:

This questionnaire has been designed to identify the small number of persons (15-69 years of age) for whom physical activity might be inappropriate. The questions are based upon the Physical Activity Readiness Questionnaire (PAR-Q), originally devised by the British Columbia Dept of Health (Canada), as revised by [1]Thomas *et al.* (1992) and [2]Cardinal *et al.* (1996), and with added requirements of the Massey University Human Ethics Committee. The information provided by you on this form will be treated with the strictest confidentiality.

Qu 1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?

Yes No

Qu 2. Do you feel a pain in your chest when you do physical activity?

Yes No

Qu 3. In the past month have you had chest pain when you were not doing physical activity?

Yes No

Qu 4. Do you lose your balance because of dizziness or do you ever lose consciousness?

Yes No

Qu 5. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or a heart condition?

Yes No

Qu 7. Do you currently or have you previously had an injury that could be made worse by strenuous physical activity?

Yes No

Qu 8. Do you know of any other reason why you should not do physical activity?

Yes No

Qu9. Have any immediate family had heart problems prior to the age of 60?

Yes No

Qu 10. Have you been hospitalised recently?

Yes No

Qu 11. Do you have any infectious disease that may be transmitted in blood?

Yes No

Qu 12. This test will include the taking of blood for measuring markers of nitrate status and hematological parameters. Do you have any objection to this?

Yes No

Qu 13. Do you have any disorder of bleeding or clotting of the blood?

Yes No

Qu 14. Do you have any family history of any bleeding disorder or disorder of blood clotting?

Yes No

Qu 15. Do you have food allergies, in particular to pomegranate?

Yes No

If yes, please list: _____

Qu 16. Are you allergic to the adhesive found on plasters/medical tape and/or alcohol used in preparing the skin for blood sampling?

Yes No

Qu 17. Do you have cultural or religious sensitivities about human body measurements?

Yes No

Qu 18. Are you currently anaemic/iron deficient?

Yes No

Qu 19. Are you currently pregnant?

Yes No

Qu 20. Do you have a history of altitude sickness?

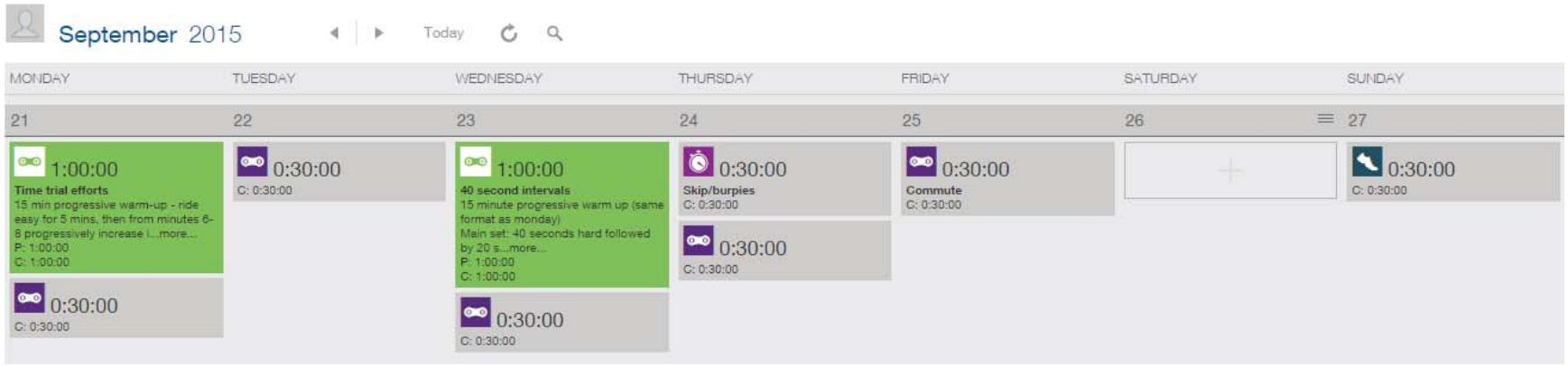
Yes No

You should be aware that even amongst healthy persons who undertake regular physical activity there is a risk of sudden death during exercise. Though extremely rare, such cases can occur in people with an undiagnosed heart condition. If you have any reason to suspect that you may have a heart condition that will put you at risk during exercise, you should seek advice from a medical practitioner before undertaking an exercise test.

I have read, understood and completed this questionnaire.

Signature: _____ Date: _____

A.4. Example of an exercise log in training peaks



A.5. Example of a diet log in My Fitness Pal

Your Food Diary For: Tuesday, April 28, 2015

	Calories kcal	Carbs g	Fat g	Protein g	Fiber g	Sugar g
Breakfast						
Multi-grain Toast - Multi-grain Toast 1 Slice, 2 slice	122	30	1	6	3	4
Watties (Nz) - Small Baked Beans Tin, 220 g	231	41	1	10	9	18
Add Food | Quick Tools	353	71	2	16	12	22
Lunch						
Add Food | Quick Tools						
Dinner						
Homemade - Baked Chicken Strips, 6 strips	168	6	15	29	0	0
Steamed Veggie Mix - Cauliflower, Brocolli, Carrots, 1 cup	40	7	0	1	3	3
Potatoes - Boiled, cooked in skin, flesh, with salt, 0.5 cup	68	16	0	1	2	1
Countdown - Sunshine Mix, 100 g	428	50	20	10	0	42
Add Food | Quick Tools	704	79	35	41	5	46
Snacks						
Griffins (Nz) - Original Snax Crackers, 12.5 Crackers (approx 19.4g)	251	30	13	3	0	3
Apples - Raw, with skin, 1 serving	80	21	0	0	4	16
Add Food | Quick Tools	331	51	13	3	4	19
During Training						
Apples - Raw, with skin, 1 serving	80	21	0	0	4	16
Add Food | Quick Tools	80	21	0	0	4	16
After Training						
Apples - Raw, with skin, 1 serving	80	21	0	0	4	16
Add Food | Quick Tools	80	21	0	0	4	16

A.6. Rating of perceived exertion scale

Rating	Descriptor
6	No exertion at all
7	Extremely light
8	
9	Very light
10	
11	Light
12	
13	Somewhat hard
14	
15	Hard (heavy)
16	
17	Very hard
18	
19	Extremely hard
20	Maximal exertion

Adapted from Borg (1998)

A.7. Gastrointestinal health questionnaire

A. Side-effects

Question 1.

Did you experience heartburn during the last week? (heartburn is a burning sensation in the stomach, rising to the upper chest and sometimes as far as the throat; it can be accompanied by an acidic taste in the mouth). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 2.

Did you experience any feelings of nausea, and/or vomiting? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms in the boxes below, if possible.

Mild symptoms < ----------> *Severe symptoms*		
Slight queasy feeling	General nausea	Strong feeling of wanting to (or actually did) vomit

Question 3.

Were you "off your food"? (i.e. you did not feel like eating, even if you were hungry)? Or did you feel unusually full-up at any point? Please tick appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 4.

Wind – did you experience any noticeable changes in your belching (or feelings of wanting to belch)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 5.

Did you experience any borborygmia? (this is unusual rumbling or gurgling sounds in your stomach, and normally occurs shortly after a meal or drink). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 6.

Did you suffer from indigestion? (indigestion can be defined as a prolonged, steady ache in your stomach following a meal, somewhat like the dull ache of a toothache). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 7.

Did you suffer from colic at any point? (colic can be defined as a painful, pinching sensation in your stomach that comes and goes in waves). Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 8.

Did you experience any noticeable changes in the frequency or consistency of your stools?

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms in the boxes below, if possible.

Frequency

More than 3 times a day	2 – 3 times a day	One stool a day	More than 2 days without passing a stool

Consistency

Watery (diarrhoea)	Loose (e.g. the consistency of porridge)	Solid, and a bit difficult to pass	Extremely hard stool; painful to pass

Please only complete these boxes if you noticed any <u>change</u> in your normal bowel habits – if your bowel habits remained much the same as usual, please do not complete

Question 9.

Flatulence – did you experience any noticeable changes in your flatulence (either in frequency, or in odour)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

B. General well-being

Question 10.

Did you feel that you had any changes in your energy levels (i.e. that you felt noticeably any more or less energetic at any stage)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 11.

Did you notice any obvious changes in your sleeping patterns (e.g. sleeping more or less soundly; sleeping for longer or shorter than usual)? Please tick appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 12.

Did you notice any marked changes in your mood? (e.g. feelings of happiness, melancholy, optimism or pessimism)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 13.

Did your weight change noticeably (either up or down)? Please tick appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 14.

Did you notice any marked changes in any pre-existing medical conditions that you might have (e.g. your hay fever/asthma, if you have this condition)? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

Question 15.

Did you notice any marked changes in your appetite at any point? Please tick the appropriate box.

NO ☐

YES ☐

If you ticked the YES box above, please state the days on which you experienced these symptoms, and also provide a written description, if possible.

_____

A.8. Foods to avoid during POMx studies

Please limit the following foods which are rich in either polyphenols or nitrates as best that you can.

Foods rich in polyphenols	**Foods rich in nitrates**
Fruits:	Pomegranate
Pomegranate	Beetroot
Berries (blackberry, raspberry, strawberry)	Spinach
Blackcurrant	Celery
Black grapes	Cress
Cherry	Lettuce
Rhubarb	Chinese cabbage
Peach	Endive
Kiwifruit	Fennel
Plum	Leek
Apricot	Parsley
Apple	Cabbage, esp. savoy cabbage
Large quantities of fruit juice	Dill
Vitamin supplements, e.g. vitamin C	Turnip
Vegetables:	Highly processed meats e.g. hot dogs, luncheon meat, bacon, poor quality sausages
Chicory	
Aubergine (Eggplant)	
Yellow onion	
Curly kale	
Leek	
Beans	
Artichoke	

Celery	
Other:	
Green tea	
Red wine	
Large quantities of dark chocolate	
Large quantities of soy products	

APPENDIX B COMPOSITION OF THE NOVEL KERATIN SUPPLEMENT

B.1. Protein drink recipe

Ingredients

Premix	**Weight (g)**
Dextrose monohydrate	9.84
Cocoa	10.41
Cekol 4000 sodium carboxymethyl cellulose	0.75
Vanilla #15960	0.426
10% anisaldehyde in EOH	0.0075
Flavouring	
Orange #13245	1.42
Mint	0.253
Stevia	0.694
Keratin powder	76.92

*makes 100 g, use 15 g in 100 mL water

Method

1. Take 21.43 g pre-mix
2. Add 1.42 g orange powder or 0.253 g mint powder, and 0.694 g stevia
3. Add to 79.62 g of keratin powder

B.2. Banana chocolate bar

Ingredients

	Mass per bar (g)
Protein powder (keratin or casein)	23
Polydextrose	11.5
Merigel modified starch 1	0.92
Glycerol	4.6
Coconut fat (kremelta)	2.76
Lecithin	0.23
Glycerol monostearate	0.46
Stevia	0.18
Tartrazine 2% solution	0.23
Water	5
Banana flavour	0.23
Chocolate coating	9.7

Method

1. Seal the mixer bowl with special plastic cover and hose with pegs
2. Combine glycerine, banana flavour, tartrazine and kremelta in a jug and leave standing in hot water until pourable. Don't have it too warm.
3. Mix all dry ingredients including lecithin in a cake mixer and mix on speed 1
4. Turn off mixer and insert small funnel - turn mixer to speed 2
5. Slowly add glycerol mix

B.3. Chocolate peanut brownie bar

Ingredients

	Mass per bar (g)
Protein powder (keratin or casein)	23
Polydextrose	9.2
Cocoa	6.2
Chopped peanuts	4.6
Peanut flavour	0.23
Lecithin	0.23
Glycerol monostearate	0.47
Stevia	0.18
Glycerol	7.4
Water	10.5
Chocolate coating	12.4

Method

1. Seal the mixer bowl with special plastic cover and hose with pegs
2. Combine glycerine and peanut flavour in a jug
3. Mix all dry ingredients including ground peanuts and lecithin in a cake mixer and mix on speed 1
4. Turn off mixer and insert small funnel - turn mixer to speed 2
5. Slowly add glycerol mix

APPENDIX C CHRONIC CONSUMPTION OF A NOVEL KERATIN SUPPLEMENT

C.1. Blood measures

C.1.1 WBC at pre-, mid- and post-supplementation time points with CAS and KER

ID	CAS			KER		
	Pre	Mid	Post	Pre	Mid	Post
CB	5.83	7.65	5.60	5.58	9.03	6.31
DF	6.42	4.19	4.44	4.58	4.96	4.78
DO	6.88	6.69	6.54	6.88	8.23	5.82
JB	6.21	6.63	5.10	7.24	5.84	5.26
JD	5.34	6.01	5.47	5.36	5.01	5.97
JE	5.59	5.06	5.55	5.52	5.37	5.27
JH	9.40	5.36	6.80	5.14	6.59	5.58
JL	6.14	6.55	6.95	6.14	5.68	6.81
PJ	4.62	5.06	3.59	4.98	5.42	5.25
PT	5.58	7.16	6.03	5.08	4.61	4.94
RL	7.36	6.90	7.29	7.11	7.87	7.37
SS	4.92	6.18	6.79	4.70	5.48	5.13
TD	5.50	6.16	6.68	7.74	5.46	6.36
TM	5.58	7.01	5.23	5.18	5.28	5.87
WO	4.92	4.94	5.63	4.33	4.34	4.76
Mean	6.02	6.10	5.85	5.70	5.94	5.70
± SD	± 1.15	± 0.95	± 0.99	± 1.03	± 1.33	± 0.74

WBC, total leukocyte count (*10^9/L); CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Mid, two weeks into the supplementation period; Post, post-supplementation.

C.1.2 RBC and Hb at pre-, mid- and post-supplementation time points with CAS and KER

	RBC ($*10^{12}$/L)						Hb ($g.dL^{-1}$)					
	CAS			KER			CAS			KER		
	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post
CB	4.55	4.80	4.80	4.60	4.67	4.67	142	146	151	140	146	142
DF	5.46	5.18	5.04	5.13	4.95	5.19	155	150	149	145	151	158
DO	4.72	4.93	5.14	5.14	4.78	4.95	150	154	161	159	155	157
JB	5.22	5.16	4.99	5.25	5.20	5.07	148	152	150	153	152	149
JD	4.72	4.80	4.65	4.72	4.56	4.92	139	137	139	142	135	148
JE	5.08	4.89	4.92	5.17	4.93	5.22	162	147	146	152	144	142
JH	5.15	4.84	5.20	5.42	5.31	4.74	156	143	152	158	150	140
JL	5.79	5.50	5.83	5.66	5.55	5.84	155	149	154	151	153	160
PJ	5.04	5.07	5.06	4.84	5.06	5.11	146	148	148	148	146	147
PT	4.98	4.67	4.89	4.76	4.84	4.81	150	146	152	148	150	149
RL	4.99	5.25	5.16	5.12	4.91	5.08	147	153	149	150	145	151
SS	4.73	5.05	4.81	4.65	4.66	4.80	146	157	147	145	147	148
TD	5.39	4.96	5.07	5.28	5.20	5.08	152	150	144	153	145	142
TM	5.17	4.99	5.21	4.94	5.17	5.34	136	149	161	148	152	155
WO	4.71	4.51	4.77	4.69	4.48	4.61	136	137	140	140	131	139
Mean	5.05	4.97	5.04	5.02	4.95	5.03	148	148	150	149	147	148
± SD	± 0.32	± 0.24	± 0.27	± 0.30	± 0.29	± 0.30	± 7	± 5	± 6	± 6	± 6	± 7

RBC, total erythrocyte volume; Hb, total haemoglobin content; CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Mid, two weeks into the supplementation period; Post, post-supplementation.

