The effects of antioxidant supplementation on exercise-induced oxidative stress in cyclists

by

Matthew Soden

A thesis submitted to

The University of Birmingham

For the degree of

DOCTOR OF PHILOSOPHY

School of Sport, Exercise & Rehabilitation Sciences

College of Life and Environmental Sciences

The University of Birmingham

March 2017

Abstract

Exercise is inherently linked with the production of reactive oxygen and nitrogen species (RONS), which are able to elicit an effect on cellular redox status and may augment oxidative stress post-exercise. Antioxidant supplements have the potential to reduce exercise-induced oxidative stress, and interact with redox status but in doing so they may have a negative effect on exercise adaptations associated with RONS mediated signalling pathways. This thesis presents novel findings on the consumption habits of antioxidant supplements in amateur cyclists and the effect of habitual supplementation on exercise-induced oxidative stress following a cycling sportive in recreational cyclists (chapters 2 and 3). The thesis goes on to assess the effect of habitual supplementation on basal levels of endogenous antioxidant proteins and the exercise-induced oxidative stress response to steady-state cycling in trained cyclists (chapter 4), and the effects of polyphenol supplementation on cycling recovery and subsequent day cycling performance, assessed via markers of oxidative stress, inflammation, and a time-trial in trained cyclists (chapter 5). Consumption of antioxidant supplements was reported in 40% of the participants sampled and was not associated with age, cycling experience or weekly training hours. The main reasons given for antioxidant supplementation were to: improve immunity; recovery; and performance. Upon completion of 100 km of cycling, antioxidant supplementation did not attenuate exercise-induced oxidative markers compared to a non-supplemented group. Both groups displayed augmented oxidative stress after cycling 100 km. In trained cyclists, habitual supplementation did not result in any differences in resting concentrations of the endogenous antioxidants superoxide dismutase-1 (SOD1), superoxide dismutase-2 (SOD2) or glutathione peroxidase-1 (GPx-1); nor the oxidative stress response to steady-state cycling. Supplementation with a polyphenol based drink had no effect on exercise-induced oxidative stress and inflammation markers at any

time-point compared to a placebo control drink. Furthermore, supplementation during the recovery period (24 hours) did not provide an ergogenic when undertaking a cycling time-trial in trained cyclists. In conclusion, the results obtained in this thesis do not support a beneficial role for the habitual consumption of commercially available antioxidant supplements in cyclists. No attenuation in exercise-induced oxidative stress in a 'real-world' or laboratory setting was observed compared to non-supplemented individuals. Furthermore, additional education may be required for cyclists to enhance their knowledge on the interactions of antioxidant supplements. The reasons given by cyclists for antioxidant consumption were not supported by the current research.

Acknowledgements

Unlike the thesis, my acknowledgments will be short and sweet. This achievement would not have been possible without the constant support from my loving family and friends. It was never going to be an easy journey, but at the same time, who could have conceptualised how difficult this was going to be. However, through the setbacks and disappointments, this thesis has finally come to fruition.

Remarkable things are always achieved as a collective. And I would like to give a special appreciation to the people behind the scenes who have made this possible. These individuals deserve to be recognised because they have had to deal with the rollercoaster ride over the last several years. On that note, I would like to give my sincerest thanks and gratitude to my mother, Teresa Soden, for her eternal belief and constant support throughout this process. You have experienced the highs and the lows, and it is impossible for me to put into words how appreciative I am for the sacrifices you have made to help me obtain my doctorate. Without your support, not just over the last few years, but throughout the whole of my academic life I would not be where I am today. Additionally, a huge thank you goes to Suzanne and Nolan Shinn. The two of you gave me the initial confidence to pursue a degree and look where it has led! Thanks for helping turn my life around for the better, you are both amazing friends whom I consider more like family.

To my supervisors, Sarah and Gareth thank you for your backing and advice. Sarah, thank you for believing in me as an undergraduate and for providing me with this opportunity. I will forever be grateful. Gareth, thank you for your support and encouragement along the way, both inside and outside of the laboratory, and for always having your door open for a chat. I hope in the near future we will have the opportunity to work with one another again.

I would like to thank members of the Exercise Metabolism Research Group for being an amazing group of people, who would always be on hand to discuss and solve any problems. It has been an immense pleasure working with each and every one of you. A big appreciation goes out to my fellow postgraduates and staff members who have made this experience extremely pleasurable. Additionally, thanks to every participant who volunteered their time, blood, muscle and sweat to my research, the Marie Curie Hospice in Solihull, and the organisers of the Wiggle Dragon Ride and the Tommy Godwin cycling sportive; without your assistance this thesis would have never come to fruition.

I would like to dedicate this thesis to my Dad. I know you would be so proud to share this accomplishment with me.

List of Contents

Chapter One

Chapter Three

Chapter Four

Chapter Five

Chapter Six

List of tables and figures

List of abbreviations

AP-1	Activator protein-1
ANOVA	Analysis of variance
BCA	Bicinchonic acid
BMI	Body mass index
BSA	Bovine serum albumin
CAT	Catalase
CEB	Cytoplasmic extraction buffer
CHL	Chlorogenic acid
CK	Creatine kinase
CRP	C-reactive protein
DEM	Diethyl maleate
DOMS	Delayed onset muscle soreness
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DNP	Dinitrophenyl
DNPH	Dinitrophenylhydrazine
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraaceitic acid

ELISA	Enzyme linked immunosorbent assay
ERK1/2	Extracellular signal related kinase 1/2
FRAP	Ferric reducing ability of plasma
GHQ	General health questionnaire
GPx	Glutathione peroxidase
GPx-1	Glutathione peroxidase-1
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GR	Glutathione reductase
HR	Heart rate
HCl	Hydrochloric acid
HPL	High performance laboratory
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IL	Interleukin
MAPK	Mitogen activated protein kinase
MC	Montmorency Tart cherry
MDA	Malondialdehyde
MEB	Membrane extraction buffer
MnSOD	Manganese superoxide dismutase

MVC	Maximal voluntary contraction
MVIC	Maximal voluntary isometric contraction
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
NF-κβ	Nuclear-factor κβ
NSAID	Non-steroidal anti-inflammatory drugs
p38MAPK	p38 mitogen activated protein kinase
PBS	Phosphate buffered saline
PCA	Protocatechuic acid
PC	Protein carbonyls
PLA	Placebo
PLFFD	Prolonged low frequency force depression
ROS	Reactive oxygen species
RONS	Reactive oxygen and nitrogen species
RNS	Reactive nitrogen species
RPE	Rate of perceived exertion
RPM	Revolutions per minute
SD	Standard deviation
SEM	Standard error mean
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD1	Superoxide dismutase-1

SOD2	Superoxide dismutase-2
TAC	Total antioxidant capacity
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive substance
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween 20
TCA	Trichloroacetic acid
TEAC	Trolox equivalence antioxidant capacity
TEMED	N,N,N,N-tetramethyl-ethylenediamine
TNF-α	Tumour necrosis factor-α
TT	Time-trial
VA	Vanillic acid
W_{max}	Maximal power output
$\dot{V}O_{2max}$	Maximal oxygen uptake

Chapter One

1. General Introduction

1.1 Introduction

The consumption of antioxidant supplements, particularly vitamin C and E, in people undertaking exercise has been greatly debated for over a decade (Carmen Gomez-Cabrera et al., 2012; Holloszy et al., 2012). Studies in the late 1970's and early 1980's identified the ability of exercise to augment the production of free radical species, resulting in what appeared to be, undesirable oxidative damage within the cellular environment (Davies et al., 1982; Dillard et al., 1978) and muscle damage sustained following exercise (Jackson et al., 1985). Furthermore, exercise undertaken in a vitamin deficient state elevated the degree of oxidative damage compared to a non-deficient state (Davies et al., 1982), and supplementation with an antioxidant compound – vitamin E – attenuated the observed oxidative damage post-exercise (Dillard et al., 1978). These initial observations could be responsible for sending exercise scientists off in pursuit of an explanation of the role and/or function of antioxidant supplements, predominately vitamin C and E, in reducing exercise-induced oxidative stress and muscle damage (Arent et al., 2010; Bailey et al., 2011; Nakhostin-Roohi et al., 2008; Naziroğlu et al., 2010; Theodorou et al., 2011).

Unbeknown at the time, the transient release of radical species from contracting skeletal muscle are essential in activating cell signalling pathways, known as redox signalling, that upregulate endogenous antioxidants and mitochondrial content. Thus, providing a greater tolerance against exercise-induced oxidative damage in future exercise bouts (Kawagishi and Finkel, 2014; Ristow, 2014). Suggesting, antioxidant supplementation could have a negative effect on the redox signalling pathways, reducing the favourable cellular adaptations gained from repeated bouts of exercise. Conversely, the reduction in oxidative damage and subsequent inflammation in muscle might be appealing to athletes who are having to repeatedly perform in events over several days or over a competitive season.

Thus, despite evidence to suggest antioxidants may reduce adaptation, supplementation may be desirable, where the emphasis is on recovery and performance rather than adaptation.

Experiments investigating the effects of vitamin C and E supplementation in both animal and human studies provide equivocal results in endogenous antioxidant synthesis and mitochondrial biogenesis. Some studies found supplementation to have no effect on redox sensitive signalling pathways (Cumming et al., 2014; Higashida et al., 2011; Yfanti et al., 2010; Yfanti et al., 2012) whereas others reported supplementation inhibited the effects of RONS signalling (Gomez-Cabrera et al., 2008; Gomez-Cabrera et al., 2005; Morrison et al., 2015; Paulsen et al., 2014; Ristow et al., 2009). Although there are methodological differences within these studies, both in study design and analysis of outcomes, most studies use supra-physiological doses of vitamin C and vitamin E that are ~11 (1000 mg/d) and ~18 times greater (400 IU/d) than the recommended daily intake (RDI) respectively. Using a dose above the RDI in experimental research enables researchers to elucidate possible mechanisms of action, but these doses may not reflect the effects that habitual supplementation might have. Especially in a real-world setting where recreational athletes consume the RDI.

Experimental research provides well controlled environments ensuring the internal validity of the scientific question; however, when the work is then applied in a real-world setting, upholding the control variables that are vital to ensuring internal validity cannot be ensured in the general population. Thus, there is a need for research studies that attempt to translate lab based controlled studies into applied real-world situations. With relevance to the topic of this thesis, research is needed to determine whether habitual supplementation in free-living athletes, is having any effect on factors which may influence exercise training and performance. Do supplements containing vitamin C and E influence the concentrations of proteins related to redox signalling or markers of oxidative stress, in a population of recreational cyclists? If so, to what extent?

The consumption of antioxidant supplements could be beneficial to individuals who are looking to improve a sporting performance and/or enhance recovery. The reduction in oxidative stress and inflammation during and post-exercise may result in attenuated muscle and cellular damage, curtailing performance decrements whilst competing and/or on consecutive days of competition. Studies investigating traditional antioxidant supplements (vitamin C, vitamin E, and co-enzyme Q10) have not yet provided substantial scientific evidence to corroborate these supplements' ability to improve performance and/or recovery by reducing oxidative stress and/or inflammation (Braakhuis, 2012; Braakhuis and Hopkins, 2015; Peternelj and Coombes, 2011). Recent attention from exercise and sport scientists has focused on a subset of metabolites derived from plant based sources called polyphenols, due to their anti-oxidant and anti-inflammatory properties (Manach et al., 2004; McCune et al., 2011; Wallace, 2011). Montmorency tart cherries containing multiple polyphenol compounds have become a popular supplement to use in an exercise setting (Bell et al., 2014a; Myburgh, 2014). Studies have reported that interventions with this supplement improve muscle recovery after completing endurance events (Bell et al., 2014c; Bell et al., 2015; Howatson et al., 2010) and resistance training (Bowtell et al., 2011; Connolly et al., 2006). There is additional evidence supporting the idea that improvements in muscle recovery can have a beneficial effect on performance in the days following fatiguing exercise (Bell et al., 2016; Bell et al., 2015; Bowtell et al., 2011). However, it is not known if these effects are due to the antioxidant properties of the supplement, or the phytochemical compounds contained within (Croft, 2016; Forman et al., 2014).

In addition, the measures of performance used in studies to date may not be sport specific i.e. not transferable or applicable to all sporting disciplines. It may not be appropriate to extrapolate measurements that assess closed circuit movements such as, maximal voluntary contractions, maximal voluntary isometric contractions, and counter movement jumps to

other sporting disciplines. Therefore, interventions need to be conducted examining the effect of supplementation on recovery and performance in specific modes of exercise such as cycling or running, to determine if these closed-circuit outcomes relate to performance outcomes in functional sporting settings.

Perhaps a point not often considered is why recreational athletes choose to consume antioxidant supplements. It is not clear what motivates amateur athletes to supplement in the first place. Previous research investigating supplement intake has combined all dietary supplements together, making it difficult to determine why an individual supplement may be consumed. For example, we might postulate that a protein supplement may be primarily used to improve recovery, a carbohydrate supplement to improve performance, and a vitamin supplement to maintain health. By grouping all dietary supplements together, it makes it difficult for a sports practitioner to either support or dismiss whether an athlete requires a specific supplement for the stated reason. The rationale for consuming one dietary supplement compared to another may or may not be supported by scientific research; therefore, more specific research must be conducted on individual dietary supplements to dismiss or support the rationale for their consumption in recreational athletes.

1.2 Oxidative Stress

1.2.1 Free Radicals

The term free radical can denote any species that is capable of independent existence and contains one or more unpaired electrons within the atomic or molecular orbit of an element or a molecule. As a consequence, free radical species have paramagnetic properties making them extremely reactive (Halliwell and Gutteridge 2007) Within a biological system,

the reactivity of free radicals can have differential effects on the surrounding cellular and molecular environment. To enable *in vivo* biological systems to systematically control the fate of free radicals a matrix of redox reactions maintains redox equilibrium. Redox reactions occur through **red**uction or **ox**idation. Oxidation results in the loss of an electron and is typically assisted by an oxidising agent that can accept electrons. Reduction results in the gaining of an electron and is typically assisted by reducing agents that can donate electrons (Figure 1.1) (Halliwell and Gutteridge 2007).

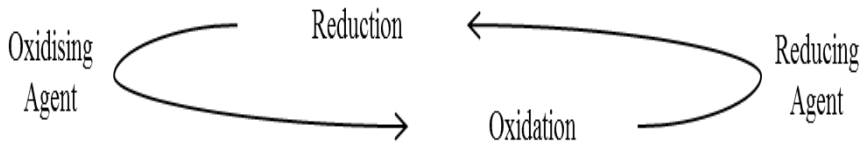


Figure 1.1 A simple schematic to demonstrate the roll of reducing and oxidising agents in transferring electrons during oxidation and reduction reactions. Oxidation results in the loss of an electron and reduction results in the gaining of an electron.

1.2.2. Reactive oxygen species and nitrogen species

Reactive oxygen species (ROS) reactive nitrogen species (RNS) is a collective term for both free radical and non-radical species derived from oxygen and nitrogen, respectively; and these species can be formed by losing or gaining a single electron. The collective term that can integrate both ROS and RNS is reactive oxygen and nitrogen species (RONS). Superoxide ($O_2^{-\bullet}$) and nitric oxide ($NO^{\bullet}$) are the main oxygen and nitrogen derived free radical species produced within RONS. The electron transport chain is one of the main producers of $O_2^{-\bullet}$ during cellular respiration through the reduction of oxygen via electron

leakage (Muller et al., 2008; Turrens, 2003), or via the specific membrane proteins nicotinamide adenine dinucleotide phosphate oxidases (NAD(P)H oxidases) (Bedard and Krause, 2007). Nitric oxide is synthesised through the amino acid L-arginine and occurs through 3 distinct NO synthases (NOS), depending on the function or location. The three NOS isoforms to produce $NO^{\bullet}$ are neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Radomski et al., 1990).

Superoxide itself is relatively unreactive but if left unregulated it can produce highly reactive radical species. The enzyme superoxide dismutase (SOD) is responsible for dismutation of $O_2^{-\bullet}$ to hydrogen peroxide (H_2O_2) and molecular oxygen (Reaction.1). Hydrogen peroxide is classed as a non-radical but in the presence of transition metals, iron (Fe^{2+}) and copper (Cu^{2+}), it can produce the hydroxyl radical ($OH^{\bullet}$) through the metal-catalysing Fenton reaction (Reaction.2). In the presence of $O_2^{-\bullet}$ the oxidised iron ion (Fe^{3+}) (copper) can be reduced allowing for the Haber-Weiss reaction to occur producing $OH^{\bullet}$ from H_2O_2 (Reaction.3)(Jackson, 2005; Powers et al., 2011). If the generation of $O_2^{-\bullet}$ and $NO^{\bullet}$ coincide with one another the irreversible formation of peroxynitrite ($ONOO^{--}$) arises (Reaction.4) and when converted into its pronated form peroxynitrous acid (ONOOH), it becomes extremely reactive (Beckman & Koppenol, 1996).

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Reaction.1)}$$

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^- \qquad \text{(Reaction.2)}$$

$$O_2^{-\bullet} + H_2O_2 \xrightarrow{\text{Metal Catalyst}} OH^{\bullet} + OH^- + O_2 \qquad \text{(Reaction.3)}$$

$$O_2^{-\bullet} + NO^{\bullet} \rightarrow ONOO^- \qquad \text{(Reaction.4)}$$

(adapted from Powers et al., 2011)

If RONS are left unregulated, due to an inefficient antioxidant system, they can create a pro-oxidant environment that leads to detrimental effects on the surrounding biomolecules. The imbalance of pro-oxidants and antioxidants, in favour of the former is referred to as "oxidative stress" that can have a negative effect on redox signalling and regulation, and the structure and functionality of lipids, proteins, and DNA (Sies and Jones, 2007). The chronic exposure of RONS throughout a lifetime has been linked to the ageing process through several different theories "the free radical theory of ageing" (Harman, 2006), "the redox stress hypothesis of aging", (Sohal and Orr, 2012) and "the cell signalling disruption theory of aging" (Vina et al., 2013); and related to several disease pathologies (Berlett and Stadtman, 1997; Hecht et al., 2016; Santilli et al., 2015). Paradoxically, the transient generation of RONS through such means as exercise has been shown to have positive physiological effects by the ability to upregulate antioxidant enzymes (Hollander et al., 2001), heat shock proteins (Khassaf et al., 2003), and mitochondrial proteins (Gomez-Cabrera et al., 2008).

Due to radical species being highly reactive and having a very short half-life, they are extremely difficult to measure *in vivo*. In human cells and tissue, stable end products are formed when radical species interact with biomolecules. These end products, known as adducts, can be used to estimate oxidative modifications to lipids, proteins, and nucleic acids (Mecocci et al., 1999); allowing an indirect measurement of radical interaction to be measured. However, they do not provide information on whether the oxidative stress markers represent beneficial or detrimental effects. Methods do exist to allow direct capture of radical species, such as electron paramagnetic resonance or electron spin resonance, however, the capture of radical species is challenging, and where studies are undertaken in human participants, especially using exercise interventions, these methodologies do not offer a realistic opportunity to assess redox status (Tanabe et al., 2006; Xu et al., 2012).

1.3 Antioxidants

1.3.1 Overview

Antioxidants are essential in controlling the fate of RONS when they have been generated and play an important role in maintaining cellular homeostasis. Antioxidants can be categorised into two main categories: exogenous and endogenous antioxidants. Exogenous antioxidants must be consumed through ingestion of foods and/or supplements that contain a specific or a variety of antioxidants. Endogenous antioxidants on the other hand must be synthesised *in vivo* with their production and concentrations dependent on the activity of RONS in specific cellular compartments (Higuchi et al., 1985; Hollander et al., 2001). Antioxidants can function in two differing ways. They can either work as a reducing agent through the donation an electron to RONS or as an oxidising agent that accept electrons from RONS.

1.3.2. Endogenous antioxidants

The endogenous antioxidants could be classed as the front-line defence in the neutralisation of RONS. As mentioned previously the endogenous antioxidant superoxide dismutase (SOD) can dismutase $O_2^{-\bullet}$ into H_2O_2. Because $O_2^{-\bullet}$ can be generated in different biological compartments, there are several isoforms of SOD to combat against $O_2^{-\bullet}$ production. Dismutation of $O_2^{-\bullet}$ in the mitochondria relies on the manganese superoxide dismutase (MnSOD/SOD2), in the cytosol by copper-zinc SOD (Cu-ZnSOD/SOD1), and in the extracellular fluid by extracellular SOD (ecSOD/SOD3) (Powers et al., 2011). To prevent or reduce the interaction of H_2O_2 with the transition metals copper and iron and the subsequent production of $OH^{\bullet}$ there are two main endogenous antioxidants responsible for its

removal, catalase (CAT) and reduced glutathione (GSH). Both compounds can be oxidised allowing the reduction of H_2O_2 to form H_2O by accepting an electron. GSH is dependent on the enzyme glutathione peroxidase (GPx) that enables its oxidation to form oxidised glutathione (GSSG), and GSSG can be recycled back to GSH through the enzyme glutathione reductase (GR) and the cofactor NADPH (Urso and Clarkson, 2003). Figure 1.2 represents a schematic illustration of the pathway for radical removal via endogenous antioxidants.

1.3.3 Exogenous Antioxidants

Fruits and vegetables offer the primary source of exogenous antioxidants in a habitual diet. Vitamin C (ascorbic acid) and vitamin E are perhaps the most well-known exogenous antioxidants and are the two exogenous antioxidants that have been most extensively studied *in vitro* and *in vivo*. The predominant reason for this is due to their ability to provide antioxidant protection in two different cellular domains.

Vitamin C is a hydrophilic antioxidant enabling it to provide antioxidant protection within extracellular fluids such as plasma and the cytoplasm (Rutkowski and Grzegorczyk, 2012). Vitamin C exerts its antioxidant effect by donating an electron to a RONS leading the formation of a stable product and dehydroascorbic acid, the oxidised form of ascorbic acid (Pohanka et al., 2012; Talaulikar and Manyonda, 2011). Vitamin C can scavenge several RONS that include $O_2^{-\bullet}$, $OH^{\bullet}$, and $ONOO^-$ (Carr and Frei, 1999).

Vitamin E is lipophilic antioxidant protecting lipid structures such as cellular membranes and lipoproteins from the lipid peroxyl radical ($LO_2^{\bullet}$) (Rutkowski and Grzegorczyk, 2012). The main function of vitamin E is to act as a chain breaking antioxidant to protect against lipid peroxidation and is comprised of eight isoforms consisting of four

tocopherols (α-tocopherol, β-tocopherol, γ- tocopherol, δ- tocopherol) and four tocotrienols (α- tocotrienol, β- tocotrienol, γ- tocotrienol, δ- tocotrienol), with α-tocopherol having the greatest bioavailability and biological activity (Niki, 2014). After vitamin E has performed its role as an antioxidant it becomes a vitamin E radical, and if in the presence of vitamin C, vitamin C can recycle this newly formed radical back to its naive form (Hamilton et al., 2000).