C.1.3 Hct and MCV at pre-, mid- and post-supplementation time points with CAS and KER

							MCV (fL)					
		CAS			KER			CAS			KER	
	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post
CB	41.7	44.6	44.9	42.2	43.3	42.9	91.5	93.0	93.6	91.7	92.8	91.9
DF	49.1	46.7	44.9	46.1	44.3	46.3	90.0	90.2	89.2	89.9	89.4	89.2
DO	41.9	43.7	45.7	47.3	41.9	43.6	88.8	88.6	88.9	92.0	87.7	88.1
JB	47.4	46.6	44.7	47.8	45.9	44.8	90.9	90.5	89.7	90.0	90.2	89.2
JD	42.1	43.1	41.2	42.2	41.1	44.3	89.2	89.9	88.5	89.4	90.3	90.0
JE	45.1	43.8	43.7	46.2	44.0	46.1	88.8	89.7	89.0	89.4	89.2	88.3
JH	45.9	43.7	46.3	48.3	45.0	42.0	89.2	90.1	89.0	89.2	84.7	88.5
JL	47.6	45.4	48.4	45.7	46.0	49.7	82.2	82.7	83.0	82.1	82.8	85.1
PJ	41.9	41.9	42.2	42.1	44.5	44.9	83.1	82.6	83.4	87.0	87.9	87.9
PT	46.9	42.5	44.5	44.0	44.5	44.2	94.1	91.0	91.0	92.4	91.9	91.9
RL	41.4	43.7	43.1	44.4	42.4	43.7	83.0	83.2	83.5	87.0	86.4	86.1
SS	44.5	47.3	45.2	43.1	43.1	44.7	94.0	93.7	94.0	92.6	92.6	93.0
TD	46.4	44.0	42.4	45.2	45.1	43.4	86.1	88.7	83.8	85.7	86.7	85.5
TM	46.5	44.8	47.1	45.1	46.4	48.1	89.9	89.7	90.4	91.2	89.7	90.1
WO	42.6	41.1	43.2	42.7	40.9	42.0	90.6	91.2	90.6	91.0	91.2	90.9
Mean	44.7	44.2	44.5	44.8	43.9	44.7	88.8	89.0	88.5	89.4	88.9	89.0
± SD	± 2.5	± 1.7	± 1.9	± 2.0	± 1.7	± 2.0	± 3.6	± 3.3	± 3.4	± 2.8	± 2.8	± 2.3

Hct, haematocrit; MCV, mean erythrocyte cell volume; CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Mid, two weeks into the supplementation period; Post, post-supplementation.

C.1.4 MCH and MCHC at pre-, mid- and post-supplementation time points with CAS and KER

	MCH (pg)						MCHC ($g.L^{-1}$)					
	CAS			KER			CAS			KER		
	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post	Pre	Mid	Post
CB	31.1	30.4	31.4	30.5	31.2	30.3	340	327	336	332	337	330
DF	28.4	29.1	29.5	28.2	30.6	30.5	316	322	331	314	342	346
DO	31.8	31.2	31.3	30.9	32.4	31.7	358	352	352	336	370	360
JB	28.4	29.4	30.2	29.1	29.2	29.4	313	325	336	319	331	333
JD	29.4	28.6	29.9	30.0	29.6	30.1	329	318	338	336	328	334
JE	31.9	30.2	29.7	29.4	29.2	27.2	359	336	334	329	327	308
JH	30.3	29.4	29.3	29.1	28.2	29.5	340	327	329	326	333	333
JL	26.7	27.1	26.5	27.2	27.6	27.4	325	328	319	331	333	322
PJ	29.0	29.2	29.2	30.6	28.8	28.7	348	353	351	351	327	327
PT	30.1	31.3	31.1	31.1	31.0	31.0	319	344	342	336	337	337
RL	29.5	29.1	28.9	29.3	29.6	29.7	355	350	346	337	342	346
SS	30.8	31.0	30.6	31.1	31.5	30.7	328	331	325	336	340	330
TD	28.2	30.2	28.5	28.9	27.9	28.0	328	340	340	337	322	327.
TM	30.23	29.89	30.88	29.93	29.44	29.05	337	333	342	328	328	322
WO	28.9	30.4	29.4	29.8	29.2	30.1	319	333	324	327	320	331
Mean	29.6	29.8	29.7	29.7	29.7	29.6	334	335	336	332	334	332
± SD	± 1.4	± 1.1	± 1.2	± 1.1	± 1.3	± 1.3	± 15	± 11	± 9	± 8	± 12	± 11

MCH, mean cell haemoglobin volume; MCHC, mean cell haemoglobin concentration relative to erythrocyte cell volume; CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Mid, two weeks into the supplementation period; Post, post-supplementation.

C.1.5 WBC pre- and post-supplementation with CAS and KER, before and after the two-hour constant-load exercise test.

	Pre-CAS		Post-CAS		Pre-KER		Post-KER	
	Pre-ex	Post-ex	Pre-ex	Post-ex	Pre-ex	Post-ex	Pre-ex	Post-ex
CB	7.17	10.76	5.60	16.30	6.36	19.60	6.31	9.71
DF	4.48	6.33	4.44	7.20	5.36	8.27	4.78	4.99
DO	6.14	7.17	6.54	8.52	9.31	8.76	5.82	7.80
JB	6.01	8.82	5.10	7.84	5.61	8.08	5.26	8.13
JD	8.87	10.78	5.47	9.52	7.46	9.29	5.97	5.52
JE	5.44	10.75	5.55	9.13	5.54	8.79	5.27	9.12
JH	6.00	11.57	6.80	16.03	5.08	18.09	5.58	13.18
JL	6.03	21.49	6.95	11.85	6.78	12.40	6.81	11.92
PJ	4.10	4.40	3.59	7.42	4.55	8.35	5.25	7.65
PT	4.67	6.61	6.03	4.60	4.87	4.98	4.94	5.33
RL	6.90	10.49	7.29	11.12	5.47	9.63	7.37	11.49
SS	4.76	15.45	6.79	10.63	4.35	11.88	5.13	16.48
TD	5.87	11.88	6.68	11.53	5.78	13.18	6.36	8.73
TM	7.45	9.90	5.23	9.62	6.31	12.34	5.87	11.13
WO	3.85	7.07	5.63	8.97	4.56	13.26	4.76	12.03
Mean	5.85	9.63	5.85	10.02	5.83	11.13	5.70	9.55
± SD	± 1.33	± 2.78[a]	± 0.99	± 3.01[a]	± 1.25	± 3.76[a]	± 0.74	± 3.09[a]

WBC, total leukocyte count; CAS, sodium caseinate; KER, novel keratin supplement; Pre-ex, immediately before the constant-load exercise test; Post-ex, five minutes after completion of the constant-load exercise test. [a] indicates $p \leq 0.05$ compared to pre-exercise.

C.1.6 RBC and Hb pre- and post-supplementation with CAS and KER, before and after the two-hour constant-load exercise test.

	RBC ($*10^{12}$/L)								Hb ($g.dL^{-1}$)							
	Pre-CAS		Post-CAS		Pre-KER		Post-KER		Pre-CAS		Post-CAS		Pre-KER		Post-KER	
	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-
CB	4.82	4.98	4.80	4.88	4.76	4.92	4.67	4.80	148	155	151	155	147	151	142	147
DF	4.90	5.14	5.04	5.33	5.32	5.53	5.19	5.50	142	149	149	157	153	161	158	163
DO	5.02	5.19	5.14	5.22	3.99	5.00	4.95	5.27	156	163	161	164	125	158	157	168
JB	4.81	5.31	4.99	5.18	5.07	5.30	5.07	5.23	142	157	150	156	151	162	149	154
JD	5.00	5.02	4.65	4.94	5.03	5.36	5.08	5.52	153	156	139	147	143	134	148	146
JE	5.04	5.01	4.92	5.32	5.44	5.26	5.22	5.46	161	168	146	161	161	157	142	153
JH	4.93	5.11	5.20	5.30	5.48	5.69	4.74	5.02	144	152	152	157	153	160	140	150
JL	5.74	5.96	5.83	5.79	5.68	5.87	5.84	5.71	155	161	154	153	156	159	160	158
PJ	5.40	5.00	5.06	5.37	4.74	4.95	5.12	4.97	164	163	148	155	145	150	147	149
PT	4.49	4.77	4.89	4.68	4.74	4.89	4.81	4.82	144	152	152	145	151	153	149	150
RL	5.35	5.58	5.02	5.00	5.18	5.37	5.08	5.40	153	163	149	160	154	159	151	158
SS	4.91	5.07	4.81	4.96	4.66	4.77	4.80	5.14	152	157	147	154	148	149	148	159
TD	5.27	5.60	5.07	5.33	5.03	5.36	5.08	5.52	146	151	144	153	141	151	142	154
TM	5.57	5.41	5.21	5.48	5.62	5.82	5.34	5.62	156	157	161	167	166	172	155	165
WO	4.59	4.85	4.77	4.92	4.50	4.65	4.61	4.86	139	140	140	146	135	138	139	144
Mean	5.06	5.20	5.03	5.18	5.02	5.20	5.03	5.21	150	156	150	155	149	154	148	154
± SD	± 0.34	± 0.31[a]	± 0.27	± 0.27[a]	± 0.45	± 0.39[a]	± 0.30	± 0.31[a]	± 7	± 7[a]	± 6	± 6[a]	± 10	± 9[a]	± 7	± 7[a]

RBC, total erythrocyte volume; Hb, total haemoglobin content; CAS, sodium caseinate; KER, novel keratin supplement; Pre-ex, immediately before the constant-load exercise test; Post-ex, five minutes after completion of the constant-load exercise test. [a] indicates $p \leq 0.05$ compared to pre-exercise.

C.1.7 Hct and MCV pre- and post-supplementation with CAS and KER, before and after the two-hour constant-load exercise test.

	Hct (%)								MCV(fL)							
	Pre-CAS		Post-CAS		Pre-KER		Post-KER		Pre-CAS		Post-CAS		Pre-KER		Post-KER	
	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-	Pre-	Post-
CB	44.7	46.0	44.9	45.5	45.5	45.0	42.9	44.6	92.9	92.4	93.6	93.2	91.4	91.4	91.9	93.0
DF	43.8	46.1	44.9	47.7	47.5	49.8	46.3	48.9	89.5	89.7	89.2	89.4	89.2	90.0	89.2	88.9
DO	45.4	46.6	45.7	46.2	35.8	44.3	43.6	46.1	90.4	89.8	88.9	88.5	89.7	88.6	88.1	87.5
JB	43.7	48.4	44.7	47.2	45.6	47.9	44.8	46.0	90.7	91.1	89.7	91.1	89.5	89.7	89.2	88.9
JD	44.8	44.8	41.2	44.2	45.6	41.4	44.3	43.7	89.6	89.2	88.5	89.4	90.5	89.5	90.0	91.0
JE	44.8	45.8	42.7	47.7	48.7	47.5	46.1	48.6	88.8	91.5	89.0	89.7	89.5	90.2	88.3	89.1
JH	44.5	46.0	46.3	47.4	48.1	49.3	42.0	44.1	90.3	90.0	89.0	89.0	87.7	86.7	88.5	87.9
JL	47.4	49.6	48.4	48.0	46.8	49.0	49.7	47.7	82.7	83.3	83.0	82.9	82.3	83.4	85.1	83.4
PJ	/	/	42.2	44.3	41.4	43.0	44.9	44.2	98.0	99.0	83.4	82.5	87.3	86.9	87.9	88.6
PT	42.7	45.5	44.5	43.0	43.9	43.9	44.2	44.2	95.2	95.3	91.0	91.9	92.6	89.8	91.9	91.7
RL	44.9	46.7	43.1	46.2	45.1	46.9	43.7	46.8	83.9	83.7	83.5	82.6	87.2	87.4	86.1	86.6
SS	45.7	46.9	45.3	46.9	43.1	44.3	44.7	47.7	93.0	92.4	94.0	94.4	92.6	93.5	93.0	92.7
TD	45.1	47.6	42.4	45.7	43.2	46.1	43.4	47.2	85.6	85.0	83.8	85.7	85.9	85.9	85.5	85.5
TM	50.3	49.1	47.1	49.7	51.2	52.7	48.1	51.0	90.2	90.8	90.4	90.8	90.9	90.5	90.1	90.9
WO	41.8	43.1	43.2	45.0	41.0	42.0	42.0	44.3	91.1	89.1	90.6	91.5	91.1	90.4	90.9	91.2
Mean	45.0	46.6	44.4	46.3	44.7	46.2	44.7	46.3	90.1	90.2	88.5	88.8	89.2	88.9	89.0	89.2
± SD	± 1.9	± 1.7[a]	± 1.9	± 1.7[a]	± 3.6	± 3.1[a]	± 2.0	± 2.1[a]	± 3.8	± 4.0	± 3.4	± 3.7	± 2.6	± 2.4	± 2.3	± 2.6

Hct, haematocrit; MCV, mean erythrocyte cell volume; CAS, sodium caseinate; KER, novel keratin supplement; Pre-ex, immediately before the constant-load exercise test; Post-ex, five minutes after completion of the constant-load exercise test. Note: analysis of participant PJ's bloods did not include Hct at two time-points. [a] indicates $p \leq 0.05$ compared to pre-exercise.

C.1.8 MCH and MCHC pre- and post-supplementation with CAS and KER, before and after the two-hour constant-load exercise test.

	MCH (pg)								MCHC ($g.L^{-1}$)							
	Pre-CAS		Post-CAS		Pre-KER		Post-KER		Pre-CAS		Post-CAS		Pre-KER		Post-KER	
	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-	Pre-ex	Post-
CB	30.67	31.20	31.43	31.75	30.92	30.77	30.34	30.57	330	338	336	341	338	337	330	329
DF	29.09	29.05	29.51	29.48	28.79	29.09	30.47	29.67	325	324	331	330	321	323	342	334
DO	31.1	31.4	31.3	31.4	31.3	31.6	31.7	31.9	344	350	353	355	349	357	360	364
JB	29.46	29.59	30.17	30.19	29.84	30.51	29.40	29.40	325	325	336	332	332	337	333	335
JD	30.50	31.08	29.88	29.67	28.41	28.87	30.05	30.42	340	348	338	332	314	323	334	334
JE	31.97	33.47	29.66	30.20	29.49	29.77	27.17	27.95	360	366	334	337	330	330	308	314
JH	29.13	29.75	29.31	29.52	27.87	28.07	29.52	29.80	323	331	329	332	319	324	333	339
JL	26.94	27.01	26.48	26.39	27.37	27.04	27.38	27.68	326	324	319	318	332	324	322	332
PJ	30.00	33.00	29.20	28.90	30.51	30.38	28.72	29.80	/	/	351	350	349	350	327	336
PT	32.06	31.87	31.10	31.00	31.90	31.30	31.00	31.10	337	335	342	337	344	349	337	339
RL	28.60	29.20	28.90	28.60	29.82	29.67	29.73	29.23	341	349	346	346	342	339	346	338
SS	30.95	31.00	30.55	31.10	31.65	31.31	30.74	30.96	333	335	325	330	343	337	330	334
TD	27.70	27.04	28.49	28.79	28.09	28.19	27.98	27.94	324	318	340	336	327	328	327	327
TM	28.06	28.95	30.88	30.46	29.56	29.59	29.05	29.37	311	319	342	336	325	327	322	323
WO	30.30	28.91	29.37	29.62	29.91	29.78	30.08	29.56	333	325	324	324	327	329	331	324
Mean	29.77	30.17	29.75	29.80	29.70	29.73	29.56	29.69	332	335	336	336	333	334	332	333
± SD	± 1.47	± 1.85	± 1.23	± 1.30	± 1.35	± 1.27	± 1.26	± 1.17	± 11	± 14	± 9	± 9	± 11	± 10	± 11	± 11

MCH, mean cell haemoglobin volume; MCHC, mean cell haemoglobin concentration relative to erythrocyte cell volume; CAS, sodium caseinate; KER, novel keratin supplement; Pre-ex, immediately before the constant-load exercise test; Post-ex, five minutes after completion of the constant-load exercise test. Note: analysis of participant PJ's bloods did not include MCHC at two time-points.

C.2. Cardiorespiratory measures

C.2.1 VO_2 during the four-stage submaximal exercise test

	Pre-CAS				Post-CAS				Pre-KER				Post-KER			
	St-1	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**
CB	20.6	26.8	32.4	39.9	22.8	27.0	33.5	65.3	23.9	30.5	36.1	43.5	20.8	25.5	30.9	37.8
DF	15.1	31.8	40.7	48.1	26.6	32.7	40.7	48.1	21.6	28.2	35.6	43.6	22.7	32.0	38.2	47.9
DO	23.6	28.8	35.3	40.9	22.6	27.1	33.7	36.4	22.0	27.5	34.4	38.2	26.3	27.2	34.4	36.6
JB	24.7	30.5	35.7	38.9	25.7	33.3	39.3	43.8	24.3	35.9	43.3	50.2	26.0	34.7	40.6	47.0
JD	25.8	32.7	41.7	44.3	26.8	36.1	49.1	51.3	22.8	35.3	45.4	46.4	32.4	39.9	48.9	48.9
JE	26.1	32.4	37.1	40.7	23.6	29.1	31.7	38.9	22.0	29.8	31.6	34.4	49.0	63.9	70.2	73.9
JH	30.7	40.1	44.2	47.7	33.5	37.7	45.5	50.0	30.5	38.2	44.0	47.6	33.5	37.6	45.7	50.0
JL	26.6	32.5	44.8	49.5	28.0	32.4	38.1	42.6	26.0	35.6	38.0	42.0	26.2	31.1	35.3	41.1
PJ	17.5	24.2	25.4	30.9	22.0	24.6	27.7	28.6	28.2	28.3	32.7	33.9	22.3	26.1	29.5	31.0
PT	29.6	34.4	37.5	41.7	25.7	31.7	35.7	39.2	23.7	32.4	35.7	39.3	23.6	30.5	34.1	36.9
RL	21.6	30.6	36.4	40.9	25.5	31.1	38.0	42.6	22.3	29.0	38.7	42.2	25.5	31.1	37.9	42.5
SS	25.2	36.0	39.8	50.5	28.0	32.9	40.9	47.4	/	34.4	41.1	46.9	24.8	32.9	40.9	44.8
TD	28.7	39.7	44.2	50.6	35.6	46.0	59.8	60.8	31.8	40.5	46.4	52.6	31.4	40.9	45.7	49.9
TM	24.6	30.9	40.7	42.9	27.2	33.2	41.6	40.0	25.8	38.0	38.25	41.8	27.7	34.3	43.0	46.8
WO	26.3	35.1	40.8	48.4	24.0	37.0	37.8	50.2	22.3	34.0	39.1	51.2	26.6	32.7	40.7	48.1
Mean	24.5	32.4	38.4	43.7	26.5	32.8	39.5	45.7	24.8	33.2	38.7	43.6	27.9	34.7	41.1	45.5
± SD	± 4.1	± 4.1[a]	± 4.9[a]	± 5.3[a]	± 3.7	± 5.0[a]	± 7.5[a]	± 9.0[a]	± 3.2	± 4.0[a]	± 4.4[a]	± 5.5[a]	± 6.6	± 9.0[a]	± 9.4[a]	± 9.4[a]

VO_2, volume of oxygen consumed in $ml.min^{-1}kg^{-1}$; CAS, sodium caseinate; KER, novel keratin supplement. [a] indicates $p \leq 0.05$ compared to previous stage.

C.2.2 VCO_2 during the four-stage submaximal exercise test

	Pre-CAS				Post-CAS				Pre-KER				Post-KER			
	St-1	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**	**St-1**	**St-2**	**St-3**	**St-4**
CB	15.5	20.9	26.4	34.3	18.6	21.8	27.9	33.4	20.9	26.9	26.6	40.8	16.8	20.4	24.8	32.0
DF	12.6	26.3	33.8	42.8	21.3	26.7	34.0	41.2	19.2	25.9	33.1	42.3	20.0	28.7	34.9	44.9
DO	18.4	23.2	28.6	33.6	18.7	22.3	27.7	30.2	18.7	24.9	31.1	34.2	23.1	23.8	30.5	32.8
JB	24.7	30.6	37.8	42.9	24.0	31.3	38.1	43.4	20.3	33.4	39.9	44.8	22.5	31.5	36.9	42.7
JD	23.2	30.1	38.5	41.7	29.3	38.8	42.8	44.8	17.4	27.9	38.2	39.3	27.6	35.1	44.1	45.2
JE	23.9	29.3	34.1	36.6	21.2	27.8	30.4	37.2	21.4	30.0	31.3	34.4	23.1	29.3	32.7	34.1
JH	24.3	32.2	35.6	39.3	27.3	30.5	37.6	41.9	25.9	34.3	37.1	43.0	27.3	30.4	37.8	41.9
JL	24.8	32.2	44.2	48.2	23.7	28.1	33.2	36.8	25.0	34.2	36.4	40.4	25.4	29.9	34.4	40.2
PJ	16.6	23.1	23.2	29.5	19.1	21.7	25.4	26.6	22.8	24.0	27.5	28.4	19.9	22.9	25.0	27.2
PT	24.7	28.4	31.6	35.3	19.3	25.4	28.5	21.4	19.6	27.3	29.8	33.3	20.4	25.8	29.4	32.5
RL	16.4	24.7	29.6	32.7	20.2	25.1	31.6	35.8	17.3	23.9	33.2	36.4	20.1	25.1	31.5	35.7
SS	21.4	31.9	35.1	43.0	21.3	26.2	31.7	38.0	/	26.7	32.4	37.8	18.1	25.5	31.7	35.4
TD	26.6	35.8	39.8	45.7	32.0	41.0	53.2	54.3	27.7	36.8	41.7	48.0	26.7	37.1	41.5	45.6
TM	19.2	24.4	34.2	36.7	23.2	28.0	34.9	34.2	19.6	29.6	29.6	33.0	22.9	27.2	35.7	39.1
WO	20.7	28.2	32.7	40.3	20.6	32.9	33.6	45.1	19.7	29.2	34.4	47.7	21.3	26.7	34.0	41.2
Mean	20.9	28.1	33.7	38.8	22.6	28.5	34.0	38.3	21.1	29.0	33.5	38.9	22.4	28.0	33.7	38.0
± SD	± 4.1	± 4.1[a]	± 5.2[a]	± 5.1[a]	± 3.9	± 5.5[a]	± 6.7[a]	± 6.8[a]	± 3.0	± 3.9[a]	± 4.3[a]	± 5.5[a]	± 3.2	± 4.3[a]	± 5.1[a]	± 5.5[a]

VCO_2, volume of carbon dioxide expired in $ml.min^{-1}kg^{-1}$; CAS, sodium caseinate; KER, novel keratin supplement. [a] indicates $p \leq 0.05$ compared to the previous stage.

C.2.3 VO_{2max} and VCO_{2max} values achieved in the ramp protocol

	VO_{2max} ($ml.min^{-1}kg^{-1}$)				VCO_{2max} ($ml.min^{-1}kg^{-1}$)			
	Pre-CAS	**Post-CAS**	**Pre-KER**	**Post-KER**	**Pre-CAS**	**Post-CAS**	**Pre-KER**	**Post-KER**
CB	56.2	59.8	64.9	55.8	65.1	68.6	75.6	56.2
DF	65.7	73.3	55.2	61.9	70.5	76.8	64.7	66.7
DO	53.4	51.7	45.1	49.1	48.8	49.0	42.5	46.6
JB	47.0*	50.5	53.7	54.9	/	53.0	53.7	55.9
JD	65.2	74.2	63.4	71.3	79.6	75.0	69.1	81.6
JE	53.4	47.9	46.3	56.3	50.3	49.5	55.3	61.6
JH	69.6	70.3	64.5	70.1	73.2	70.3	69.4	70.1
JL	66.3	66.4	52.4	54.9	69.2	61.7	58.4	63.3
PJ	39.2	41.6	52.8	45.0	43.5	47.1	49.1	45.5
PT	52.8	60.6	57.3	60.7	51.4	59.5	/	62.0
RL	60.0	61.2	56.1	61.3	60.7	61.2	57.2	61.3
SS	81.3	80.6	79.4	81.9	85.7	83.2	79.8	81.9
TD	67.0	72.7	69.7	66.7	70.8	76.4	76.0	74.2
TM	61.3*	65.1	64.6	66.0	/	64.3	59.0	69.3
WO	71.9	67.9	71.2	73.3	76.4	74.7	85.0	76.8
Mean	60.7	62.9	59.8	62.0	65.0	64.7	63.9	64.9
± SD	± 10.3	± 10.7	± 9.2	± 9.5	± 12.6	± 11.1	± 11.9	± 10.8

VO_{2max}, maximal volume of oxygen consumed in $ml.min^{-1}kg^{-1}$ VCO_{2max}, maximal volume of carbon dioxide expired in $ml.min^{-1}kg^{-1}$; CAS, sodium caseinate; KER, novel keratin supplement. * estimated value calculated from the ACSM equation (Glass *et al.* 2007). The corresponding VCO_{2max} values were not estimated.

C.3. Other measures during the two-hour, constant load exercise test

C.3.1 Heart rate

	Pre-CAS				Post-CAS				Pre-KER				Post-KER			
	30	**60**	**90**	**120**	**30**	**60**	**90**	**120**	**30**	**60**	**90**	**120**	**30**	**60**	**90**	**120**
CB	155	148	152	159	148	152	153	163	143	150	160	168	135	140	138	155
DF	118	124	127	129	127	134	138	139	148	150	139	142	130	130	136	141
DO	121	128	129	129	134	131	134	140	128	127	135	137	117	117	122	120
JB	143	146	144	151	141	136	146	146	140	155	157	163	148	165	164	168
JD	143	143	143	148	128	132	131	133	132	134	132	138	143	148	143	136
JE	125	129	136	133	126	126	132	129	130	142	134	149	128	132	130	142
JH	147	141	143	155	146	146	147	154	161	153	151	/	141	138	147	148
JL	145	147	148	159	138	136	135	146	141	142	138	148	144	143	137	134
PJ	125	129	136	133	126	126	132	129	130	142	134	149	128	132	130	142
PT	102	105	106	116	120	113	111	112	103	101	105	111	121	118	109	111
RL	148	138	137	146	132	132	135	137	142	147	157	147	143	141	144	155
SS	158	158	162	167	150	152	158	159	140	148	153	156	163	174	173	184
TD	146	144	152	156	135	124	130	136	142	138	137	137	135	142	134	145
TM	139	147	149	148	142	143	147	151	54	60	155	160	158	166	167	167
WO	133	141	144	152	139	144	146	146	148	154	156	156	160	168	172	168
Mean	138	139	142	147	137	137	140	144	140	143	144	147	140	144	145	148
± SD	± 15	± 13[a]	± 13[a]	± 13[a]	± 9	± 11[a]	± 12[a]	± 13[a]	± 13	± 14[a]	± 14[a]	± 14[a]	± 13	± 17[a]	± 18[a]	± 19[a]

HR, heart rate in bpm; CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Post, post-supplementation. Participant JH was unable to complete the pre-KER trial due to fatigue. [a] indicates $p \leq 0.05$ compared to previous time point.

C.3.2 RPE during the steady state exercise test

	Pre-CAS				Post-CAS				Pre-KER				Post-KER			
	30	**60**	**90**	**120**	**30**	**60**	**90**	**120**	**30**	**60**	**90**	**120**	**30**	**60**	**90**	**120**
CB	12	12	14	14	13	16.5	16	17	15	15	17	18	12	12	13	13
DF	14	15	17	17	12	13	14	15	15	19	15	16	10	14	16	15
DO	13	14	15	16	13	15	16	17	9	11	14	16	11	13.5	15	16
JB	12	12	14	14	13	16	16	15	12	13	15	15	12	12	14	14
JD	10	9	11	11.5	10	10	10	10	11	11	11	12	10	11	12	12
JE	13.5	14	15	15	12	13	13	15	13	13	15	17	12	13	14	16
JH	12	12	12	12	12	12	12	13	13	14	18	/	12	12	12	12
JL	14	16	17	19	13.5	13.5	14	18	13	14	14.5	15	12	12.5	13	17
PJ	12	12	12	12	11	11	11	11	10.5	12	12	12	10	11	11	13
PT	10	12	14	13	15	13	14	18	12	12	13	12	13	14	15	15
RL	13	13	14.5	16	13	15	16	17	12	13	15	16	13	13	15	16
SS	15	15	15	15	14	14	15	13	13	13	14	15	16	16	17	18
TD	13	14	15	17	10	10	10	11	10	12	13	12	10	11	11	10
TM	12	12	14.5	12.5	11.5	11.5	12.5	13	12	12	13	15	12	13	14	16
WO	12	13	13	15	12	12	13	13	14	15	15	15	15	15	16	16
Mean	12.5	13.0	14.2	14.6	12.3	13.0	13.5	14.4	12.3	13.3	14.3	14.7	12.0	12.9	13.9	14.6
± SD	± 1.3	± 1.8[a]	± 1.6[a]	± 2.1[a]	± 1.3	± 1.9[a]	± 2.0[a]	± 2.6[a]	± 1.7	± 1.9[a]	± 1.7[a]	± 1.9[a]	± 1.7	± 1.4[a]	± 1.8[a]	± 2.1[a]

RPE, perceived exertion; CAS sodium caseinate; KER, novel keratin supplement; Pre, pre-supplementation; Post, post-supplementation. Participant JH was unable to complete the pre-KER trial due to fatigue. [a] indicates $p \leq 0.05$ compared to pre-exercise.