More recently, greater attention has focused on a subset of antioxidant compounds called polyphenols, derived predominantly from plant extracts. To distinguish between the several thousand compounds that contribute to the polyphenol family, each polyphenol compound can be classified by the amount and arrangement of their aromatic rings, and the structures used to connect them. The four main sub-categories of polyphenols are phenolic acids, flavonoids, stilbenes, and lignans; table 1.1 identifies the major subclasses and their food derivatives. The greatest volume of research has focused on the subset of polyphenols called flavonoids. These compounds are comprised of two aromatic rings with at least one aromatic hydroxyl on each. The aromatic rings are referred to as the A and B rings, connected by a carbon bridge, with 3 carbon atoms linked to an oxygen and a further 2 carbons from the aromatic ring (ring A) to form a third ring, referred to as the C ring (Figure 1.3) (Beecher, 2003; Manach et al., 2004). *In vitro* and *in vivo* studies have demonstrated these compounds have antioxidant and anti-inflammatory properties that could prevent the progression of disease pathologies associated with oxidative stress (Graziani et al., 2005; Mitjavila and Moreno, 2012) and inflammation (Decendit et al., 2013).

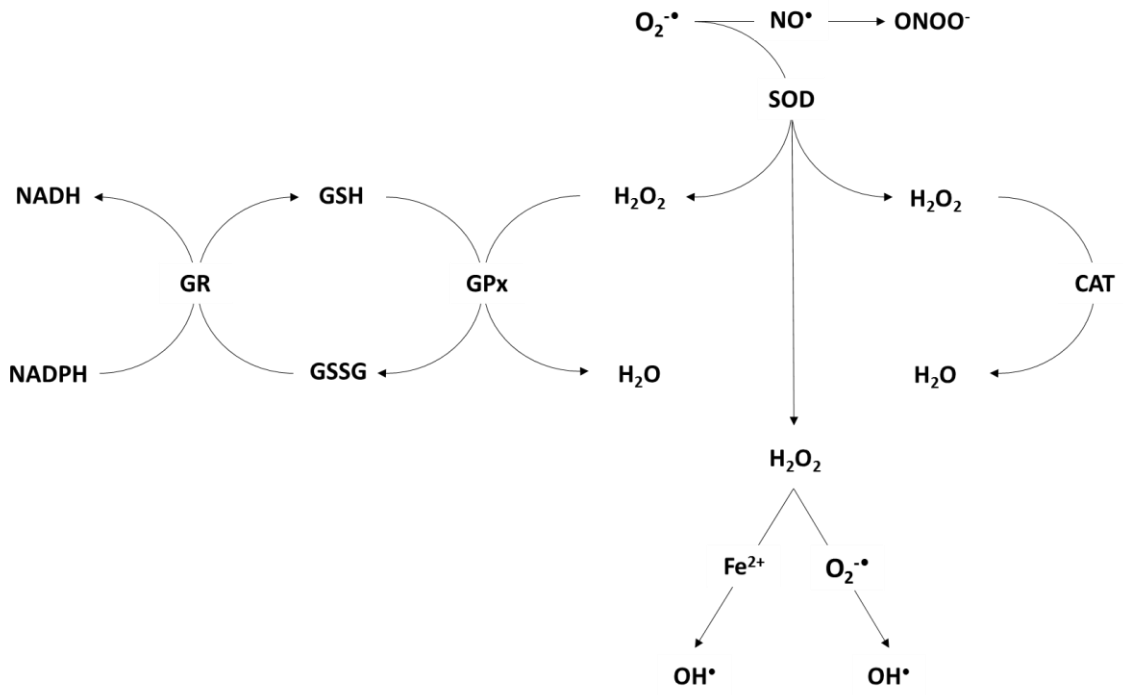


Figure 1.2 The endogenous antioxidant response to superoxide generation. Superoxide ($O_2^{-\bullet}$) is produced from several cellular sources. Superoxide dismutase (SOD) is responsible for the dismutation of $O_2^{-\bullet}$ to hydrogen peroxide (H_2O_2). In the presence of nitric oxide ($NO^{\bullet}$) $O_2^{-\bullet}$ will convert $NO^{\bullet}$ to peroxynitrite ($ONOO^-$) at a faster rate than SOD. H_2O_2 can be converted by catalase (CAT) or reduced glutathione (GSH) in the presence of glutathione peroxidase (GPx) to form water ($H_{2+}O$). GSH can be resynthesized from its oxidised form (GSSG) through the electron donor nicotinamide adenine dinucletode phosphate-oxidase (NADPH) and glutathione reductase (GR). If H_2O_2 is in the presence of transition metals (e.g. Fe^{2+}) or $O_2^{-\bullet}$ the Fenton and Haber-Weiss reactions occur producing the highly reactive hydroxyl radical ($OH^{\bullet}$).

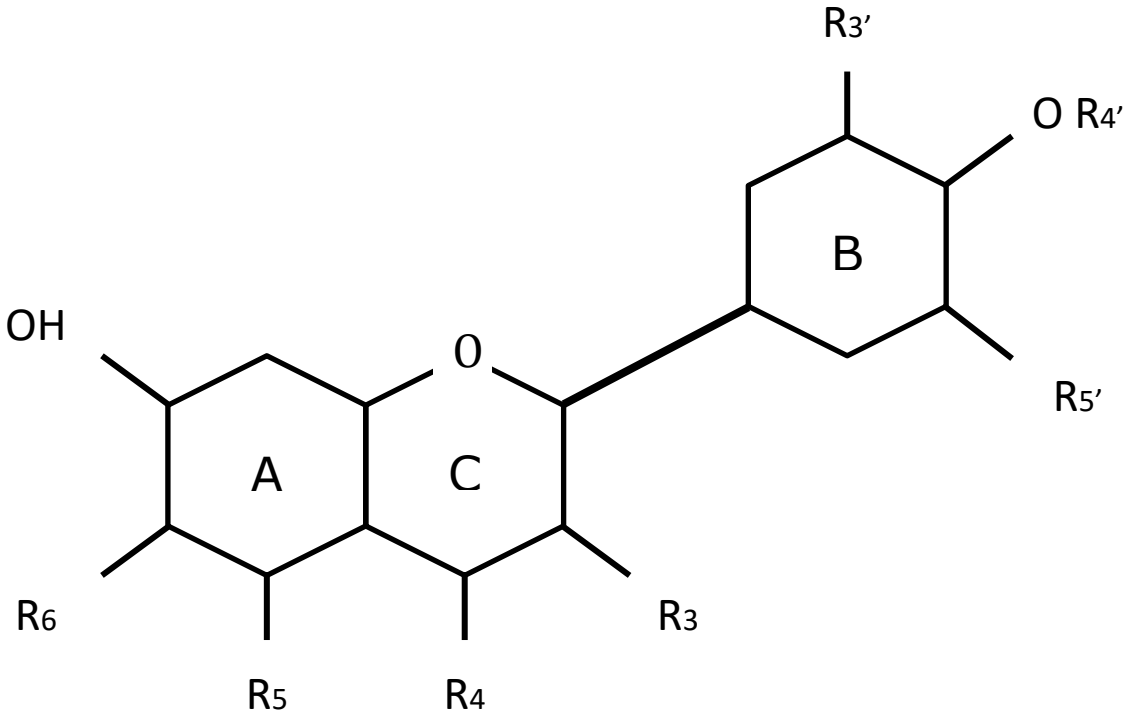


Figure 1.3 The general structure of a flavonoid. The aromatic rings (A & B) are connected by a carbon bridge that creates a third ring (C). The R groups indicate where different arrangements of H, OH, and CH_3, attach to the basic flavonoid structure to create the numerous subclasses in the flavonoid family, Adapted from Beecher, 2003.

1.4 Exercise and Oxidative Stress

1.4.1 Sources of RONS during skeletal muscle contractions

It is well established that exercise is responsible for the generation of reactive oxygen and nitrogen species (RONS) within skeletal muscle due to metabolic and mechanical stresses during skeletal muscle contractions (Bloomer et al., 2005). Several cellular sites have been identified as the main producers of RONS within skeletal muscle: complex I (NADH dehydrogenase) and complex III (cytochrome c) of the electron transport chain in the mitochondria, nicotinamide adenine dinucleotide phosphate oxidases (NAD(P)H oxidases)

enzymes located within the sarcolemma, transverse tubules, sarcoplasmic reticulum, and the mitochondria; contraction stimulated phospholipase A2-dependent pathways (PLA_2) leading to the activation of lipoxygenases and cyclooxygenases; and xanthine oxidases (XO) located within endothelial cells (Mason and Wadley, 2014; Sakellariou et al., 2014). These species have been identified as important regulators of cell signalling pathways in response to exercise (Barbieri and Sestili, 2012; Jackson, 2005; Silveira et al., 2006) and at low levels essential for contractile activity (Hernández et al., 2012; McKenna et al., 2006; Powers and Jackson, 2008; Westerblad and Allen, 2011).

1.4.2 Reactive oxygen and nitrogen species are involved in exercise adaptations

Early exercise studies undertaken in rats identified that endurance exercise was responsible for an increase in SOD activity after 3 months of endurance training (Higuchi et al., 1985). However, this initial study did not identify whether RONS were part of the mechanism responsible for the observed elevations in SOD activity. It is now widely accepted that RONS are important regulators of redox-states within cells, enabling the activation of signalling pathways involved in biological processes (Finkel, 2011; Peternelj and Coombes, 2011). The release of RONS during exercise has been well documented (Davies et al., 1982; Jackson et al., 1985) with skeletal muscle being a main source of RONS production during and immediately following exercise (McArdle et al., 2001). Hollander et al., (2001) identified that an acute bout of treadmill running in rats at 65% $\dot{V}O_{2max}$ until exhaustion, generated sufficient stress to activate the redox-sensitive transcription factors involved in MnSOD mRNA transcription post-exercise, compared to a non-exercising control group. Binding of the redox-sensitive activator-protein 1 (AP-1) and nuclear factor - κβ (NF-κβ), both downstream targets of the mitogen-activated protein kinase pathway (MAPK), were

identified as the targets for RONS mediated endogenous antioxidant generation. It is now well accepted that RONS generated during exercise are responsible for the activation of signalling pathways that up-regulate endogenous antioxidants post-exercise (Ho et al., 2005; Hollander et al., 2001).

A hormesis paradigm has been suggested to explain how RONS produce advantageous adaptations in response to exercise. Hormesis is presented as a dose/response relationship to a stress and it has been suggested that a small dose of biological stress, in this case caused by RONS, can activate stress response signalling pathways leading to favourable adaptations. However, if a large dose of RONS is generated, the reactive species become maladaptive causing molecular damage and/or hinder adaptational processes (Radak et al., 2008).

1.4.3 Factors influencing oxidative stress post-exercise

To determine the relationship between exercise and oxidative modifications, indirect markers of RONS production can be measured in plasma. The type of measurement is dependent on the macromolecule of interest. To determine oxidative changes in lipids several measurements are predominantly assessed: lipid hydroperoxides (LOOH), thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), and F_2-isoprosrtanes (F_2-IsoP); and protein modifications are mainly assessed by changes in protein carbonyl (PC) concentrations. Section 1.4.6 describes the limitations of measuring oxidative stress *in vivo*.