C.4. Performance measures

C.4.1 Maximal power output achieved in the ramp protocol

	Pre-CAS	Post-CAS	Pre-KER	Post-KER
CB	501	511	480	465
DF	399	404	421	394
DO	346	361	315	357
JB	258	291	285	317
JD	398	397	346	373
JE	385	381	388	397
JH	410	415	360	381
JL	319	321	319	321
PJ	303	317	331	347
PT	321	344	/	369
RL	411	378	356	384
SS	461	503	458	509
TD	341	362	354	360
TM	401	419	383	420
WO	426	412	448	442
Mean	379	388	375	389
± SD	± 62	± 60	± 56	± 50

Power measured in W; CAS, sodium caseinate; KER, novel keratin supplement; Pre, pre-intervention; Post, post-intervention. Participant PT was unable to complete the pre-KER trial due to fatigue.

APPENDIX D Acute pomegranate extract supplementation

D.1. Resting measures

D.1.1 Pre-exercise blood NO_3^- values at SEA

	SEAPLAC	SEAPOM
AW	19.2	30.4
CG	2.5	22.0
HW	23.4	30.5
JH	33.6	27.7
JS	34.5	50.8
LS	28.1	41.5
NL	/	/
RS	/	/
Mean	23.6	33.8
± SD	±10.8	±9.5[a]

NO_3^-, nitrates in µmol, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; [a] indicates $p \leq 0.05$ compared to SEAPLAC, [b] p≤0.05 compared to SEAPOMx, [c] p≤0.05 compared to ALTPLAC. Notes: Blood analysis was unsuccessful for participant NL, blood could not be collected from participant RS.

D.1.2 Pre-exercise SBP, DBP and Hct

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
	SBP	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**
AW	119	69	46	132	70	45	129	78	48	150	89	48
CG	120	65	50	121	63	49	124	72	49	126	82	49
HW	112	71	41	132	78	43	126	81	39	134	80	40
JH	135	83	46	129	85	47	152	91	48	152	92	46
JS	121	67	46	122	73	46	125	78	52	134	80	49
LS	97	59	42	93	61	40	108	77	42	117	75	42
NL	141	85	47	156	95	45	124	78	46	120	74	48
RS	119	65	45	113	62	45	130	72	46	112	53	46
Mean	120	71	45	125	73	45	127	80	46	130	86	46
± SD	± 12	± 8	± 3	± 17	± 11	± 3[a]	± 11	± 8	± 4	± 14	± 8	± 3[b]

SBP, systolic blood pressure in mmHg, DBP, diastolic blood pressure in mmHg, Hct, haematocrit as %; SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] indicates $p \leq 0.05$ compared to SEAPLAC. [b] indicates $p \leq 0.05$ compared to ALTPLAC.

D.1.3 Post-exercise SBP, DBP and Hct

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
	SBP	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**
AW	132	77	48	128	64	43	160	76	49	166	83	48
CG	130	73	51	131	86	50	134	74	51	124	98	49
HW	133	80	42	142	85	40	129	69	43	142	80	40
JH	133	82	48	135	89	47	155	87	49	157	96	48
JS	131	79	46	139	80	48	133	94	51	136	89	49
LS	102	74	43	106	68	42	109	77	42	113	77	46
NL	122	88	48	134	82	46	136	85	52	143	89	50
RS	118	76	46	122	72	45	127	78	49	134	77	47
Mean	120	78	46	129	78	45	135	80	48[a]	139	86	47[b]
± SD	± 12[c]	± 5[c]	± 3[c]	± 11[c]	± 9[c]	± 3[ac]	± 15[c]	± 8[c]	± 4[c]	± 16[c]	± 8[c]	± 3[bc]

SBP, systolic blood pressure in mmHg, DBP, diastolic blood pressure in mmHg, Hct, haematocrit as %; SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract, [a] p≤0.05 compared to SEAPLAC, [b] p≤0.05 compared to SEAPOMx, [c] p≤0.05 compared to ALTPLAC. [a] indicates $p \leq 0.05$ compared to SEAPLAC. [b] indicates $p \leq 0.05$ compared to ALTPLAC. [c] indicates $p \leq 0.05$ compared to pre-exercise values.

D.2. Measures during submaximal exercise

D.2.1 VO_2 during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	27.2	42.4	55.1	28.3	43.5	56.1	24.4	38.0	50.6	28.7	42.4	56.5
CG	34.9	44.2	53.2	41.9	53.1	61.6	36.0	45.2	53.4	41.1	52.4	60.2
HW	32.6	39.2	51.0	30.1	38.3	48.8	35.8	41.3	53.9	33.6	39.9	47.7
JH	31.6	40.8	52.3	30.2	39.6	47.8	26.9	33.6	47.2	30.9	38.0	49.8
JS	34.4	44.1	52.4	33.7	43.1	54.6	35.9	45.1	50.4	33.0	42.1	50.8
LS	29.1	37.0	44.3	30.8	37.5	43.2	32.2	41.5	45.3	28.5	38.7	48.2
NL	41.8	50.6	62.8	40.5	50.3	59.6	45.9	53.4	57.5	42.5	48.2	58.0
RS	40.6	51.9	66.3	41.3	51.0	62.3	39.6	50.2	57.5	38.0	48.6	59.9
Mean	34.0	43.8	54.7	34.6	44.5	54.2	34.6	43.5	52.0	34.6	43.8	53.9
± SD	± 4.8	± 4.9[a]	± 6.5[a]	± 5.3	± 5.7[a]	± 6.5[a]	± 6.4	± 6.0[a]	± 4.2[a]	± 5.1	± 5.0[a]	± 5.0[a]

VO_2, oxygen consumption in $ml.min^{-1}kg^{-1}$; SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] indicates $p \leq 0.05$ compared to previous intensity.

D.2.2 VCO_2 during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	25.0	40.6	53.2	26.6	41.8	54.2	23.7	36.5	50.1	27.8	40.8	55.0
CG	30.9	39.8	50.5	37.4	48.4	57.3	33.1	42.7	54.2	40.3	50.9	64.7
HW	29.1	34.6	46.0	28.5	37.2	45.5	35.6	40.1	53.3	32.3	37.2	45.8
JH	24.3	37.9	51.9	27.8	36.9	47.3	26.8	33.1	49.7	29.8	36.4	49.6
JS	27.5	38.3	46.7	25.3	35.3	45.7	30.6	40.1	46.5	28.8	37.9	47.7
LS	25.5	32.0	39.3	26.9	33.5	40.3	28.4	36.2	39.5	24.1	34.3	42.5
NL	37.7	48.1	58.9	37.8	46.3	57.0	41.9	49.4	47.5	39.5	45.3	57.8
RS	33.6	43.3	58.0	40.7	45.0	58.4	37.2	46.3	54.8	36.5	46.9	58.3
Mean	29.2	39.3	50.6	31.4	40.6	50.7	32.2	40.6	49.4	32.4	41.2	52.7
± SD	± 4.4	± 4.7[a]	± 6.1[a]	± 5.8	± 5.2[a]	± 6.4[a]	± 5.6	± 5.1[a]	± 4.7[a]	± 5.5	± 5.5[a]	± 7.0[a]

VCO_2, carbon dioxide production in ml.min^{-1}kg^{-1}; VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] indicates $p \leq 0.05$ compared to previous intensity.

D.2.3 SmO_2 during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
$\%VO_{2max}$	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	60	55	53	60	59	49	61	55	50	69	41	/
CG	52	57	52	52	53	49	49	49	50	41	47	41
HW	73	76	68	76	74	71	61	71	67	64	69	65
JH	82	82	77	84	82	79	70	70	62	52	/	/
JS	60	62	56	65	67	55	61	66	61	63	67	66
LS	64	66	77	80	70	75	68	65	68	60	63	66
NL	53	45	44	56	55	50	50	/	/	51	54	44
RS	60	59	54	52	56	56	52	56	56	57	56	/
Mean	63	63	60	66	65	61	59	62	59	57	57	56
± SD	± 9	± 11	± 12	± 12	± 10	± 12	± 7	± 8	± 7	± 8	± 10	± 11

SmO_2, muscle oxygen saturation as %; VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. Note: data is incomplete due to loss of battery life of the device during testing in the mobile laboratory.

D.2.4 THb during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	13.0	13.1	13.1	13.1	13.2	13.2	13.0	12.9	12.9	12.9	13.0	/
CG	12.8	12.7	12.7	12.8	12.7	12.7	12.9	12.8	12.8	13.0	12.9	12.9
HW	12.2	12.1	12.0	12.3	12.2	12.2	12.3	12.2	12.1	12.72	12.2	12.1
JH	12.3	12.3	12.3	12.2	12.2	12.2	12.3	12.3	12.3	12.7	/	/
JS	12.6	12.5	12.5	12.4	12.3	12.3	12.4	12.3	12.3	12.6	12.6	13.0
LS	12.2	12.0	12.0	12.2	12.1	12.0	12.2	12.3	12.2	12.1	12.1	12.0
NL	13.1	13.0	13.1	13.4	13.3	13.3	13.3	/	/	13.4	13.3	13.2
RS	13.0	13.0	13.0	12.2	12.1	12.0	12.2	12.3	12.2	12.1	12.1	12.0
Mean	12.7	12.6	12.6	12.7	12.6	12.6	12.7	12.6	12.5	12.8	12.7	12.6
± SD	± 0.3	± 0.4[a]	± 0.4[a]	± 0.4	± 0.5[a]	± 0.5[a]	± 0.4	± 0.4[a]	± 0.4[a]	± 0.4	± 0.4[a]	± 0.5[a]

THb, total haemoglobin in $g.dL^{-1}$; VO_{2max}, maximal oxygen consumption in $ml.min.kg^{-1}$, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. , [a] indicates $p \leq 0.05$ compared to previous intensity. Note: data is incomplete due to loss of battery life of the Moxy device during testing in the mobile laboratory.

D.2.5 HR during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	106	132	149	109	134	151	117	130	147	106	133	157
CG	100	140	160	110	147	166	117	139	167	122	143	167
HW	121	141	158	123	139	160	113	137	157	122	142	160
JH	104	121	143	106	125	139	102	120	148	105	124	148
JS	123	144	164	125	147	165	134	157	172	135	157	176
LS	110	127	147	119	133	151	119	132	147	120	141	159
NL	138	159	176	132	154	171	134	156	181	130	156	175
RS	116	133	155	129	139	163	113	144	161	120	141	159
Mean	115	137	157	119	140	158	119	139	160	119	141	163[b]
± SD	± 12	± 11[a]	± 10[a]	± 9	± 9[a]	± 10[a]	± 10	± 12[a]	± 12[a]	± 10	± 10[a]	± 9[a]

HR, heart rate in bpm; VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract, , [a] indicates *p* indicates ≤ 0.05 compared to previous intensity.

D.2.6 $[La^-]_b$ during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	1.5	1.0	2.1	0.8	1.3	1.3	2.8	1.5	1.4	1.6	1.4	3.0
CG	1.0	1.2	2.7	3.1	2.3	4.4	2.1	1.8	3.0	1.4	7.9	7.1
HW	1.9	1.9	5.0	1.5	2.8	2.3	5.1	1.6	3.1	2.9	2.7	2.4
JH	1.1	2.7	2.8	1.7	2.0	2.5	5.2	3.9	3.8	2.3	3.0	4.7
JS	0.8	1.0	1.5	0.7	1.1	2.0	1.3	1.2	2.3	2.5	1.8	3.0
LS	2.2	3.0	2.1	1.3	1.5	3.5	1.8	3.8	1.3	1.0	1.1	3.0
NL	1.0	2.7	2.5	2.3	3.6	3.0	1.0	1.4	3.0	7.7	4.6	3.3
RS	1.8	1.6	1.5	4.5	1.2	3.5	1.8	3.8	1.3	1.0	1.1	3.0
Mean	1.4	1.9	2.5	2.0	2.0	2.7	2.8	2.3	2.5	2.8	3.1	3.7
± SD	± 0.5	± 0.8	± 1.0	± 1.2	± 0.8	± 0.9	± 1.5[a]	± 1.0[a]	± 0.8[a]	± 2.0[b]	± 2.1[b]	± 1.4[b]

$[La^-]_b$, blood lactate concentration in µmol; VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] *p* indicates ≤ 0.05 compared to SEAPLAC, [b] *p* indicates ≤ 0.05 compared to ALTPLAC.

D.2.7 RPE during submaximal cycling exercise

	SEAPLAC			SEAPOMx			ALTPLAC			ALTPOMx		
%VO_{2max}	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
AW	6	10	14	10	12	14	8	12	16	8	12	16
CG	8	13	15	8	11	14	7	12	16	7	13	15
HW	11	13	15	11	12	14	12	14	16	10	14	16
JH	7	9	12	9	10	12	6	13	15	7	10	12
JS	9	12	16	10	12	14	8	12	16	8	12	16
LS	11	12	14	10	12	15	10	12	14	10	13	15
NL	8	12	15	9	12	15	8	14	18	6	9	13
RS	8	12	15	8	11	14	8	11	14	9	11	16
Mean	8	12	15	9	11	14	8	12	15	8	11	14
± SD	± 2	± 1	± 1	± 1	± 1	± 1	± 2	± 2	± 2	± 1	± 2	± 2

RPE, rating of perceived exercise (6-20; Borg, 1999); VO_{2max}, Maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract.