An increase in oxidative stress in response to exercise has predominantly been studied after an acute bout of exercise. Perturbations in exercise-induced oxidative stress can be affected by an individual's training status, exercise duration, intensity and modality. Perhaps

one of the most striking differences in oxidative stress following exercise has been reported between trained and untrained populations in response to different exercise intensities. In untrained individuals, the oxidative stress response post-exercise is largely independent of exercise intensity. Wadley et al., (2016a) reported significant changes in post-exercise LOOH and PC concentrations compared to baseline upon completion of cycling trials at 60% $\dot{V}O_{2max}$, 80% $\dot{V}O_{2max}$, and a low-volume, high-intensity interval exercise (LV-HIIE); with no significant differences between any of the trials. However, trained individuals have a greater threshold to exercise-induced oxidative stress, demonstrated by an attenuation in oxidative stress at lower exercise intensity compared to exercise at higher intensity (Munoz Marin et al., 2010)

Intervention studies have demonstrated that exercise training can have a positive impact on reducing oxidative stress when exercise at the same intensity is completed throughout intervention period (Fatouros et al., 2004; Shin et al., 2008). In order to stimulate a post-exercise oxidative stress, exercise intensity must then increase. It is hypothesized that the production of exercise-induced RONS must surpass a biological threshold, even in trained individuals to cause oxidative stress (Radak et al., 2017).

In addition to exercise intensity, exercise duration can directly affect the levels of exercise-induced oxidative stress. Bloomer et al., (2007) demonstrated the augmentation of PC after steady cycling at 70% $\dot{V}O_{2max}$ for 30, 60, and 120 minutes. Although PC significantly increased from baseline in all conditions 30 minutes post-exercise, the 120-minute trial was the only condition to remain significantly elevated 60 minutes post-exercise. The PC response was significantly pronounced throughout the post-exercise period after the 120-minute trial compared to both the 30 and 60-minute trials, further supporting the theory that a threshold exists at which RONS generation can overwhelm antioxidant defences.

Within this study, PC reached their maximal concentrations immediately post-exercise, indicating transient oxidative stress occurred via metabolic stress rather than mechanical stress. Oxidative stress markers have been reported to remain elevated for periods between 1 and 7 days after completing ultra-endurance running events lasting for > 30 hours (Machefer et al., 2004; Skenderi et al., 2008; Turner et al., 2011). The combination of metabolic and mechanical stress could account for the prolonged oxidative stress response reported in these studies. To support the notion of post-exercise oxidative stress occurring from two separate pathways, Bloomer et al., (2005) reported anaerobic exercise, consisting of squatting for 30 minutes, caused a more robust and prolonged oxidative stress response post-exercise compared to aerobic exercise, cycling at 70% $\dot{V}O_{2max}$. The additional loading of the muscle fibres during resistance exercise provides greater mechanical stress, compared to cycling that is non-weight bearing, causing greater perturbations of the myofibril filaments resulting in enhanced oxidative stress.

These studies highlight the importance of selecting exercise protocols that are able to stimulate an oxidative stress post-exercise capable of stimulating adaptation. In untrained individuals, such an oxidative stress response can be propagated at relatively low-exercise intensities, which reflects the untrained individuals' physiological ability to combat exercise-induced RONS. However, in trained individuals exercise must be undertaken at a higher-intensity and/or for longer durations to cause an oxidative stress response post-exercise capable of stimulating adaptation. This threshold is hypothesized to increase as enhanced physiological defence mechanisms are accrued from performing repeated bouts of exercise. Ultra-endurance events provide novel insights into the oxidative stress response following extended and often extreme bouts of exercise, but these events are not undertaken by the majority of recreational athletes. This thesis focuses on the oxidative stress response to exercise in events that are frequently undertaken by recreational athletes.

1.4.4 Antioxidant response to exercise

To establish the antioxidant response during exercise several antioxidant markers can be measured in plasma. The most common way to measure the antioxidant activity within plasma is to measure total antioxidant status (TAS) or total antioxidant capacity (TAC). More specific markers of antioxidant status can also be measured such as SOD, CAT, GSH, GSSG, and enzymes involved in the recycling of specific antioxidants.

The antioxidant response during exercise is dependent on a combination of exercise intensity, duration, and fitness levels. Berzosa et al., (2011) observed that exhaustive and submaximal cycling in untrained individuals elevated the concentrations of SOD, CAT, GPx, GR, and TAS; compared to baseline levels. In contrast to these results Knez et al., (2007) reported a decrease in CAT and SOD activity after an ultra-endurance in recreationally fit individuals. Indicating that the constant generation of RONS over an extended period can potentially reduce the antioxidant pool, regardless of fitness level. There is evidence to indicate that repeated exposure to an exercise stimulus can augment the antioxidant response to the same relative workload after a training intervention (Fatouros et al., 2004; Shin et al., 2008) and that the addition of a detraining period returns the antioxidant response back to pre-training levels (Fatouros et al., 2004). An observation study carried out by Serrano et al., (2010) in professional cyclists before and after a four-day competition is a good example of this adaption. The erythrocyte glutathione pool after the race had no changes in GSH, GSSG, concentrations, allowing the riders to maintain the GSSG:GSH ratio.

1.4.5 Relationship between exercise-induced inflammation and oxidative stress

The production of pro and anti-inflammatory cytokines can be exacerbated by a single bout of exercise. The appearance of the anti-inflammatory cytokines interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-1ra (IL-1ra); the pro-inflammatory cytokines tumour necrosis factor α (TNF-α), interleukin-1β (IL-1β); and the chemokine interleukin-8 (IL-8) have all been reported to increase after exercise (Nieman et al., 2001; Wadley et al., 2016a). The mechanisms attributed to the inflammatory response are myofibrillar damage (Pereira Panza et al., 2015) and elevations in stress hormones (cortisol, adrenaline, human growth hormone) (Nieman et al., 2001; Peake et al., 2007; Wadley et al., 2016a). In response to these events, the immune system responds by mobilising and activating phagocytes (Nieman et al., 2001; Suzuki et al., 1999) and lymphocytes (Wadley et al., 2016a) in the affected area. Cytokine formation can be directly affected by the generation of RONS during exercise. RONS can activate redox-sensitive pathways controlling cytokine expression in skeletal muscle (Aoi et al., 2004; Kosmidou et al., 2002) and lymphocytes (Vider et al., 2001), augmenting the inflammatory response. The combination of these factors are responsible for the low-grade inflammation following exercise.

The inflammatory response is primarily driven by the recruitment, activation, and co-localisation of neutrophils to the affected cellular location (Wang and Arase, 2014). Neutrophils make up approximately 70% of the white blood cell population and function as the first responders to invading bacteria and in the removal of tissue debris. They perform these functions through the production of $O_2^{\bullet-}$ via NADPH oxidase subunits located within their cellular membrane, in a process known as the respiratory burst (Tiidus, 1998). The initial neutrophil response post-exercise mainly consists of the removal of muscle tissue debris through phagocytosis (Butterfield et al., 2006). This is then followed by a secondary response within the 24 hours of exercise cessation and is more cytotoxic, containing a greater

number of neutrophils with the ability to produce $O_2^{\bullet-}$ (Quindry et al., 2003) leading to the formation of H_2O_2 and hydrochlorous acid (HOCl) (Tidball, 2005).

The induction of a more cytotoxic neutrophil phenotype is less discriminate than the phagocytic phenotype, resulting in untargeted cytotoxic attacks on healthy structures in the area where muscle injury occurred. This cytotoxic response results in a more oxidative cellular environment where muscle fibres and cell membranes encounter undesirable oxidative stress. Generation of $O_2^{\bullet-}$, H_2O_2, and HOCl indiscriminately attacks healthy tissue and cells, propagating secondary muscle damage (Brickson et al., 2001; Brickson et al., 2003; Pizza et al., 2001; Tiidus, 1998) and undesirable oxidative modifications to functional cells during recovery (Butterfield et al., 2006; Dahlgren and Karlsson, 1999). The transient increase in oxidative stress after neutrophil activation can generate a secondary inflammatory response through the activation of the redox sensitive transcription factor NF-κβ (Aoi et al., 2004; Kosmidou et al., 2002).

The extent of the inflammatory and oxidative stress response post-exercise is dependent on the perturbations in cellular, metabolic and mechanical stress during exercise. Any form of exercise that disrupts cellular homeostasis will promote stress signals initiating the inflammatory and oxidative stress response. Ultimately the extent of this response will be dictated by exercise duration, intensity, and modality. Markers of inflammation and oxidative stress increase when exercise intensity is amplified (Ostrowski et al., 1998; Wadley et al., 2016a) and exercise is undertaken for a prolonged period (Bell et al., 2014c; Howatson et al., 2010). Furthermore, the modality of exercise has a pronounced effect on post-exercise inflammation and oxidative stress. Protocols involving muscle lengthening such as, eccentric-loading or downhill running promote greater skeletal muscle damage through increased mechanical stress compared to running and cycling protocols, shown by prolonged and elevated inflammation and oxidative stress immediately post-exercise and in the days

following exercise cessation (Bell et al., 2015; Howatson et al., 2010; Levers et al., 2015; Peake et al., 2007; Tiidus, 1998). A combination of these factors will influence the amount of time it takes a muscle to recover after exercise. Muscle recovery will be inhibited when exercise incorporates muscle loading and is performed at maximal intensity for a prolonged period, compared to, submaximal exercise that incorporates non-muscle loading exercise for a short period.

In summary, exercise is responsible for the initial inflammatory response observed post-exercise through elevated stress hormones, myofibrillar damage and RONS. The secondary inflammatory response in the hours and days of exercise cessation is related to elevated cytotoxic neutrophil activation that enhances the concentration of RONS, propagating secondary myofibrillar damage, oxidative damage, and inflammation. The initial inflammatory response is related to exercise duration, intensity, and modality; and will dictate the amount of time it takes a muscle to recover. For athletes competing in multi-day events finding a strategy to reduce exercise-induced inflammation or oxidative stress post-exercise could be imperative for subsequent days performance. Studies are emerging indicating a beneficial effect of antioxidant supplementation on exercise-induced inflammation and muscle recovery (Bell et al., 2016; Bell et al., 2014c; Bell et al., 2015; Bowtell et al., 2011; Howatson et al., 2010), but further research needs to be conducted to elucidate if these promising results can improve exercise performance on subsequent days.

1.4.6 Methodological Limitations of Assessing Markers of Oxidative Stress

As previously stated in section 1.2.2 the direct measurement of reactive species *in vivo* is extremely difficult due to their high reactivity resulting in their short half-life (Halliwell and Whiteman, 2004). To determine their production in a biological system a

unique chemical fingerprint (adduct) can be measured on lipids, proteins, and nucleic acids that is related to oxidative modifications of these macromolecules. In addition, the enzymatic and non-enzymatic antioxidant response can be measured as a guide to indicate the presence of reactive species requiring quenching.

One of several limitations to measuring adducts in biological samples is the lack of specific information that can be determined on the identity, or amount of reactive species that has been generated. Most adducts are stable and thus there is no specific information on when the adduct was formed, or even which cell or cellular compartment was the source of the radical generation (Cobley et al., 2017; Nikolaidis et al., 2012b). Therefore, adducts provide limited information on the mechanisms responsible for their generation, they merely suggest radical species have been present and have interacted with a biomolecule.