D.3. Measures during the TTE100%

D.3.1 VO_2 and VCO_2 during the TTE100%

	SEAPLAC		SEAPOMx		ALTPLAC		ALTPOMx	
	VO_2	VCO_2	VO_2	VCO_2	VO_2	VCO_2	VO_2	VCO_2
AW	75.4	79.0	73.6	78.3	68.4	75.5	73.8	81.8
CG	73.3	79.9	77.3	78.4	64.0	72.4	66.7	78.8
HW	63.1	67.0	64.0	66.1	62.4	72.1	66.1	67.7
JH	67.1	70.1	57.4	58.7	58.5	66.7	61.8	69.4
JS	67.1	70.9	70.3	72.2	53.6	58.0	60.3	68.6
LS	60.1	57.5	57.8	57.4	60.3	62.1	61.2	59.9
NL	74.1	75.2	72.5	73.5	65.2	70.4	70.7	77.9
RS	80.0	79.9	75.1	80.0	72.9	79.9	74.8	81.8
Mean	70.0	72.4	68.5	70.6	63.2	69.6	66.9	73.2
± SD	± 6.3	± 7.3	± 7.3	± 8.3	± 2.0[a]	± 6.6	± 1.9[bc]	± 7.5

TTE100%, time trial to exhaustion at a workload calculated to elicit 100% of maximal oxygen consumption; VO_2, oxygen consumption in ml.min.kg^{-1}, VCO_2, carbon dioxide production in ml.min.L^{-1}; VO_{2max}, Maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] *p* indicates ≤ 0.05 compared to SEAPLAC, [b] *p* indicates ≤ 0.05 compared to SEAPOMx, [c] *p* indicates ≤ 0.05 compared to ALTPLAC.

D.3.2 Time to exhaustion at 100% VO_{2max}.

	SEAPLAC	SEAPOM	ALTPLAC	ALTPOM
AW	9.30	7.93	7.52	7.93
CG	12.53	13.28	7.38	5.07
HW	13.45	32.32	11.78	19.00
JH	16.80	16.55	8.27	7.10
JS	9.55	7.35	5.63	6.88
LS	7.38	9.02	5.32	8.72
NL	10.53	11.32	5.88	6.00
RS	9.13	6.20	7.18	7.00
Mean	11.09	13.00	7.37[a]	8.46[b]
± SD	± 2.82	± 7.97	± 1.93	± 4.12

Time to exhaustion measured in minutes, VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] *p* indicates ≤ 0.05 compared to SEAPLAC, [b] *p* indicates ≤ 0.05 compared to SEAPOMx.

D.3.3 Muscle oxygenation, heart rate and $[La^-]_b$ during the TTE100%

ID	SEAPLAC				SEAPOMx				ALTPLAC				ALTPOMx			
	SmO_2	THb	HR	$[La^-]_b$	SmO_2	THb	HR	$[La^-]_b$	SmO_2	THb	HR	$[La^-]_b$	SmO_2	THb	HR	$[La^-]_b$
AW	43	13.2	175	11.5	38	13.3	165	7.7	38	13.0	178	10.6	/	/	178	15.0
CG	45	12.6	183	13.9	41	12.7	185	12.7	54	13.2	187	15.8	37	12.9	182	10.7
HW	60	12.1	177	12.9	66	12.1	179	11.9	58	12.1	175	12.2	57	12.2	177	15.6
JH	71	12.3	176	9.7	71	12.1	162	8.4	43	12.4	175	17.6	/	/	176	11.7
JS	48	12.4	184	11.6	65	12.4	184	12.5	48	12.5	187	12.0	55	13.1	189	14.9
LS	75	12.0	170	3.5	81	12.1	174	3.5	62	12.0	183	3.8	60	12.3	173	3.0
NL	40	13.1	191	9.0	45	13.3	191	13.2	/	/	192	12.1	32	13.3	191	10.2
RS	41	13.0	180	15.5	31	13.1	183	10.1	41	13.3	185	13.0	/	/	185	11.5
Mean	53	12.6	180	11.0	55	12.6	178	10.2	49	12.6	183	12.2	48	12.8	181	11.7
± SD	± 13	± 0.4	± 6	± 3.4	± 17	± 0.5	± 9	± 2.7	± 8[a]	± 0.5	± 6[a]	± 2.0	± 11[b]	± 0.4	± 6[b]	± 3.6

TTE100%, time trial to exhaustion at a workload calculated to elicit 100% of maximal oxygen consumption; SmO_2, muscle oxygen saturation as %; THb, Total haemoglobin in $g.dL^-$, HR, Heart rate in beats per min; $[La^-]_b$, Blood lactate concentration in µmol; SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract. [a] *p* indicates ≤ 0.05 compared to SEAPLAC, [b] *p* indicates ≤ 0.05 compared to SEAPOMx. Note: data is incomplete due to loss of battery life of the Moxy device during testing in the mobile laboratory. Data was collected five minutes into the TTE100%.

D.4. Performance measures

D.4.1 Time to exhaustion at 100% VO_{2max}.

	SEAPLAC	SEAPOM	ALTPLAC	ALTPOM
AW	9.30	7.93	7.52	7.93
CG	12.53	13.28	7.38	5.07
HW	13.45	32.32	11.78	19.00
JH	16.80	16.55	8.27	7.10
JS	9.55	7.35	5.63	6.88
LS	7.38	9.02	5.32	8.72
NL	10.53	11.32	5.88	6.00
RS	9.13	6.20	7.18	7.00
Mean	11.09	13.00	7.37[a]	8.46[b]
± SD	± 2.82	± 7.97	± 1.93	± 4.12

Time to exhaustion measured in minutes, VO_{2max}, maximal oxygen consumption in ml.min.kg^{-1}, SEAPLAC, sea-level placebo; SEAPOMx, sea-level pomegranate extract; ALTPLAC, high-altitude placebo; ALTPOM, high-altitude pomegranate extract.[a] *p* indicates ≤ 0.05 compared to SEAPLAC, [b] *p* indicates ≤ 0.05 compared to SEAPOMx.

APPENDIX E The combination of thiol and polyphenol supplementation

E.1. Resting measures

E.1.1 Resting pre-exercise NO_3^-

	PLAC	POMx	NAC	POMx + NAC
BF	43.6	63.7	28.7	43.8
DC	21.0	22.1	21.7	24.5
EC	18.5	42.3	27.9	52.4
EV	5.2	32.8	30.9	24.8
JL	/	/	/	/
JM	12.1	31.8	0	17.7
OJ	/	/	/	/
ST	3.5	13.2	4.7	3.5
Mean	17.3	34.3	19.0	27.8
± SD	± 13.4[b]	± 16.0[a]	± 12.2[b]	± 16.2

NO_3^-, plasma nitrate concentration in µmol; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. Analysis was unsuccessful for participants JL and OJ. [a] indicates $p \leq 0.05$ compared to PLAC. [b] indicates $p \leq 0.05$ compared to POMx.

E.1.2 Pre-exercise SBP, DBP and Hct

	PLAC			POMx			NAC			BOTH		
	SBP	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**
BF	143	94	42.4	144	86	43.1	131	82	42.8	134	85	39.1
DC	130	87	46.1	135	83	43.3	130	88	47.4	117	75	44.2
EC	113	63	45.5	101	62	43.1	102	63	38.8	114	70	41.2
EV	94	65	39.2	95	60	39.0	104	56	40.8	96	67	38.8
JL	124	74	47.4	116	67	48.4	129	80	48.1	125	77	46.5
JM	120	85	46.5	131	81	46.0	131	89	48.1	141	91	45.7
OJ	121	84	47.1	123	85	45.0	132	82	44.8	114	76	45.0
ST	116	70	45.0	114	112	42.7	119	70	42.4	116	77	43.6
Mean	120	78	45.0	120	79	43.8	122	77	44.1	119	77	43.0
± SD	± 13	± 11	± 2.6	± 16	± 16	± 2.6	± 12	± 11	± 3.3	± 13	± 7	± 2.8

SBP, systolic blood pressure in mmHg, DBP, diastolic blood pressure in mmHg, Hct, haematocrit as %; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC.

E.1.3 Post-exercise SBP, DBP and Hct

	PLAC			POMx			NAC			BOTH		
	SBP	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**	**SBP**	**DBP**	**Hct**
BF	149	85	42.9	150	81	46.8	143	88	44.0	148	84	44.1
DC	159	79	45.6	140	80	45.7	135	82	48.5	132	83	44.0
EC	118	71	43.4	111	71	41.2	113	62	41.3	104	74	41.3
EV	104	60	40.1	107	75	40.5	101	64	41.8	111	69	39.4
JL	118	64	49.6	121	66	52.7	129	64	52.9	124	71	52.0
JM	140	78	48.7	154	92	48.5	138	70	50.6	122	72	50.0
OJ	132	81	50.0	122	75	48.7	129	79	44.8	129	76	48.8
ST	134	76	46.1	121	70	48.1	118	72	46.2	116	68	48.2
Mean	132	74	45.8	128	75	46.5	125	72	46.3	123	75	46.0
± SD	± 17[a]	± 8	± 3.3[a]	± 16[a]	± 8	± 3.8[a]	± 13[a]	± 9	± 3.9[a]	± 13[a]	± 6	± 4.1[a]

SBP, systolic blood pressure in mmHg, DBP, diastolic blood pressure in mmHg, Hct, haematocrit as %; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to pre-exercise values.

E.1.4 Pre-exercise HR, SmO_2 and THb

	PLAC			POMx			NAC			BOTH		
	HR	**SmO_2**	**THb**	**HR**	**SmO_2**	**THb**	**HR**	**SmO_2**	**THb**	**HR**	**SmO_2**	**THb**
BF	62	78	12.4	59	70	12.1	51	63	12.3	53	83	12.1
DC	55	58	12.1	62	58	12.5	56	71	12.4	57	49	12.4
EC	52	63	12.2	48	50	12.4	47	52	12.3	44	48	12.4
EV	55	60	12.2	59	69	12.1	56	60	12.1	61	67	12.0
JL	57	50	12.6	54	60	12.5	72	57	12.7	54	56	12.5
JM	68	66	12.5	73	63	12.4	76	62	12.3	83	65	12.4
OJ	70	52	12.5	72	52	12.6	71	62	12.3	71	58	12.4
ST	40	54	13.4	48	55	13.3	45	55	13.3	51	58	13.4
Mean	57	60	12.5	59	60	12.5	59	59	12.5	59	60	12.5
± SD	± 9	± 8	± 0.4	± 9	± 7	± 0.4	± 11	± 5	± 0.3	± 12	± 11	± 0.4

HR, heart rate in bpm; SmO_2, muscle oxygenation as %, THb, total haemoglobin content in dL; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC.

E.1.5 Post-exercise HR, SmO_2 and THb

ID	PLAC			POMx			NAC			BOTH		
	HR	SmO_2	THb	HR	SmO_2	THb	HR	SmO_2	THb	HR	SmO_2	THb
BF	99	83	12.6	106	79	12.4	88	78	12.3	88	89	12.4
DC	98	81	12.2	91	83	12.7	102	82	12.6	87	70	12.2
EC	79	79	12.1	71	74	12.2	69	75	12.1	79	74	12.2
EV	80	82	12.0	86	89	12.2	83	83	12.3	87	84	12.1
JL	109	90	12.6	111	86	12.6	120	86	12.9	111	88	12.7
JM	91	87	12.6	102	87	12.5	97	87	12.5	100	86	12.4
OJ	109	84	12.7	110	83	12.7	114	85	12.4	108	85	12.7
ST	75	68	13.3	85	71	13.4	77	62	13.4	82	72	13.4
Mean	92	82	12.5	95	82	12.6	94	80	12.6	93	81	12.5
± SD	± 13[a]	± 6[a]	± 0.4[a]	± 13[a]	± 6[a]	± 0.3[a]	± 17[a]	± 8[a]	± 0.4[a]	± 11	± 7[a]	± 0.4[a]

HR, heart rate in bpm; SmO_2, muscle oxygenation as %, THb, total haemoglobin content in dL PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to pre-exercise values.

E.2. Measures during submaximal exercise

E.2.1 VO_2 during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity (%VO_{2max})	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	27.5	37.6	45.3	24.5	28.4	35.8	26.7	37.3	47.3	24.9	37.5	47.3
DC	24.5	32.2	37.6	22.9	25.0	36.4	22.9	28.8	36.5	20.4	26.8	32.8
EC	33.5	40.7	51.4	30.1	39.6	51.6	33.4	44.0	53.8	31.6	42.1	51.5
EV	29.6	40.3	50.7	27.6	37.5	45.4	29.5	35.5	44.9	36.0	42.1	52.9
JL	26.2	32.6	45.2	26.8	33.5	42.0	28.6	36.7	46.6	27.4	35.3	44.7
JM	20.2	26.6	33.0	19.5	23.7	29.0	22.2	27.2	34.9	17.6	24.7	32.6
OJ	23.0	29.4	34.3	22.3	28.7	33.1	24.6	30.1	36.3	23.6	30.7	35.7
ST	38.4	45.6	58.6	31.0	46.0	59.7	42.3	53.4	72.1	35.4	47.1	59.6
Mean	27.8	35.6	44.5	25.6	32.8	41.6	28.8	36.6	46.5	27.1	35.8	44.6
± SD	± 5.5[b]	± 6.0[ab]	± 8.4[ab]	± 3.7[b]	± 7.3[a]	± 9.6[a]	± 6.2[b]	± 8.2[ab]	± 11.5[ab]	± 6.3[b]	± 7.4[ab]	± 9.4[ab]

VO_2, oxygen consumption in $ml.min^{-1}kg^{-1}$; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared previous intensity. [b] indicates $p \leq 0.05$ compared to POMx.

E.2.2 VCO_2 during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity ($\%VO_{2max}$)	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	26.6	36.1	48.3	25.6	31.7	44.1	24.9	37.4	52.0	24.7	37.1	50.1
DC	21.8	32.7	36.4	23.6	24.6	35.7	21.8	29.4	38.9	22.9	28.2	34.6
EC	30.7	38.4	48.0	28.6	37.9	51.2	29.0	39.6	50.1	28.1	38.6	48.7
EV	26.7	36.6	50.1	28.0	39.4	48.8	36.9	42.5	57.1	29.5	35.6	46.5
JL	24.4	31.2	45.2	25.5	31.7	43.0	24.8	33.3	43.7	25.7	35.5	45.6
JM	18.4	24.9	32.3	18.7	23.8	29.2	20.4	25.0	32.2	15.4	22.7	30.7
OJ	22.7	27.4	32.6	20.6	27.0	30.4	22.9	30.8	35.4	22.6	27.9	32.0
ST	30.7	38.4	48.0	28.6	37.9	51.1	29.0	39.6	50.1	28.1	38.6	48.7
Mean	26.2	34.0	44.5	25.5	33.0	43.0	26.9	35.3	46.6	25.2	33.9	43.8
± SD	± 5.7	± 5.9[a]	± 9.8[a]	± 4.4	± 7.7[a]	± 10.3[a]	± 5.7[b]	± 6.4[ab]	± 10.3[ab]	± 4.9	± 6.7[a]	±10.1[a]

VCO_2, expired carbon dioxide in $ml.min^{-1}kg^{-1}$; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to PLAC. [b] indicates $p \leq 0.05$ compared to POMx.