This is the main limitation in using adducts as a measure of oxidative stress as it is impossible to answer questions about the specific pathways of redox signalling involved in the reactions and the consequences of the oxidative modifications formed. For example, whether a modification might be beneficial/adaptational and part of a signalling process designed to stimulate favourable change versus detrimental/non-functional designed to promote degradation of the biomolecule or stimulate cell death (Margaritelis et al., 2016). To address these questions with more specificity in humans, the use of new methodological protocols such as proteomics and lipidomics could provide the break through required in the field of redox signalling and exercise (Margaritelis et al., 2016; McDonagh et al., 2014). The use of these techniques in an exercise setting is going to require new collaborations between sport scientists and redox biologists to fully elucidate the effects of exercise on redox signalling due to the expertise and equipment required to perform these analyses.

The use of specific assays and enzyme-linked immunosorbent assay (ELISA) are primarily used to determine adduct quantity in any given biological sample and can be used to specifically quantify adducts to lipids, proteins and nucleic acids. Spectrophotometric assays, high-performance liquid chromatography (HPLC) and liquid/gas chromatography mass spectrometry (LC-MS, GC-MS) can also be used to quantify changes in antioxidant defences. Although these latter techniques are more sensitive and detect specific molecules of interest, they are expensive and require qualified technicians to operate. Therefore, the use of assays and commercially available ELISA kits have mainly been used by sport and exercise scientist due to their reproducibility, ease of use, especially when undertaking sample collection in the field, and cost effectiveness.

The use of some of these well-established techniques has come under criticism as new methodological advances have been developed (Cobley et al., 2017; Halliwell and Whiteman, 2004; Sies, 2007). For example, the determination of lipid peroxidation through the detection of malondialdehyde (MDA) via the thiobarbituric acid reactive substances (TBARS) assay has come under criticism because of its lack of specificity in biological systems (Forman et al, 2015). In simple or purified systems, the addition of thiobarbituric acid (TBA) can give an accurate account on the amount of MDA as a measure of lipid peroxidation in a sample.

However, in more complexed systems, such as human plasma, TBA can react with a multitude of different compounds (sugars, amino acids, bilirubin, albumin, and non-related aldehydes) in addition to MDA, thus resulting in the non-specific measurement of TBA reactions within these biological samples (Forman et al., 2015; Gutteridge and Halliwell, 1990; Sachdev and Davies, 2008). In addition to this, the boiling of samples during the TBARS assay has been proposed to induce lipid decomposition resulting in extraneous lipid peroxidation that does not reflect MDA generated *in vivo* (Cobley et al., 2017; Gutteridge and Halliwell, 1990). The gold standard measurement for lipid peroxidation is the quantification

of F_2-isoprostanes using GC-MS. F_2-isoprostanes are the result of free radical oxidation of arachidonic acid (Milne et al., 2013). However, even determining F_2-isoprostane concentrations using GC-MS has come under some criticism. This is due to the peroxidation of arachidonic acid occurring either enzymatically or chemically, producing prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) respectively; thus, leading to the overestimation of lipid peroxidation using this technique (van't Erve et al., 2015). These examples indicate the difficulty that currently exists in accurately measuring adduct formation within biological samples. To help combat these issues, using several different markers of oxidative change can provide a more robust indication of systemic oxidative stress.

An overlooked limitation to assessing oxidative stress, especially post-exercise, are the sampling time-points. After an acute bout of aerobic exercise, oxidative stress markers have been reported to reach their maximal/minimum concentrations from anywhere between immediately post-exercise up until 4 hours post-exercise (Michailidis et al., 2007). These time-points can be further affected depending on the modality of exercise, and whether the exercise is non-muscle damaging versus muscle damaging. In non-muscle damaging exercise, there is a monophasic response with oxidative stress markers reaching their maximal values immediately post-exercise up until 4 hours post-exercise. However, in muscle damaging exercise, there is a biphasic response. In this biphasic response, oxidative stress markers initially respond immediately post-exercise, in the same manner to non-muscle damaging exercise, and then a secondary response occurs between 24 and 48 hours post-exercise (Nikolaidis et al., 2012a). There is a possibility that some research studies could miss the maximal/minimum response of oxidative stress markers due to restricted sampling time-points post-exercise. It is therefore important to choose sampling time-points that co-inside with the maximal/minimal concentrations of the marker of interest.

1.5 Exercise and Antioxidant Supplementation

1.5.1 Impact of antioxidant supplementation on RONS mediated signalling post-exercise

A growing area of research is now focused on the effect of consumption of antioxidant supplements on exercise-induced adaptations (Carmen Gomez-Cabrera et al., 2012; Holloszy et al., 2012). It has been hypothesized that a potential decrease in RONS via antioxidant quenching could impact redox-sensitive signalling pathways. As discussed in section 1.4.2, RONS are responsible for the upregulation of genes associated with numerous processes of 'exercise adaptation' including upregulation of the endogenous antioxidant system. RONS act through the redox-sensitive transcription factors NK-κβ and AP-1, and the master regulator of mitochondrial biogenesis, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (Kang et al., 2009). Therefore, it is possible that the use of antioxidant supplements in an exercise setting could impact redox-sensitive signalling pathways via quenching exercise-induced RONS.

The effects of antioxidant supplementation on exercise-induced redox signalling have been studied in humans (Cumming et al., 2014; Paulsen et al., 2014; Ristow et al., 2009; Yfanti et al., 2010; Yfanti et al., 2012) and animals (Gomez-Cabrera et al., 2008; Gomez-Cabrera et al., 2005; Higashida et al., 2011), and to date results from these studies have not determined whether antioxidant supplementation affects exercise adaptation.

Initial studies conducted in rats reported antioxidant supplementation immediately before exercise [allopurinol (32 mg/kg)] inhibited the transcriptional activation of NF-κβ following a bout of exhaustive exercise (Gomez-Cabrera et al., 2005), and supplementation for a period of 3 weeks [vitamin C (500 mg/kg/d)] inhibited PGC-1α (Gomez-Cabrera et al., 2008) compared to non-supplemented animals. Inhibition of these pathways resulted in diminished training-induced adaptations as shown by decreased gene expression of the

endogenous antioxidant system (SOD2, GPx-1), genes associated with mitochondrial biogenesis (mitochondrial transcription factor A (mTFA), nuclear respiratory factor 1 and 2 (NRF1/NRF2)), and mitochondrial subunit protein content (cytochrome C (CyC)) (Gomez-Cabrera et al., 2008; Gomez-Cabrera et al., 2005).

In contrast to these findings, Higashida et al., (2011) reported no differences in endogenous antioxidant (SOD1, SOD2) or mitochondrial electron transport chain protein content (e.g. cytochrome oxidase subunit 4 COXIV) after rats were supplemented for 8 weeks with vitamin C (750 mg/kg/d) and vitamin E (150 mg/kg/d) and submitted to 3 weeks of exercise. The differences observed in these studies could be explained by the duration of the exercise bouts, because increasing exercise duration has been shown to propagate the oxidative stress response in a time-dependent manner (Bloomer et al., 2007). The protocol undertaken in the study by Higashida et al., (2011) exposed animals to 6 hours of exercise per day, whereas, Gomez-Cabrera et al., (2008) exposed animals to 85 minutes of exercise per day. Therefore, the extended exposure of exercise-induced RONS could have overwhelmed the endogenous and exogenous antioxidant systems, even though a higher dose of vitamin C was administered (750 mg/kg/d versus 500 mg/kg/d), resulting in augmented redox signalling that was not observed in the Gomez-Cabrera et al., (2008) study where exercise duration was considerably less.

Human studies, similarly, have given mixed outcomes. A number of studies have indicated that antioxidant supplementation can curtail training-induced adaptations through inhibition of genes associated with the endogenous antioxidant system and mitochondrial biogenesis (Morrison et al., 2015; Paulsen et al., 2014; Ristow et al., 2009). Ristow et al., (2009) demonstrated concurrent exercise training with vitamin C (1000 mg/d) and vitamin E (400 IU/d) supplementation for 4 weeks blunted the expression of genes associated with the endogenous antioxidant system (SOD1, SOD2, GPx-1) and mitochondrial biogenesis (PGC-

1α/β, peroxisome proliferator-activated receptor gamma (PPARγ) in trained and untrained individuals. In another study consisting of trained participants, Paulsen et al., (2014) observed that 11 weeks vitamin C (1000 mg/d) and vitamin E (235 mg/d) supplementation combined with multi-intensity running 4 times per week, attenuated the increased protein concentrations of COXIV, cytosolic PGC-1α, and mRNA expression of the mitogen-activated protein kinase 1 (MAPK1) that was observed in the non-supplemented group. Furthermore, Morrison et al., (2015) reported 8 weeks of vitamin C (1000 mg/d) and vitamin E (400 IU/d) supplementation attenuated the training-induced upregulation in skeletal muscle mTFA and SOD2 protein content upon completion of 4 weeks endurance training that coincided with the last 4 weeks of supplementation.

Interestingly, the reductions in gene expression and protein content associated with the endogenous antioxidant system and mitochondrial biogenesis did not affect endurance performance (Morrison et al., 2015; Paulsen et al., 2014). Antioxidant supplementation, even at supra-physiological doses, did not influence changes in $\dot{V}O_{2max}$, performance in a 20m shuttle test (Paulsen et al., 2014), $\dot{V}O_{2peak}$, or maximal power output (Morrison et al., 2015); with both training groups demonstrating significant improvements from baseline. These studies highlight the difficulties of extrapolating changes in the cellular domain to performance outcomes in response to redox-sensitive signalling.

In contrast to these studies, research conducted by Yfanti et al., (2010; 2012) reported 16 weeks vitamin C (500 mg/d) and vitamin E (400 IU/d) supplementation combined with 12 weeks of multi-intensity cycling 5 times per week did not have a negative effect on exercise-induced training adaptations. Augmentation in the endogenous antioxidant system enzymes SOD1, CAT, GPx-1 (Yfanti et al., 2012)l. 2012) and SOD2 (Yfanti et al., 2010) occurred in both training groups post-intervention. Additionally, the mitochondrial enzymes citrate

synthase (CS) and β-hydroxyacyl-CoA dehydrogenase (β-HAD) were elevated in both training groups, suggesting an increase in endurance capacity through increased mitochondrial enzyme content (Perry et al., 2008). Further, in a study conducted by Cumming et al., (2014) which used the same training intervention and supplementation period described previously by Paulsen et al., (2014), no negative effect of supplementation was reported for vitamin C (1000 mg/d) or vitamin E (235 mg/d) on NF-κβ activation after an acute bout of exercise 5 weeks into the 11-week intervention. These preliminary findings persisted until the end of the intervention, with no differences in training-induced adaptations reflected by the same directional changes in SOD2, GPx-1, and GSH.

The lack of agreement in cellular adaptations between the studies could be related to the dose and timing of the supplement, although studies administering the same doses have reported opposing outcomes. None the less, there is a lack of consistency in the dose, frequency and timing of supplement in research studies. For example, participants in the Paulsen et al., (2014) study were instructed to consume 500 mg of vitamin C and 117.5 mg of vitamin E in the 3 hours preceding exercise, and to consume the remaining dose (500mg Vitamin C and 117.5mg Vitamin E) within 1 hour of exercise cessation. Contrastingly, in the study conducted by Yfanti et al., (2010; 2012) participants consumed 500 mg of vitamin C each morning, and it is feasible that plasma concentrations of vitamin C would start to decline in the hours following consumption (Padayatty et al, 2004). Although the studies by Ristow et al., (2009) and Morrison et al., (2015) do not approximately state the timing of the supplements before exercise training, both studies reported consumption of 500 mg of vitamin C twice daily; tentatively supporting the theory that the dose and timing of the supplement in the Yfanti et al., (2010; 2012) study was not sufficient to alter exercise-induced adaptations related to redox signalling. However, the supplementation period used in these studies is not greater than 16 weeks. It could be questioned whether these

supplementation periods reflect the habitual consumption in the general exercising population and/or what affect commercially available doses could have on exercise-induced adaptations that are related to redox-sensitive signalling pathways.