E.2.3 SmO_2 during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity (%VO_{2max})	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	68	58	42	59	53	33	58	65	42	83	78	59
DC	58	63	56	51	51	49	53	57	64	47	47	42
EC	49	49	54	50	49	50	46	48	48	42	46	45
EV	47	43	41	57	56	56	47	42	31	47	48	47
JL	37	34	22	37	35	24	49	44	28	35	25	20
JM	51	40	37	56	53	44	52	43	31	47	53	61
OJ	27	40	45	29	23	16	38	30	29	23	17	17
ST	50	40	26	50	44	33	47	41	32	49	41	29
Mean	49	46	38	49	46	38	47	44	40	48	46	40
± SD	± 10	± 11[a]	± 13[a]	± 5	± 10[a]	± 12[a]	± 16	± 17[a]	± 15[a]	± 12	± 9[a]	± 11[a]

SmO_2, muscle oxygen saturation as %; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to previous intensity.

E.2.4 THb during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity (%VO_{2max})	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	12.2	12.2	12.2	12.2	12.1	12.2	12.3	12.2	12.1	12.0	11.9	11.8
DC	12.0	11.9	11.9	12.4	12.3	12.3	12.4	12.3	12.2	12.4	12.2	12.2
EC	12.2	12.2	12.1	12.3	12.3	12.3	12.4	12.3	12.2	12.4	12.4	12.2
EV	12.2	12.0	12.0	12.0	11.9	11.8	12.3	12.2	11.9	12.2	12.0	11.9
JL	12.5	12.3	12.3	12.5	12.4	12.3	12.7	12.6	12.5	12.5	12.4	12.4
JM	12.3	12.3	12.3	12.2	12.2	12.1	12.3	12.2	12.2	12.3	12.2	12.3
OJ	12.6	12.5	12.4	12.7	12.6	12.6	12.3	12.3	12.3	12.5	12.4	12.5
ST	13.4	13.4	13.4	13.4	13.4	13.4	13.4	13.4	13.4	13.3	13.4	13.4
Mean	12.4	12.4	12.3	12.5	12.4	12.4	12.5	12.4	12.4	12.4	12.4	12.3
± SD	± 0.4	± 0.4[a]	± 0.4[a]	± 0.4	± 0.4[a]	± 0.4[a]	± 0.3	± 0.4[a]	± 0.4[a]	± 0.4	± 0.4[a]	± 0.5[a]

THb, total haemoglobin content in dL; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to previous intensity.

E.2.5 HR during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity (%VO_{2max})	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	114	134	154	109	132	157	125	144	161	105	131	152
DC	104	114	130	103	116	129	92	110	133	94	110	128
EC	106	118	146	102	129	152	104	127	152	107	128	152
EV	113	133	156	107	137	151	119	136	156	127	144	161
JL	111	138	171	102	128	160	122	150	177	106	142	172
JM	106	121	135	108	118	133	105	122	137	107	122	136
OJ	108	126	150	110	125	148	115	143	162	113	140	153
ST	100	114	132	90	124	141	95	118	136	100	120	142
Mean	108	125	147	104	126	146	110	131	152	107	130	150
± SD	± 4	± 9[a]	± 13[a]	± 6	± 7[a]	± 10[a]	± 12	± 13[a]	± 14[a]	± 9	± 11[a]	± 13[a]

HR, heart rate in bpm; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to previous intensity.

E.2.6 RPE during submaximal exercise

	PLAC			POMx			NAC			BOTH		
Intensity (%VO_{2max})	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**	**50**	**65**	**80**
BF	8	11	15	7	11	16	9.5	13.5	17	8	11	17
DC	9	11	13	9	12	14	8	13	15	9	12	14
EC	9	12	14	9	12	15	9	13	16	8	21	15
EV	9	11	13	9	10.5	13	9	11	14	11	13	15
JL	10	12	13.5	10	11	12.5	10	11	11.5	8	11	13.5
JM	11	13	15	10	13	15	11	13	15	10	13	15
OJ	6	8	12	6	10	15.5	7	9.5	14	7	10	14
ST	11	12.5	13	11	12	13	10	12	13	10	11	13
Mean	9	11	14	9	11	14	9	12	14	9	12	15
± SD	± 2	± 1[a]	± 1[a]	± 2	± 1[a]	± 1[a]	± 1	± 1[a]	± 2[a]	± 1	± 1[a]	± 1[a]

RPE, perceived exertion, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. [a] indicates $p \leq 0.05$ compared to previous intensity.

E.3. Measures during the time trial

E.3.1 SmO_2 and THb during the time trial

	SmO_2 (%)				THb (dL)			
	PLAC	**POMx**	**NAC**	**BOTH**	**PLAC**	**POMx**	**NAC**	**BOTH**
BF	39	33	33	53	11.9	12.2	12.2	11.8
DC	44	33	44	36	12.2	12.5	12.2	12.2
EC	48	46	40	37	12.0	12.1	12.11	12.1
EV	44	53	29	51	12.5	11.7	11.9	11.9
JL	25	23	27	25	12.3	12.3	12.6	12.4
JM	40	41	28	54	11.9	12.1	12.2	12.3
OJ	38	20	36	24	12.5	12.5	12.2	12.4
ST	21	28	28	24	13.5	13.4	13.5	13.4
Mean	37	35	33	38	12.3	12.4	12.4	12.3
± SD	± 9	± 11	± 6	± 12	± 0.5	± 0.5	± 0.4	± 0.5

SmO_2, muscle oxygenation; THb, total haemoglobin content, PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. Data represents average values across the entire time trial.

E.3.2 SmO_2 and THb during the last minute of the time trial

	SmO_2 (%)				THb (dL)			
	PLAC	**POMx**	**NAC**	**BOTH**	**PLAC**	**POMx**	**NAC**	**BOTH**
BF	37	31	33	51	12.4	12.2	12.2	11.9
DC	35	28	39	32	11.9	12.5	12.3	12.2
EC	40	41	36	31	12.0	12.1	12.2	12.2
EV	42	50	24	50	11.9	11.7	12.0	11.9
JL	22	20	23	21	12.3	12.4	12.6	12.4
JM	35	35	2	51	12.2	12.1	12.2	12.3
OJ	35	13	33	17	12.4	12.5	12.2	12.5
ST	15	27	26	20	13.4	13.5	13.5	13.4
Mean	33	31	29	34	12.3	12.4	12.4	12.3
± SD	± 9	± 11	± 6	± 14	± 0.4	± 0.5	± 0.4	± 0.5

SmO_2, muscle oxygenation; THb, total haemoglobin content, PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC.

E.3.3 HR during the time trial

ID	PLAC	POMx	NAC	BOTH
BF	154	156	163	149
DC	157	151	158	145
EC	165	163	162	163
EV	161	164	163	160
JL	183	175	183	175
JM	146	156	155	160
OJ	169	166	166	170
ST	145	149	146	150
Mean	160	160	162	159
± SD	± 12	± 8	± 10	± 10

SmO_2, muscle oxygenation; THb, total haemoglobin content, PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. Data represents average values during the time trial.

E.3.4 HR during the last minute of the time trial

ID	PLAC	POMx	NAC	BOTH
BF	168	171	174	163
DC	168	161	169	154
EC	170	171	168	168
EV	172	175	176	170
JL	189	190	196	192
JM	161	168	169	173
OJ	185	182	180	184
ST	155	163	160	163
Mean	171	173	174	171
± SD	± 11	± 9	± 10	± 11

SmO_2, muscle oxygenation; THb, total haemoglobin content, PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. Data represents average values during the time trial.

E.4. Performance measures

E.4.1 Energy expenditure during the time trial

	PLAC	POMx	NAC	BOTH
BF	106.2	102.7	97.5	101.9
DC	102	93.5	97.1	90.1
EC	76.5	75.2	74.8	78.9
EV	69.7	72.0	73.5	63.7
JL	79.1	80.9	76.7	75.1
JM	59.1	65.8	66.4	67.5
OJ	69.7	70.3	64.0	70.1
ST	111.4	110.7	115.2	112.9
Mean	84.5	83.9	83.2	82.5
± SD	± 18.1	± 15.4	± 16.9	± 16.4

Energy expenditure measured in kJ; PLAC, placebo; POMx, pomegranate extract; NAC, *N*-acetylcysteine; BOTH, POMx + NAC. Data represents average values during the time trial.

APPENDIX F Published research papers

F.1. Validity and reliability of the Moxy oxygen monitor during incremental cycling exercise

European Journal of Sport Science

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Validity and reliability of the Moxy oxygen monitor during incremental cycling exercise

E. M. Crum , W. J. O'Connor, L. Van Loo, M. Valckx & S. R. Stannard

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MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Emma Crum

Name/Title of Principal Supervisor: Prof. Steve Stannard

Name of Published Research Output and full reference:

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Crum, E. M., O'Connor, W. J., Van Loo, L., Valckx, M., & Stannard, S. R. (2017). Validity and reliability of the Moxy oxygen monitor during incremental cycling exercise. European Journal of Sport Science, 1-7.

In which Chapter is the Published Work: Appendix F

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 50%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  The candidate too the primary role in designing the study, contributed significantly to the data collection, and was primarily responsible for drafting the manuscript.

Emma Crum Digitally signed by Emma Crum Date: 2017.07.10 15:49:10 +12'00'
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Date

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Principal Supervisor's signature

11th July, 2017
Date

GRS Version 3– 16 September 2011

F.2. The effect of acute pomegranate extract supplementation on oxygen uptake in highly-trained cyclists during high-intensity exercise in a high altitude environment

Crum et al. Journal of the International Society of Sports Nutrition (2017) 14:14
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Journal of the International Society of Sports Nutrition

RESEARCH ARTICLE Open Access

The effect of acute pomegranate extract supplementation on oxygen uptake in highly-trained cyclists during high-intensity exercise in a high altitude environment

Emma May Crum[1*], Ahmad Munir Che Muhamed[2], Matthew Barnes[1] and Stephen Robert Stannard[1]

Abstract

Background: Recent research has indicated that pomegranate extract (POMx) may improve performance during aerobic exercise by enhancing the matching of vascular oxygen (O_2) provision to muscular requirements. POMx is rich in ellagitannin polyphenols and nitrates (NO_3), which are both associated with improvements in blood flow and O_2 delivery. Primarily, this study aimed to determine whether POMx improves performance in a cycling time trial to exhaustion at 100%VO_{2max} (TTE100%) in highly-trained cyclists. In addition, we investigated if the O_2 cost (VO_2) of submaximal exercise was lower with POMx, and whether any changes were greater at high altitude where O_2 delivery is impaired.

Methods: Eight cyclists exercised at three submaximal intensities before completing a TTE100% at sea-level (SEA) and at 1657 m of altitude (ALT), with pre-exercise consumption of 1000 mg of POMx or a placebo (PLAC) in a randomized, double-blind, crossover design. Data were analysed using a three way (treatment x altitude x intensity) or two-way (treatment x altitude) repeated measures ANOVA with a Fisher's LSD post-hoc analysis. Significance was set at $p \leq 0.05$. The effect size of significant interactions was calculated using Cohen's d.

Results: TTE100% performance was reduced in ALT but was not influenced by POMx ($p > 0.05$). Plasma NO_3 were 10.3 μmol greater with POMx vs. PLAC (95% CI, 0.8, 19.7,$F_{1,7} = 7.83$, $p < 0.04$). VO_2 measured at five minutes into the TTE100% was significantly increased in ALTPOMx vs. ALTPLAC (+3.8 mLmin ^{1}kg ^{1}, 95% CI, −5.7, 9.5, $F_{1,7} = 29.2$, $p = 0.001$, ES = 0.6) but unchanged in SEAPOMx vs. SEAPLAC ($p > 0.05$). Submaximal VO_2 values were not affected by POMx ($p \geq 0.05$).

Conclusions: The restoration of SEA VO_2 values at ALT is likely driven by the high polyphenol content of POMx, which is proposed to improve nitric oxide bioavailability. Despite an increase in VO_2, no change in exercise performance occurred and therefore this study does not support the use of POMx as an ergogenic supplement.

Keywords: Nitrates, Polyphenols, Ergogenic aids, Exercise performance

Background

Pomegranate (*Punica granatum*) (POM), is a seeded, red, fleshy fruit of Middle East origin which was used in traditional medicine to treat a variety of inflammatory conditions [1]. In modern-day research, the health benefits of POM have been attributed to its high concentration of nitrates (NO_3^-) and polyphenol compounds, and consumption of POM juice (POMJ) or extract (POMx) has been linked to a decline in cancer proliferation [2], the amelioration of cardiovascular disease markers [3] and decreases in gut and joint inflammation [4, 5]. Recent research has indicated that POM-based supplements can also improve performance during aerobic exercise by enhancing the matching of vascular O_2 provision to muscular requirements [6, 7].

Polyphenols are a group of phytochemicals with antioxidant properties that contain one or more aromatic rings and at least two hydroxyl groups [8]. POMJ

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contains a greater concentration of polyphenols (~3.8 mg.ml^{-1}) than other polyphenol-rich beverages such as red wine (~3.5 mg.ml^{-1}), Concord grape juice (~2.6 mg.ml^{-1}) and cranberry juice (~1.7 mg.ml^{-1}) [9]. These are predominantly from the ellagitannin (ET) subclass (80–90%) with smaller amounts of anthocyanins (8–15%) [10]. Consumption of ET or anthocyanin-rich foods is associated with a decrease in systolic blood pressure (SBP) and an increase in vessel diameter and blood flow [6, 11, 12] suggesting a link between POM consumption and O_2 delivery.

Supplementation with dietary NO_3^- influences O_2 delivery during exercise through its conversion to the potent vasodilator, nitric oxide (NO). During resting conditions, NO is primarily produced endogenously by NO synthases (NOS) [13]. However, in hypoxic conditions, such as those present locally in the muscles during exercise, activity of this pathway is limited, placing greater importance on the secondary NO_3^--nitrite (NO_2^-)-NO pathway [14]. Polyphenols further enhance the effects of dietary NO_3^- by promoting their conversion into NO [15, 16], and protecting NO from damage caused by reactive oxygen species (ROS) [17].

Previous research involving NO_3^- supplementation has predominantly used beetroot juice (BRJ), which contains ~11 mmol.L^{-1} NO_3^- [18] in comparison to 12.93 ppm.L^{-1} (~0.2 mmol.L^{-1}) in POMJ and 109 ppm.L^{-1} (~1.76 mmol.L^{-1}) in POMx, as reported by Roelofs et al. [6]. Acute or short-term BRJ supplementation in low to moderately-trained individuals is associated with a large increase (>92%) in plasma NO_2^-, a 3–5% reduction in O_2 uptake (VO_2) during submaximal exercise and a 15–25% improvement in performance during cycling, running and knee extension time to exhaustion protocols [18–22]. In contrast, BRJ has little or no benefit on these parameters in highly-trained athletes (VO_{2max} >60 ml.min^{-1}kg^{-1}), who may have greater NOS activity and consequently a lower reliance on NO production via NO_3^- [23–29]. However, BRJ appears to benefit these athletes during exercise at high altitude, where the lower atmospheric pressure of O_2 (PO_2) impairs O_2 transport and places greater reliance on the non-aerobic pathway to produce NO [30, 31]. This is significant because exercise at high altitude features in a number of major sporting competitions, such as the Tour de France.

While BRJ clearly has an effect on VO_2, the high concentration of ET in POM may provide a source of dietary NO_3^- with greater bioavailability, despite the lower total NO_3^- content. Currently, only two studies have investigated the effect of POM supplementation on endurance exercise performance at sea-level, with no previous research investigating its effects in low PO_2 conditions. In moderately-trained individuals, acute POMx intake (1000 mg) increased pre-exercise blood flow and time to exhaustion during treadmill running at 90-100% of peak velocity [7]. Conversely, following seven days of POMJ supplementation (1000 ml.day^{-1}) in a trained cohort, no change in performance was observed during a ten minute cycling time trial or time to exhaustion protocol in hot conditions [32]. Thus, further research is warranted to determine whether POM improves O_2 transport and endurance exercise performance.

The outcomes of the current study are primarily to further explore the effect of acute POMx supplementation on endurance exercise performance, and secondarily, to determine the effect of POMx on O_2 transport parameters. In addition, the study will investigate whether any observed effects are greater during exercise in a high altitude environment. We hypothesize that acute POMx supplementation will reduce the submaximal O_2 cost of exercise, and in doing so, improve performance in an environment (altitude) where O_2 availability may be limiting.

Methods

Participants

All participants provided written consent after being informed about the study requirements and benefits and risks of participating. A health questionnaire was also completed. Eight highly-trained cyclists, including seven males and one female, were recruited from the regional cycle community. This sample size was chosen based on previous studies which have successfully shown a decrease in VO_2 following NO_3^- supplementation with a sample size of 8 participants [18, 21]. While the sample size is small, this was unavoidable given the highly-trained status of participants that we wished to study. All participants were current or past members of the national cycling or triathlon junior development programmes. Their age, height, body mass and peak aerobic capacity (VO_{2max}) were 17–18 years, 67.6 ± 7 kg, 180 ± 9 cm and 74.4 ± 6.2 ml.min^{-1}kg^{-1} respectively. The study was approved by the Massey University Human Ethics Committee (Southern A 15/54) in accordance with the Declaration of Helsinki.

Experimental design

VO_{2max} test

In order to determine workloads for the supplemented trials, participants completed a VO_{2max} test in thermoneutral conditions (18–20 °C) at sea-level, on an electronically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands), which was set up as closely as possible to the participant's own bike. This session doubled as an initial familiarization session to ensure participants were familiar with the equipment and protocols involved in the experimental sessions. Following a five minute warm-up at 100 W, participants completed four × 7-min stages of increasing workload (e.g., 150, 200, 250, 300 W) with expired air being

collected into Douglas Bags during the last minute of each stage and analysed for O_2 and CO_2 concentrations and volume. Following a five minute active rest period, an incremental "ramp" protocol was used to determine VO_{2max}. Power began at 100 W and increased linearly with time (25 $W.min^{-1}$). Participants cycled for as long as possible and verbal encouragement was given to elicit maximal effort. As the participant's VO_{2max} was approached (as indicated by a change in breathing pattern), expired air was captured in Douglas bags until exhaustion. Analysis of Douglas bags was done using a calibrated gas analysing system (AD Instruments, Dunedin, New Zealand). The gas analyser was calibrated using gases of known concentration (15.01% O_2, 5.01% CO_2). Minute ventilation (V_E) and concentrations of O_2 and CO_2 values were used to calculate the volume of inspired air (V_I) using the Haldane transformation, where V_E was corrected for barometric pressure, ambient temperature and atmospheric water saturation. Subsequently, VO_2 and expired CO_2 (VCO_2) could be determined and are reported as standard temperature and pressure dry (STPD). The respiratory exchange ratio (RER) was calculated using VCO_2/VO_2 and attainment of VO_{2max} was confirmed with RER ≥1.1. A relationship between steady-state workload and VO_2 values was drawn through creation of a power curve and generation of a linear line equation $y = mx + c$, where m = gradient, x = power and c = start point. The equation was used to estimate power output at 50, 65 and 80% of VO_{2max}.

Experimental protocol

The experimental protocol (Fig. 1) was a randomized, double-blind, crossover design, which was completed on four occasions: twice in sea-level conditions (SEA) and twice in high-altitude conditions (ALT, 1657 m, ~17% O_2). The testing location was the Turoa ski-field carpark, Ohakune, which was chosen due to being the highest road accessible to our mobile laboratory in the North Island of New Zealand. Prior to each trial, participants ingested 1000 mg of either a POMx supplement (POM Wonderful LLC, USA) in capsule form or a placebo capsule of the same colour, size and shape as POMx (PLAC, brown sugar). The quantity of POMx was based on Trexler et al. [7] who showed an association between acute POMx supplementation and improved running performance. Previous analysis of the POM Wonderful LLC supplement have shown it to contain 1800 ppm polyphenols, comprised of 95.5% ellagitannins, 3.5% ellagic acid and 1% anthocyanins [32]. Participants completed a POMx trial and a PLAC trial in each environmental condition in a randomized order; the supplement blinded to the participant and the researcher. The participant was instructed to swallow the capsule whole, without tasting it, to avoid an expectation bias. In accordance with previous POMx research, the supplement was ingested 2.5 h prior to each experimental trial to allow maximal absorption prior to exercise [9]. During the 48 h prior to each session, participants were asked to limit consumption of NO_3^- and polyphenol-rich foods and avoid strenuous exercise and antibacterial substances, such as mouthwash, which destroy NO_3^- -NO_2^- converting bacteria on the tongue [33]. Participants consumed a standardized meal (524 cal, 8 g protein, 11 g fat, 51 g carbohydrate) three hours prior to exercise. Participants were asked to arrive at the testing session in a hydrated state, and consumed water ad libitum throughout the trial.

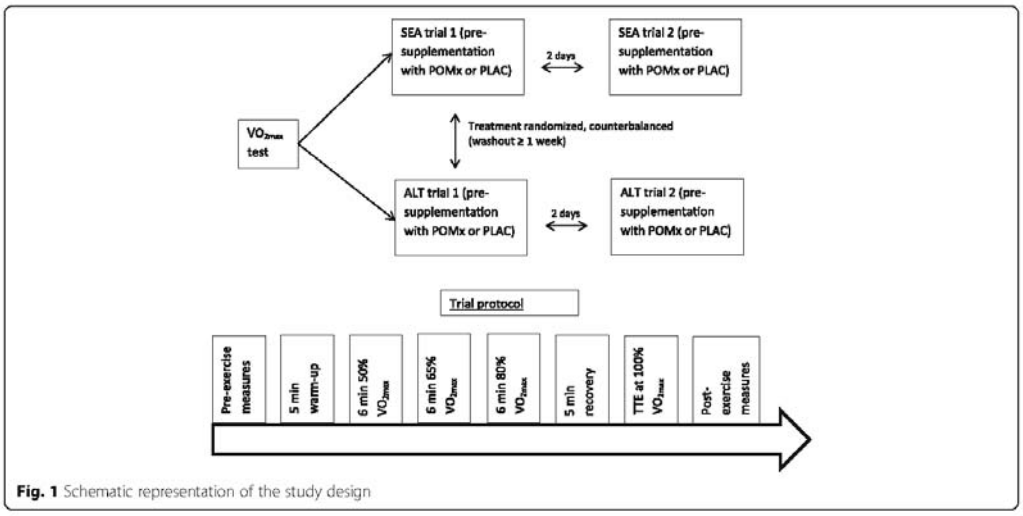

Fig. 1 Schematic representation of the study design

On arrival to the laboratory, body mass was obtained and a heart rate (HR) monitor (Garmin, Kansas, USA) was applied and recorded HR at a sampling rate of two seconds throughout the trial. After sitting in a supine position for five minutes, blood pressure of the brachial artery was measured using an automated sphygmomanometer (Japan Precision Instruments, Gumma, Japan). Blood was collected via fingerprick sample (~5 μL) and analysed for blood lactate concentration ($[La^-]_b$) using a blood lactate test meter (Lactate Pro, Arkray KDK, Japan). To determine haematocrit (Hct), additional blood (~35 μL) was collected from the fingertips in heparinized capillary microtubes and immediately spun in a microhaematocrit centifuge (Thermo IEC MB, Bellport, USA) at 14,000 rpm for two minutes. Hct was calculated as the length of red blood cells as a percentage of the total length of blood in the tube. Samples were taken in duplicate to allow calculation of an average value. In the SEA trials, a venous blood sample was also collected via venepuncture from the antecubital vein in heparinized vacutainers and immediately spun in a centrifuge (Eppendorf AG, Hamburg, Germany) at 2500 rpm for 12 min at 4 °C. Plasma was transferred into epindorphs and stored at −80 °C until analysis. The venous sample was not collected in ALT as samples could not be stored or analysed in the mobile laboratory.

Following the pre-exercise measures, the participant mounted the ergometer to begin cycle exercise. The experimental protocol began with three x six minute stages of stationary cycling exercise at power outputs corresponding to 50, 65 and 80% of their previously determined VO_{2max}. In the last minute of each stage, VO_2 was measured as previously described. Perceived exertion (RPE) was also recorded using Borg G [34] Scale of Perceived Exertion. Then, following a five minute rest, the load on the ergometer was increased to a workload calculated to elicit VO_{2max} and participants were instructed to ride at this intensity, at a cadence of ≥ 80 rpm for as long as possible, with the trial being terminated once the participant could not maintain the required cadence (for the previous 10 s) or at volitional exhaustion. Time to fatigue at 100%VO_{2max} (TTE100%) was chosen in this study as a performance measure rather than a self-paced time trial to enable physiological data to be collected and compared at the five minute point. Accordingly, five minutes into the TTE100% VO_2 was measured.

Blood analysis

Plasma samples were analysed using a Nitric Oxide Colorimetric Assay Kit (BioVision Incorporated, Milpitas, California, USA) to measure NO_3^- and NO_2^-. This method is a two-step process, in which nitrate reductase is used to convert NO_3^- to NO_2^-, and then Greiss Reagents convert NO_2^- to a deep purple azo compound. Absorbance is read at 540 nm and plotted as a function of NO_3^- and NO_2^- concentration.

Statistical analyses

Statistical analyses to compare the values of all variables measured were done using statistical computer software (SPSS Statistics, Version 23, IBM Corporation, New York). Normal distribution of data was confirmed with the Shapiro-Wilks test. Variables measured during the submaximal exercise stages were analysed using three-way repeated measures ANOVA to test the significance level for main effects of, and interactions between, altitude (SEA or ALT), workload (50, 65 and 80%VO_{2max}) and treatment (PLAC or POMx). Similarly, two-way (altitude x treatment) repeated measures ANOVA was performed on all variables measured during the TTE100%. Where significant interactions ($p \leq 0.05$) were observed, two-tailed paired t-tests with a Fisher's LSD post-hoc analysis were used to identify the location of the significance. The effect sizes (ES) of significant interactions were calculated using Cohen's d (0.1 small; 0.5 medium; 0.8 large) [35]. The relationship between VO_2 and performance during the TTE100% was analysed using the Pearson correlation coefficient. Data is presented as mean ± SD or mean change and 95% CI, as appropriate.

Results

Participants' self-reported adherence to supplement intake was 100% and no side effects of supplementation were reported. There was no order effect present for any of the significant results ($p > 0.05$).

TTE100% performance

Average performances during each condition were: SEAPLAC: 10.7 ± 2 min, SEAPOMx: 12.6 ± 8.7 min, ALTPLAC: 7.0 ± 2.3 min and ALTPOMx: 8.2 ± 4.5 min. There was no main effect of treatment on performance ($F_{1,7} = 0.776$, $p > 0.05$). Performance was significantly decreased at ALT compared to SEA (−4.1 min, 95% CI, −6.9, −1.3, $F_{1,7} = 12.3$, $p < 0.02$, ES = 0.8). The individual performance responses in the TTE100% to POMx in SEA and ALT are displayed in Fig. 2. Despite no overall significant effect of POMx, there appear to be two participants (HW and LS) who increased their performance with POMx in both altitudes.

Resting measures

Plasma NO_3^- was greater following POMx compared to PLAC (+10.3 μmol, 95% CI, 0.8, 19.7, $F_{1,7} = 7.83$, $p < 0.04$, ES = 0.9). However, the assay used was not sensitive enough to detect changes in NO_2^-. SBP was not significantly affected by POMx or ALT ($p > 0.05$).

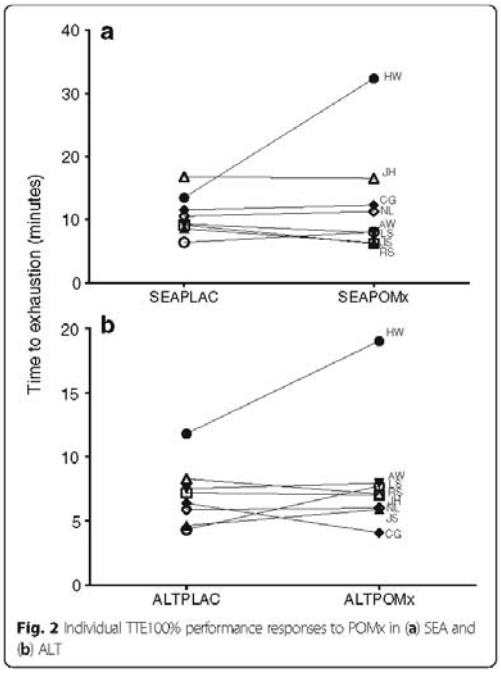

Fig. 2 Individual TTE100% performance responses to POMx in (**a**) SEA and (**b**) ALT

However, there was a trend towards an increase in SBP with POMx vs. PLAC (+3.9 bpm, 95% CI, −0.6, 8.5, $F_{1,7} = 4.28$, $p = 0.08$, ES = 0.3). There was a strong trend towards an increase in DBP with ALT, which showed a moderate effect size (+5 mmHg, $F_{1,7} = 1.18$, $p = 0.054$, ES = 0.6) and a significant treatment x altitude x time interaction ($F_{1,7} = 7.64$, $p < 0.03$). However, post-hoc analyses revealed no significant differences between pre or post-exercise DBP with either treatment at either altitude ($p > 0.05$). Hct was significantly increased by ALT (+1.4%95% CI, 0.2, 2.6, $F_{1,7} = 7.56$, $p < 0.03$, ES = 0.4) and significantly decreased by POMx, although the effect size was small (−0.76%, 95% CI, −1.3, −0.2, $F_{1,7} = 10.4$, $p < 0.02$, ES = 0.2).

Pulmonary gas exchange, ventilatory and blood lactate responses to submaximal exercise

There was no significant main effect of POMx nor any significant altitude x treatment interaction on VO_2, VCO_2, HR or $[La^-]_b$ ($p > 0.5$). There was a trend towards an increase in HR with POMx, but this had a small effect size (+2.3 bpm, 95% CI, −5.0, 0.5, $F_{1,7} = 3.80$, $p = 0.09$, ES = 0.1). There was a significant main effect of altitude on $[La^-]_b$ (−0.8 μmol,95% CI, 0.3, 1.3, $F_{1,7} = 2.95$, $p < 0.01$, ES = 0.6).