1.5.2 Polyphenol supplementation on exercise-induced oxidative stress and inflammation

As introduced in section 1.2.3. polyphenol compounds have been identified as compounds with antioxidant and anti-inflammatory properties. This potential dual role has led researchers to investigate the effects of consuming polyphenols during exercise with a view to determine whether polyphenol compounds can attenuate exercise-induced oxidative stress and inflammation. Thus, hypothesizing that polyphenols could potentially improve recovery and/or have an ergogenic effect.

Fruit and vegetables are the main food sources containing varying combinations and amounts of polyphenols. Several thousand polyphenol compounds can be found in a variety of foods, and can be divided into four distinct groups according to the arrangement of their aromatic rings and the structures connecting them (Manach et al., 2004; Myburgh, 2014). The four main classes of polyphenols are: phenolic acids, flavonoids, stilbenes, and lignans. These groups can then further be sub-divided into 10 or more subclasses of polyphenols, with these subclasses containing hundreds of derivatives (Manach et al., 2004; Myburgh, 2014). Flavonoids have the greatest number of subclasses that are categorised according to their structural properties: flavonoids, flavones, isoflavones, flavanones, flavanols, and anthocyanins. Phenolic acids consist of two additional subclasses made up of benzoic acid and cinnamic acid derivatives. Stilbenes are comprised of one major subclass, resveratrol, which is responsible for the production of the derivatives in this class (Chong et al., 2009; Jeandet et al., 2010). Lignans are the least abundant polyphenol found in foods, with no

major subclasses. Derivatives of lignans found in food are all converted into the metabolite enterolignan in the human intestine (Kuijsten et al., 2005; Peterson et al., 2010)). Table 1.1 provides an overview of the main polyphenol classes, their derivatives and food sources.

At present, there is a great deal of interest in functional foods that exert a nutraceutical affect. One such food group believed to have nutraceutical properties are cherries. Cherries contain a variety of polyphenol compounds that include phenolic acids (gallic acid, neochlorogenic acid, 3-coumaroylquinic acid, chlorogenic acid and ellagic acid), flavonols (quercetin, kaempferol, isoramnetin. and their glucosides), flavanols (catechin, epicatechin and procyanidins) and anthocyanins (malvidin, cyanidin, pelargonidin, peonidin, delphinidin, petunidin) (Howatson et al., 2010; Howatson et al., 2012). The polyphenol compounds believed to exert the main antioxidant and inflammatory response within cherries are the subclasses of anthocyanins (Blando et al., 2004; McCune et al., 2011).

Research has suggested that supplementation with a cherry based supplement can have a beneficial effect on reducing oxidative stress and inflammation after exercise. Howatson et al., (2010) was the first to demonstrate that cherry supplementation before, on the day of and 48 hours after a marathon resulted in a significant attenuation in the production of interleukin-6 (IL-6) upon completion of the race, and C-reactive protein (CRP) 24 and 48 hours after the race compared to a control drink. These differences could be attributed to the significantly elevated levels of the plasma total antioxidant status (TAS) throughout the intervention period, and the maintenance of TBARS levels in the cherry supplemented runners. Levers et al., (2016) reported similar findings after participants completed a half-marathon. A cherry supplemented group produced significantly lower levels of IL-6, interleukin-13 (IL-13), and interleukin-2 (IL-2) 60 minutes, 24 and 48 hours after the half-marathon, coupled with significantly elevated TAS for the same time periods, when compared to a non-supplemented group of runners.

Table 1.1 Classification of polyphenol compounds found in fruit and vegetables, arranged by their chemical structures into subclasses, derivatives, and potential food sources (Beecher, 2003; Manach et al., 2004)

Class	Subclass	Derivatives	Food Source
Phenolic acids	Benzoic acid	Protocatechuic acid Gallic acid	Raspberries/blackberries, tea leaves
	Cinnamic acid	Caffeic acid Chlorogenic acid Coumaric acid Sinapic acid	Coffee beans, cherries, plums, apples
Flavonoids	Flavones	Apigenin Luteolin Chrysin	Green leafy spices, parsley, celery
	Flavanones	Hesperetin Naringenin Eridodictyol	Citrus fruits and juices
	Isoflavones	Daidzein Genistein Glycitein	Soybeans, soy foods, legumes
	Flavonols	Quercetin Kaempferol Myricetin Isorhamnetin	Yellow onions, kale, leeks
	Flavanols	Catechin Gallocatechin Epicatechin Epigallocatechin Epigallocatechin Gallate	Teas, red wines, red grapes, cocoa, tannins
	Anthocyanins	Cyanidin Pelargonidin Peonidin Delphinidin Malvidin	Red, purple and blue berries – strawberries, blueberries, black grapes, red grape juice

		Petunidin	
Stilbenes	Resveratrol	Trans and cis-resveratrol Trans and cis-piceid Trans-arachidin-3	Grapes Pines Peanuts
Lignans		Secoisolariciresinol Matairesinol Lariciresinol	Chickpeas, flax seeds, sesame seeds

The only disparity between the studies was the effect of cherry supplementation on TBARS concentrations. Levers et al., (2016) did not find any differences in TBARS at any time-point during the intervention period, whilst Howatson et al., (2010) reported significant differences after 48 hours. The additional workload during a marathon compared to a half-marathon could explain this difference, but it is highly speculative. Both studies identified an effect of cherry supplementation in attenuating exercise-induced inflammation, but its effectiveness in reducing oxidative stress is still unclear. The elevation in TAS observed in the supplemented group did not seem to have a beneficial effect on reducing exercise-induced oxidative stress, since the markers of oxidative stress used were not significantly altered. Whether augmented TAS contributes to the perturbations in inflammation still needs to be elucidated.

A study by Bell et al., (2014c) has demonstrated that the consumption of a cherry supplement before (4 days) and during 3 days of prolonged, high-intensity, stochastic cycling was sufficient to reduce oxidative stress over the intervention period compared to a non-supplemented group. Throughout the intervention period the non-supplemented group had significantly higher levels of lipid hydroperoxides compared to the supplemented group, before and upon completion of each cycling trial. The inflammatory response was also

significantly higher in the non-supplemented group across the intervention period. Unfortunately, there was no data pertaining to TAS in the study, making it difficult to conclude whether the antioxidant capacity of the supplement was responsible for the differences in the groups. The use of additional oxidative stress markers could have provided a more concise interpretation of how the supplement effects systemic oxidative stress.

Bowtell et al., (2011) reported ingestion of a cherry supplement before (7 days), on the day of, and for 48 hours after intensive strength training had a trend to reduce the increase in protein carbonyls (PC) 24 and 48 hours post-exercise compared to a control group. When the raw values were adjusted to the percentage change from baseline, a significant trial effect was reported, with the supplemented group displaying a less pronounced increase in PC 24 and 48 hours post-exercise. Interestingly, and in contrast to previous studies, CRP (Bell et al., 2014c; Bell et al., 2015; Howatson et al., 2010) and TAS (Howatson et al., 2010) was not different between the two conditions at any time-point during the study. Furthermore, McCormick et al., (2016) reported that cherry supplementation had no effect on reducing the inflammatory markers IL-6 and CRP after completing a week of intensified water-polo training compared to a placebo group.

The use of between-subject (Bell et al., 2014c; Bell et al., 2015; Howatson et al., 2010) versus a within-subject design (Bowtell et al., 2011; McCormick et al., 2016) could explain the differences between these studies. Although groups can be matched for age, sex, fitness levels, and stature; one variable that cannot be controlled for is the individual response to exercise, potentially leading to interindividual variability between two independent groups. An individual's baseline level of oxidative stress can influence the subsequent oxidative stress response to exercise. Margaritelis et al., (2014) have provided evidence suggesting individuals with higher baseline oxidative stress levels have a less pronounced change in these markers following exercise, whereas, individuals with low baseline levels have a

greater increase. And in contrast, individuals with greater baseline glutathione levels demonstrated a greater reduction in glutathione post-exercise, with the reverse effect happening with lower baseline glutathione levels. Furthermore, some individuals encountered reductive stress after exercise, where 13% exhibited a decrease in oxidative biomarkers and 10% exhibited an increase in glutathione levels that were in opposite directions of the main group findings. This highlights how exercise intervention studies with a between-subject design could be influenced by an individual's response to exercise, rather than the intervention per se. A within-subject study design avoids co-founding variables, such as interindividual variability, and allows researchers to fully elucidate a treatment effect when assessing oxidative markers post-exercise.

1.5.3 Polyphenol supplementation on exercise recovery and subsequent days performance

A hypothesized benefit of consuming a cherry supplement in the days leading up to, on the day of, and the days following strenuous exercise, is in its potential ability to promote recovery and therefore improve performance. Several studies have reported a beneficial effect of cherry supplementation on muscle recovery in the days following strenuous aerobic exercise (Bell et al., 2014c; Howatson et al., 2010), resistance training (Bowtell et al., 2011; Connolly et al., 2006), and prolonged intermittent exercise (Bell et al., 2016). The use of maximal voluntary isometric contractions (MVIC) and maximal voluntary contractions (MVC) have primarily been performed to assess muscle recovery. Studies have indicated that cherry supplementation can maintain or result in a lower percentage decrease in MVC/MVIC after completing strenuous exercise for a period of 24 to 72 hours (Bell et al., 2015; Bowtell et al., 2011; Connolly et al., 2006; Howatson et al., 2010). In addition to improved muscle recovery, cherry supplementation can further reduce muscle soreness associated with

strenuous exercise (Bell et al., 2016; Connolly et al., 2006; Kuehl et al., 2010; Levers et al., 2016). Taken together, the combination of improved muscle recovery and a reduction in muscle soreness following strenuous and/or muscle damaging exercise could allow for better performance on subsequent days of exercise when consuming a cherry supplement.

The use of MVC and MVIC have provided an initial indication that cherry supplementation could be beneficial in improving exercise performance on subsequent days. This has been shown either by a less pronounced reduction, or maintenance in MVC or MVIC after performing strenuous exercise, in participants consuming cherry compared to a control drink. However, MVC and MVIC are not measures of exercise performance per se, which makes it difficult to interpret these findings, and apply them to individual sports and/or sporting events. For example, Bell et al., (2015) reported a significant group effect over a 72-hour period for MVIC after completing a high-intensity cycling protocol. The cherry supplemented group maintained MVIC over the 72-hour period, whereas, the placebo group failed to maintain MVIC compared to baseline measures. However, when a 6 second maximal power test was performed over the same period, there was no difference in power output between the two groups. These results indicate that MVIC and MVC can provide a good indication of muscle recovery, but translating these findings to applied sport performance may not be applicable. There is therefore a need to investigate the effects of a supplement on a validated performance outcome in applied sport settings, or in validated measures of performance more applicable to sporting settings, such as exercise to exhaustion, time-trial, or protocols that relate to the demands of a specific sport.

Studies that have used applied performance outcomes in the setting of a particular sport when assessing the effects of cherry supplementation on subsequent days of exercise performance have provided equivocal results. Performance benefits have been reported after completing a modified version of the Loughborough intermittent shuttle test (LIST) in semi-

professional footballers (Bell et al., 2016). In the days following the LIST protocol, counter movement jump (CMJ) was decreased from baseline in both the cherry supplemented and placebo groups, however, the reduction in the cherry supplemented group was significantly attenuated at 24 and 48 hours post-LIST compared to placebo. Furthermore, the agility performance improved by 3% over a 72-hour period in the supplemented group, and 20m sprints were performed significantly quicker at 48 hours post supplementation when compared to the placebo group (Bell et al., 2016). The same authors have also reported the benefits of cherry supplementation on cycling efficiency 24 hours after completing a high intensity cycling protocol compared to placebo, but as mentioned previously there was no difference between the groups when assessing 6 second maximal power output over a 72-hour period (Bell et al., 2015).

The null findings in performance outcomes have also been reported in repeated days of cycling (Bell et al., 2014c) and in water-polo players (McCormick et al., 2016). In the study by Bell et al., (2014c), the authors found pre-supplementation (4 days) and supplementation on the day of 3 high-intensity cycling trials, did not affect the amount of work performed during 9 minutes of simulated time-trial efforts throughout the 3 cycling trials compared to a control group. McCormick et al., (2016) used a battery of performance measures related to water-polo to assess the effects of cherry supplementation after completing a week of intensified training. In their crossover design study, the authors reported no significant differences in vertical jump height, water-polo intermittent swim test, repeated swim test or 10 m swim sprints.

Although initial findings using cherry supplementation provided promising evidence that post-exercise recovery and exercise performance on subsequent days could be improved through its consumption, studies using applied outcome measures do not seem to support this view at present. Study design could potentially explain some of the differences observed

between studies, in relation to the effects of cherry supplementation on exercise-induced inflammation and oxidative stress as previously explained. To fully elucidate the effects of cherry supplementation on post-exercise recovery and subsequent days performance, within-subject design studies with applied performance outcomes should be conducted in appropriate populations.

1.6 Aims of the thesis

The overarching aim of this thesis was to examine antioxidant supplementation in free living cyclists. Numerous studies had been conducted in laboratory settings, with strict control of diet, training, and supplement dose. However, it was not clear if the findings from these studies were applicable to recreational cyclists. Thus, this thesis aimed to explore, and gain a greater understanding of, the use and effects of antioxidant supplementation in cyclists. Polyphenol consumption is becoming increasingly popular in athletes, but little is known about the efficacy in free living individuals, and thus a further aim was to explore the effects of a popular cherry concentrate in cyclists.

Data collated within this thesis presents information on the reasons why cyclists choose to consume antioxidant supplements and includes data on the effects of supplementation on the exercise-induced oxidative stress response to cycling.

The study presented in ***Chapter two*** aimed to identify the rationale for supplementation, and to examine the supplementation habits of recreational cyclists competing in an organised cycling sportive. ***Chapter three*** aimed to investigate the effects of 100 km cycling sportive on markers of oxidative stress in non-competitive but recreationally fit cyclists. ***Chapter four*** aimed to explore the effects of habitual antioxidant supplementation (≥ 6 months) on endogenous antioxidant content at rest, and to assess whether

supplementation affected the response to steady-state cycling compared to a non-supplemented group in well-trained cyclists, and finally, ***Chapter five*** aimed to investigate the effectiveness of a polyphenol supplement on exercise-induced oxidative stress, and inflammation, during steady-state cycling; and to determine its effectiveness on recovery and subsequent performance in trained cyclists.

Chapter Two

2. The prevalence of antioxidant supplementation in amateur cyclists undertaking the Wiggle Dragon Ride

2.1 Abstract

In the United Kingdom, cycling participation has had the largest increase in popularity compared to any other sport since 2013. Both elite and amateur athletes regularly consume dietary supplements, perceived to maintain a balanced diet and immune function, and to improve performance and recovery. Studies reporting on the consumption of dietary supplements often combine supplement types, and in doing so the reasons for consuming a specific supplement is unclear. Similarly, by not assessing supplement use in specific sporting disciplines, studies have been unable to tease out and relate supplement use to the particular demands of a specific sporting discipline. Therefore, it is difficult to extrapolate whether an athlete is consuming a dietary supplement for its intended purpose. The aim of the present study was to assess the use of antioxidant supplements in cyclists undertaking the 2013 Wiggle Dragon Ride Sportive, and to determine whether the reasons for consuming antioxidant supplements in this population is supported from current empirical findings. On-line questionnaires were completed by 75 male participants with an age range between 18 to 69, in the 2 months before and after the event. Out of the 75 male responders, 40% reported taking an antioxidant supplement, with 63% stating that they had been consuming antioxidant supplements for a period $\geq$ 12 months. The main reasons given for consuming supplements were: to improve immunity; improve performance; and reduce fatigue. Multivitamins and vitamin C were the antioxidant supplements used most frequently by the current cohort. Weekly training hours ($p = 0.85$) were not related to the intake of supplementation, as has previously been reported in elite athletes. Although studies have identified the use of multivitamins and vitamin supplements in national and international athletes (Luc et al, 2012; Douscombe, et al, 2010), there is no evidence to support the consumption of these supplements will improve immunity, performance, or recovery. Until such evidence emerges individuals should consider whether antioxidant supplementation is entirely necessary.

1.2 Introduction

Over the past 10 years in the United Kingdom (UK) there has been an increased participation in cycling, with a reported 2,000,000 people now undertaking at least 30 minutes of cycling once per week. This has resulted in cycling becoming the 3rd most participated in sport within the UK behind athletics and swimming (Sport England, Active People Survey 7 October 2012 - October 2013). Cycling sportives are organised road cycling and mountain biking events held for amateur cyclists, covering distances from 30 km to 300 km, and offering a multitude of terrains to challenge the rider's ability level. The increased popularity of cycling has given rise to a large number of cycling sportives being held each year, with a 29% increase in 2013 (British Cycling, 2012), and a 900% increase in organised cycling sportives from 2002 to 2012 (Wood, 2012). The Wiggle Dragon Ride is an annual cycling sportive in South Wales, attracting cyclists to attempt the mountainous routes covering distances of 120 km, and 210 km.

Dietary supplement consumption is prevalent in elite (Braun et al., 2009; Dascombe et al., 2010; Heikkinen et al., 2011; Lun et al., 2012; Ronsen et al., 1999) and non- elite athletes (Burns et al., 2004; Giannopoulou et al., 2013; Knez and Peake, 2010; Tian et al., 2009; Tsitsimpikou et al., 2011), amongst differing age groups (Braun et al., 2009; Knez and Peake, 2010), genders (Braun et al., 2009; Knez and Peake, 2010), and sporting disciplines (Braun et al., 2009; Dascombe et al., 2010; Giannopoulou et al., 2013; Knez and Peake, 2010; Ronsen et al., 1999). Dietary supplements are often used by athletes of varying ability levels as they are perceived to maintain and/or improve health, boost immunity, prevent and/or reduce symptoms of upper respiratory tract infections (URTI), reduce fatigue, supplement poor diets, and improve performance and/or recovery (Braun et al., 2009; Dascombe et al., 2010; Knez and Peake, 2010; Tian et al., 2009). There is further evidence to suggest athletes who compete in individual events are more likely to consume dietary supplements compared to

team based sports (Giannopoulou et al., 2013; Heikkinen et al., 2011); however, the greatest proportion of research into supplementation use has been conducted in multi-sporting disciplines and not focused on a single mode of sport.

Dietary supplements can be categorised in to two distinct categories: macronutrient and micronutrient supplements. The macronutrient supplements primarily consist of proteins, and carbohydrates; whilst micronutrient supplements consist of minerals, vitamins, and antioxidants. Macronutrients are associated with improved recovery and performance, and these supplements have been extensively researched in cyclists. Protein consumed in combination with carbohydrates after prolonged cycling can promote myofibrillar protein synthesis (Breen et al., 2011; Howarth et al., 2009); whilst carbohydrate ingestion before, during, and after competition can result in improved cycling performance (Burke et al., 2011; Jeukendrup, 2010; Smith et al., 2013).

Micronutrients function primarily as coenzymes and cofactors in metabolic processes rather than being metabolic substrates themselves. Coenzymes and cofactors are essential in metabolism and provide components required for the efficient running of metabolic pathways. Vitamins are important macronutrients owing to their function in energy metabolism and as antioxidants (Huskisson et al., 2007; Maughan, 1999). Not all vitamins are capable of being an antioxidant because they do not have the capability to quench reactive oxygen and/or nitrogen species (RONS). The main vitamins classed as antioxidants are vitamin C and vitamin E, and together they provide protection against RONS in aqueous and lipid environments respectively (Niki, 2014; Padayatty et al., 2003).

Multivitamin and vitamin supplements are the most prevalent dietary supplements consumed by athletes. The frequency of vitamin and antioxidant use in elite athletes ranges from 76% to 20% (Braun et al., 2009; Dascombe et al., 2010; Heikkinen et al., 2011; Lun et

al., 2012), collegiate athletes 73% to 50% (Burns et al., 2004; Tian et al., 2009), well-trained athletes 60% (Knez and Peake, 2010), and recreational exercisers 50% (Tsitsimpikou et al., 2011). Athletes who undertake large volumes of training and/or compete at a high level of competition, such as national or international competitions, consume greater amounts of dietary supplements compared to athletes with lower overall training volumes and/or compete at an inferior competition level.

Giannopoulou et al., (2013) reported that dietary supplementation was prevalent in 44.8% of athletes training for $\geq$ 5 times per week, 26.1% of athletes training for $\geq$ 3 times per week, and 16.7% of athletes training for 1-2 days per week. These results were consistent with findings from a study conducted in university students from Singapore, with students training $\geq$ 5 hours per week reporting increased intake of multivitamin and antioxidant supplements, and those who competed in $\geq$ 3 sports per week being more likely to consume a vitamin C or multivitamin supplement, compared to those who reported undertaking less exercise (Tian et al., 2009). Furthermore, a study conducted in young elite German athletes discovered that 100% of athletes who had achieved top level positions in the Olympic Games or World Championships consumed dietary supplements; with a decrease in frequency between 70% and 80% for athletes competing at international, national, and regional levels (Braun et al., 2009). These studies suggest the amount of time spent training and/or the competition level of an athlete could influence dietary supplementation, however this has not been investigated in amateur sporting events or in a single mode of exercise.

The aim of the present study was to investigate the use of antioxidant supplements in recreational cyclists competing in an organised sportive. Furthermore, the study aimed to determine whether there are any differences in weekly training hours, cycling experience, or age between those cyclists who do, and do not, consume antioxidant supplements; and to

explore the reasons for why supplementation occurs. This is the first study to report these factors within participants competing in an amateur cycling event.

1.3 Methods

Seventy-five male amateur cyclists (aged 18-69) volunteered to participate in this study. They were all registered in the 2013 Wiggle Dragon Ride Sportive. Participants were identified by their use of antioxidant supplements as non-supplemented cyclists or supplemented cyclists. The Wiggle Dragon Ride is a cycling sportive held near the town of Neath in the South of Wales, United Kingdom; incorporating routes of 120 km (Medio Fondo) and 210 km (Gran Fondo) with altitude gains of 1850 m and 3350 m respectively. All participants were informed of the risks and benefits of the study and individual consent was obtained before the participant could complete the questionnaire. Ethical approval for the study was obtained through the University of Birmingham's Science, Technology, Engineering and Mathematics Ethical Review Committee.