Pulmonary gas exchange, ventilatory and blood lactate responses to the time trial to exhaustion at 100%VO_{2max}

The variables measured during the TTE100% are summarized in Table 1. All data was recorded five minutes into the TTE100%. The two-way ANOVA showed a significant altitude x treatment interaction for VO_2 ($F_{1,7} = 12.5$, $p = 0.01$). Post-hoc analysis identified that POMx significantly increased VO_2 at ALT (+3.8 $ml.min^{-1}kg^{-1}$, 95% CI, −5.7, 9.5, $F_{1,7} = 29.2$, $p = 0.001$) but not at SEA ($F_{1,7} = 0.95$, $p > 0.05$, ES = 0.2). Cohen's d showed a moderate effect size of 0.6. There was no correlation between VO_2 during the TTE and performance ($r^2 = 0.0001$, $p \geq 0.05$). There was a significant altitude x treatment interaction for VCO_2 ($F_{1,7} = 6.32$, $p = < 0.04$). However, post-hoc analysis identified no significant differences between treatments at SEA ($F_{1,7} = 1.76$, $p > 0.5$, ES = 0.2), and a trend towards an increase in VCO_2 with POMx at ALT with a moderate effect size (+3.6, 95% CI, −0.665, 7.82, $F_{1,7} = 3.98$, $p = 0.09$, ES = 0.5). HR and $[La^-]_b$ were not affected by POMx or altitude ($p > 0.5$).

Discussion

Although acute POMx supplementation was associated with a 10.3 μmol increase in plasma NO_3^- (95% CI, 0.8, 19.7, $p < 0.04$, ES = 0.9), its use as an acutely-ingested ergogenic supplement by highly-trained athletes is not supported by the current study, as neither performance nor submaximal VO_2 were significantly altered by POMx ingestion. However, our data indicated that POMx does allow maintenance of VO_2 at a workload prescribed to elicit 100% VO_{2max} (at sea level) during high intensity exercise under low PO_2 conditions, despite no significant performance effect.

The absence of changes in performance during the TTE100% is in agreement with Trinity et al. [32] who demonstrated no effect of acute POMJ supplementation on a cycling time trial or time to exhaustion protocol in hot conditions in moderately-trained individuals. However, Trexler et al. [7] found an increase in time to exhaustion during treadmill running at 90–100% of peak

Table 1 Variables measured during the TTE100%

	SEA-PLAC	SEA-POMx	ALT-PLAC	ALT-POMx
VO_2 (ml.min.kg $^{-1}$)	70.0 ± 6.3	68.5 ± 7.2	63.2 ± 5.6[a]	66.9 ± 5.3[bc]
VCO_2 (ml.min.kg $^{-1}$)	72.4 ± 7.8	70.6 ± 8.9	69.6 ± 7.1	73.2 ± 8.0
HR (bpm)	186 ± 8	175 ± 8	184 ± 7	184 ± 5
$[La^-]_b$ (μmol.L $^{-1}$)	11.0 ± 3.7	10.2 ± 2.9	12.2 ± 3.9	11.7 ± 3.9

Data are presented as means ± SD
VO_2 oxygen uptake, *VCO_2* expired carbon dioxide, *HR* heart rate, *$[La^-]_b$* blood lactate concentration, *SEA-PLAC* sea-level placebo, *SEA-POMx* sea-level pomegranate extract, *ALT-PLAC* high-altitude placebo, *ALT-POMx* high-altitude pomegranate extract
[a]indicates P < 0.05 compared to SEA-PLAC
[b]indicates $p < 0.05$ compared to SEA-POMx
[c]indicates $p < 0.05$ compared to ALT-PLAC

velocity, in moderately trained participants, following acute supplementation of POMx (1000 mg, 30 min before exercise) at sea-level. In addition, POMx has been shown to improve performance and recovery from resistance and sprint cycling exercise [36, 37]. The current study is the first study to test POMx supplementation in highly-trained endurance athletes and is in accordance with previous research involving acute BRJ supplementation in this cohort, which found no change in performance in a running or cycling time trial in hypoxic conditions [28, 29]. However, two studies involving multi-day periods of NO_3^- supplementation in low PO_2 conditions (11–13% O_2) showed changes in both O_2 parameters and exercise performance in moderately-trained individuals (VO_{2max} 58–61 $mL.min^{-1}kg^{-1}$). Kelly et al. [31] found that three days of BRJ supplementation (~8.4 $mmol.day^{-1}$) decreased steady-state VO_2 during a bout of moderate-intensity cycle exercise by 7.6% and this resulted in an 8% improvement in a subsequent high intensity time to exhaustion protocol, compared to exercise done following the intake of a placebo. Masschelein et al. [30] found that six days of BRJ supplementation (~5 $mmol.day^{-1}$) increased arterial O_2 saturation by 2.7% and the muscle tissue oxygenation index in the vastus lateralis by 4% during submaximal cycling. This resulted in a 5% increase in performance during a subsequent cycling graded exercise test. Thus, the small increase in VO_2 with acute supplementation of POMx may not be sufficient to produce an ergogenic effect during a 100%TTE in highly-trained athletes and a longer period of supplementation may be necessary. Alternatively, the absence of change in performance despite an increase in VO_2 may reflect the lack of correlation between VO_2 and performance times ($r^2 = 0.0001$), which indicates that VO_2 was not the determining factor in the TTE100%. Rather, it is likely that other factors, such as anaerobic capacity, contribute more greatly to performance in a high-intensity TTE.

To the authors' knowledge, the current research is the first study to have measured VO_2 during cycling exercise following supplementation with POMx. The lack of effect of POMx on submaximal VO_2 or VCO_2 values in either environment despite an increase in plasma NO_3^- is in accordance with previous research involving acute supplementation with NO_3^--rich BRJ in highly-trained athletes (VO_{2max} >66 $ml^{-1}min^{-1}kg^{-1}$) conducted at sea-level [23, 24, 26, 38] or in low PO_2 conditions (13–15% O_2) [28, 29]. In addition, the absence of change in VO_2 during the TTE100% at SEA correlated with Boorsma et al. [39] who conducted a running protocol involving similar intensities to the current study, and found no significant difference in VO_2 values during a 1500 m TT. The restoration of an ALT-induced lowering in VO_2 during intense exercise following POMx compared to SEA (+3.8 $Lmin^{-1}$, 95% CI, −5.7, 9.5, $p = 0.001$, ES = 0.6) differed from previous NO_3^--based research involving highly-trained participants, although no other studies using hypoxia and VO_2 measurement have utilised a performance test protocol similar to ours. Masschelein et al. [30] found no change in VO_{2max} recorded during a graded exercise test to exhaustion in hypoxic conditions (11% O_2, ~5000 m altitude) despite a 4% increase in the muscle tissue oxygenation index. However, in that study, exercise intensity was not stable, as it was in the current study and VO_2 was measured at exhaustion, rather than part-way into the exercise. In addition, MacLeod et al. [29] recorded no change in VO_2 during a 10 km cycling time trial completed in a normobaric hypoxic chamber (~2440 m altitude). However, the duration of this test was significantly longer than ours, which may have influenced potential effects on VO_2.

The changes in VO_2 in the current study were likely driven by the high polyphenol content of POMx, as POMx has a lower NO_3^- concentration than BRJ. While BRJ-based research in highly-trained athletes has recorded increases in plasma NO_3^- of 31–1907% [27, 31, 39], the current study measured a 44% increase in NO_3^- with POMx. Although polyphenolic compounds are also present in BRJ, these are predominantly from the quercetin subclass, in comparison to the high ET component in POMx [40]. Previous research has generally found no link between quercetin intake, and changes in VO_2 or performance parameters [41, 42]. However an increase in pre or post-exercise blood flow and vessel diameter has been observed with consumption of ET or anthocyanin-containing fruit juices and extracts [6, 7, 11]. Further, Ignarro et al. [17] demonstrated that POMJ is more effective at protecting NO from breakdown than other polyphenol-containing juices and red wine, with significant antioxidant actions occurring at dilutions greater than 1000-fold. Thus, the increased VO_2 during the TTE100% following POMx supplementation may be due to a polyphenol-induced greater NO bioavailability.

Despite the large amount of research indicating a relationship between a supplement-induced increase in NO and changes in VO_2 during exercise, presumably through an increase in the efficiency of O_2 transport, the mechanisms behind these changes are currently irresolute. Although NO is known to mediate vasoactivity, an overall increase in vasodilation does not explain the reduction in VO_2 during submaximal exercise in normoxia which has been observed in other studies [18–22]. Rather, the predominant mechanistic theories to explain the effects of NO on VO_2 involve an increase in either the efficiency of mitochondrial O_2 usage or in the muscular use of ATP, affording a lower VO_2 requirement to sustain a given work rate [21, 43]. However, during exercise under hypoxic conditions, a NO-induced augmentation in vasodilation has

been shown to be important in maintaining blood flow to the active muscles [44]. Thus, it is likely that under hypoxic conditions, the vasoactive role of NO contributes more greatly to the level of VO_2 which can be achieved during intense exercise. In accordance with previous research [26, 28], POMx did not affect $[La^-]_b$, indicating that any changes observed were probably not due to a change in fuel usage.

Several reasons have been proposed for the lower efficacy of NO_3^- supplementation on submaximal VO_2 and performance in trained compared to untrained individuals. Firstly, as a response to training, athletes tend to have elevated NOS activity [45], and higher resting NO_2^- and NO_3^- values [46]. Consequently, they may have a lower requirement for NO_3^--NO_2^--NO pathway, and have sufficient NO_3^- present in the blood to use it when needed. Further, training adaptations which aid in O_2 transport and energy production, such as increased capillarization and mitochondrial density, may reduce the incidence of acidic and hypoxic muscular environments, which decrease NOS activity, and increase reliance on the NO_3^- pathway [25, 47]. Finally, research in rats has suggested that the NO_3^- pathway is predominantly used in type II muscle fibres, which work more frequently under acidic or hypoxic conditions [48]. If this is the case, NO_3^- supplementation may be more effective in untrained individuals who tend to have a greater percentage of type II fibres [49]. However, currently, there is no direct evidence that type II fibres are preferentially affected in humans. Alternatively, the lack of overall performance response may be due to the presence of 'responders' and 'non-responders' to POMx. Despite no overall significant effect of POMx on performance in the current study, there were two participants who increased performance in both altitudes. Thus, a larger sample size of participants may be needed to determine the ratio of responders vs. non-responders in a highly-trained population.

In addition to the results already presented in the discussion, the current study produced two results which are difficult to explain. Firstly, in contrast to previous research which has reported a lowering in SBP following acute BRJ or ET polyphenols from grapes in sedentary or moderately-trained individuals [11, 18, 50], and no effect on SBP following BRJ supplementation in highly-trained individuals [25, 27, 29, 38], this study found a trend towards an increase in SBP with POMx (+3.9 bpm, 95% CI, −0.6, 8.5, $p = 0.08$). The only explanation we can give for this result is that the increase in SBP was a reactive response by the vasculatare to ensure that the mean arterial pressure and thus, vessel perfusion and blood flow, were maintained, despite the NO-induced vasodilation. Further, being an acute intervention, we did not anticipate changes in Hct with POMx, but surprisingly, there was a significant decrease in Hct following POMx compared to PLAC (−0.76%, 95% CI, −1.3, −0.2), which indicated a small reduction in the O_2-carrying capacity of the blood. However, despite being significant, this change had a small effect size (ES = 0.2) and considering the increase in VO_2 with POMx at ALT, did not appear to affect VO_2 capacity. Hct varies from day to day by ~3% [51] and it is possible that this result was due to differences in hydration between tests, as we did not measure pre-exercise hydration status, or standardize water intake during exercise. However both these explanations are purely speculative and further research is required to determine whether POMx supplementation consistently results in similar effects on SBP and Hct.

A limitation to the current study is the relatively low altitude used in the hypoxic condition compared to the altitude generally associated with physiological changes (2500–3000 m) [52] and that simulated in previous studies involving NO_3^- supplementation in hypoxia (~2500–5000 m) [28, 30, 31]. Due to our study being conducted in a mobile laboratory, we were restricted to areas with vehicle access in the North Island of New Zealand, and conducted the study at the highest altitude possible under these conditions. However, future studies could investigate the potential ergogenic benefit of POMx at a higher altitude of >2500 m. In addition, the current study was limited by a relatively small sample size (n = 8), which was unavoidable due to the research taking place during a high-performance junior training camp. While previous research has demonstrated a reduction in VO_2 during submaximal cycling exercise [18, 21], it is acknowledged that the lack of effect of POMx on submaximal VO_2 in the current study may have been due to an insufficient number of participants.

Conclusion

In conclusion, acute POMx supplementation allowed a partial restoration of VO_2 during intense exercise in a hypoxic environment. However, no significant changes in VO_2 occurred during submaximal exercise and there was no effect of POMx on performance in either environment. Thus, the results from the current study do not support POMx as an ergogenic supplement when ingested acutely prior to exercise.

Abbreviations

$[La^-]_b$: Plasma lactate concentration; ALT: High altitude conditions; BRJ: Beetroot juice; CO_2: Carbon dioxide; DBP: Diastolic blood pressure; ET: Ellagitannin polyphenols; Hct: Haematocrit; HR: Heart rate; NO: Nitric oxide; NO_2^-: Nitrites; NO_3^-: Nitrates; NOS: Nitric oxide synthases; O_2: Oxygen; PLAC: Placebo; POM: Pomegranate; POMJ: Pomegranate juice; POMx: Pomegranate extract; RER: Respiratory exchange ratio; ROS: Reactive oxygen species; RPE: Rating of perceived exertion; SBP: Systolic blood pressure; SEA: Sea level conditions; TTE100%: Time trial to exhaustion at 100%VO_{2max}; VCO_2: Volume of expired carbon dioxide; V_E: Volume of expired air; V_I: Volume of inspired air; VO_2: Volume of oxygen consumption; VO_{2max}: Maximal oxygen consumption

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
The study was designed by EC and SS; data were collected and analysed by EC, AM and SS, data interpretation and manuscript preparation were undertaken by EC with the assistance of SS and MB. All authors read and approved the final version of the paper.

Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
The participants were notified of all the potential risks and benefits associated with the study before written consent was obtained. The study was approved by the Massey University Human Ethics Committee (Southern A 15/54) in accordance with the Declaration of Helsinki, and written and verbal consent was obtained prior to commencement of the study.

Publisher's Note

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DRC 16

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Emma Crum

Name/Title of Principal Supervisor: Prof. Steve Stannard

Name of Published Research Output and full reference:

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In which Chapter is the Published Work: Appendix F

Please indicate either:

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  The candidate contributed to the research design, conducted the data collection with minimal assistance from the supervisory team, and took the primary role in drafting the manuscript.

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