Data Collection and Questionnaire

All data was obtained using an online questionnaire (Appendix 1). The questionnaire was advertised in monthly news emails sent to participants undertaking the 2013 Wiggle Dragon Ride from the organisers of the event. Participants were invited to complete the study via a link to the questionnaire either in the 2 months preceding the event or up to a period of 2 months following the completion of the event The online questionnaire collected categorical data on participants age (18-24, 25-29, 30-34...65-69, 70-74, 75+), years cycled ($\leq$ 3 years, 4-7 years, 8-11 years, $\geq$ 12 years), training hours per week (0-3 hours, 4-6 hours, 7-9 hours, $\geq$ 10 hours), route undertaken (Medio Fondo, Grand Fondo), the frequency of consuming foods containing antioxidants (more than once a day, once a day, 4-6 days a week, 1-3 days a week, at least once a month, less than once a month, never), use of antioxidant supplements (Yes/No). If answered no the questionnaire was completed; however if the participant

answered yes, then they were asked additional questions related to their supplementation: period consumed for (< 6 months, 6-12 months, > 12 months), reasons for consuming antioxidant supplements (boost immunity/improve health, peer recommendations, improve performance, reduce fatigue/recovery time, improve training adaptations, dietician/sport nutritionist recommendation, maintain a balanced diet), type(s) of antioxidant/vitamin supplements consumed (vitamin C, vitamin E, multi-vitamin….other-please specify additional supplements not on list) and frequency of consumption (more than once a day, once a day, 4-6 days a week, 1-3 days a week, at least once a month, less than once a month, never). If questions relating to supplementation usage were not completed, the participant was excluded from the study. In total, 88 questionnaires were collated at the end of the data collection, 2 questionnaires were excluded due to gender, and a further 11 questionnaires due to incomplete data sets. This resulted in 85% of the total number of questionnaires being used for data analysis.

Analysis of results

The results were electronically transferred to Microsoft Excel where they were collated and analysed. Differences between groups was analysed using SPSS software for Windows 22 (SPSS Inc, Chicago, IL, USA). The data was analysed using descriptive statistics, including medians, modes, and frequency in percentages. To determine differences between the supplemented and non-supplemented groups for the variables of interest (age, years cycled, training hours per week, and route undertaken on the day of the race) a chi-squared (χ^2) analysis was performed. Significance was accepted at $p \leq 0.05$.

1.4. Results

The average age of the participants fell within the 40-44 years of age group category, and this finding was replicated in both the supplemented and non-supplemented groups, with no significant differences for age between the groups ($\chi^{(1)} = 6.46$, $p = 0.692$) . When participants were grouped according to age, the largest number of non-supplemented participants (25%, n = 11) was recruited in the 45-49 years' age range, and the largest number of supplemented participants (23%, n = 7) was in the 40-44 years age range (Figure 2.1).

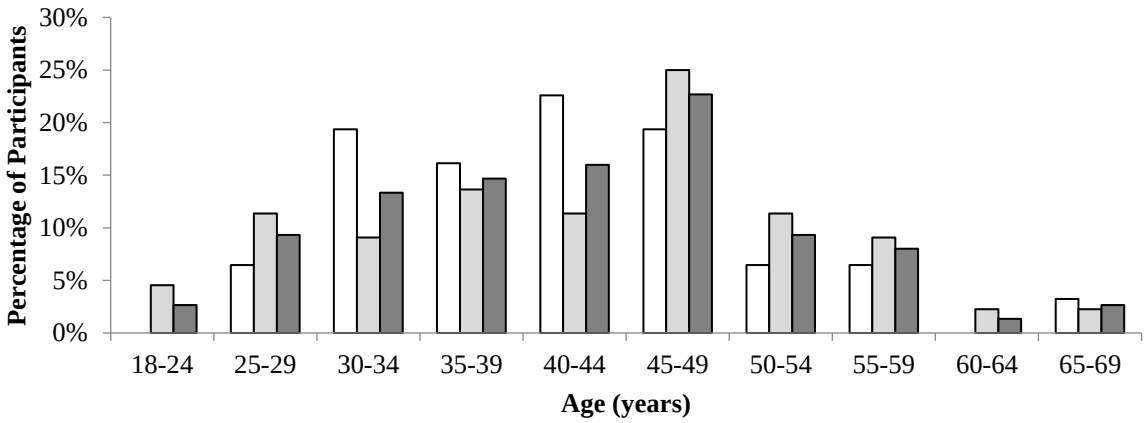


Figure 2.1 The distribution of age ranges for all of the participants and for the supplemented and non-supplemented groups. All values are expressed as percentages. White bars = supplemented group, light grey bars = non-supplemented group, and dark grey = total participants.

The average amount of years spent cycling by the participants was in the 4-7 years' category, and this finding was replicated in both groups, with no significant differences between the groups for years spent cycling ($\chi^{(1)} = 2.06$, $p = 0.56$) . Thirty-nine percent (n = 29) of participants reported cycling for 12+ years, 32% (n = 10) of the supplemented group and 43% (n = 19) of the non-supplemented group (Figure 2.2).

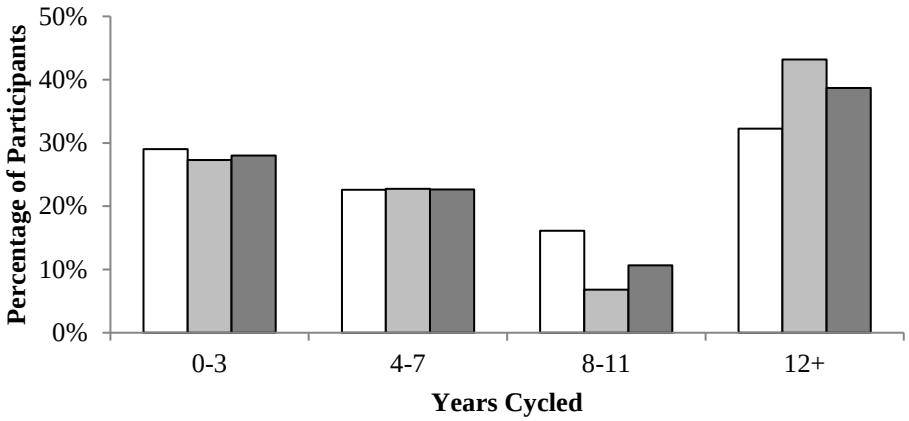


Figure 2.2 Cycling experience measured in years cycled. All values are expressed as percentages. White bars = supplemented group, light grey bars = non-supplemented group, and dark grey = total participants.

There was no significant difference between the groups for the amount of hours spent training per week ($\chi^{(1)} = 0.781$, $p = 0.854$) . The category with the greatest number of participants in was the 4-6 hours of cycling per week (44%, n = 33) for all of the participants, and this finding was replicated for the supplemented (42%, n = 13) and non-supplemented groups (45%, n = 20) (Figure 2.3).

The Gran Fondo was undertaken by 68% (n = 51) of the participants, and the Medio Fondo 28% (n = 21). Four percent (n = 3) did not specify a route. The Medio Fondo was undertaken by 19% (n = 6) of the supplemented group and 34% (n = 15) of the non-supplemented group; with the Gran Fondo undertaken by 77% (n = 24) of the supplemented group and 61% (n = 27) of the non-supplemented group. There was no significant difference between the routes undertaken by the groups, ($\chi^{(1)} = 2.09$, $p = 0.148$) (Figure 2.4).

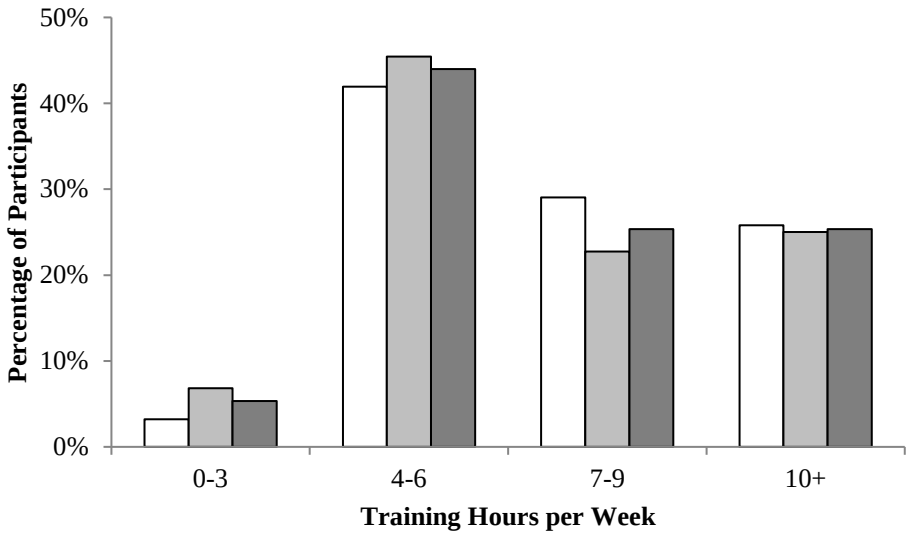


Figure 2.3 The distribution of weekly training hours undertaken by the participants. All values are expressed as percentages. White bars = supplemented group, light grey bars = non-supplemented group, and dark grey = total participants.

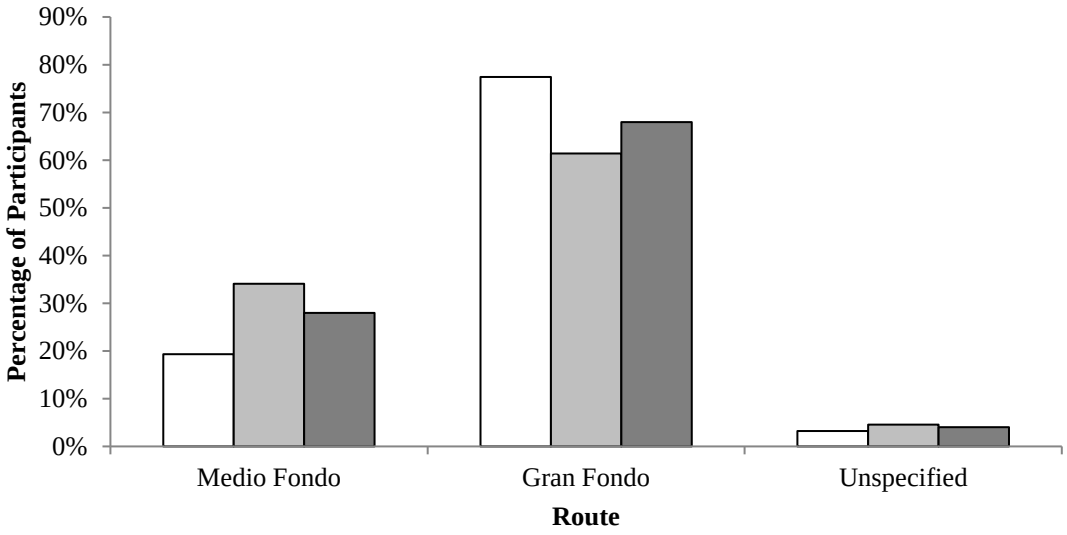


Figure 2.4 The routes undertaken by the participants at the Wiggle Dragon Ride. All values are expressed as percentages. White bars = supplemented group, light grey bars = non-supplemented group, and dark grey = total participants.

Antioxidant supplementation

In total, 41% (n = 31) of the participants reported consuming an antioxidant supplement and 59% (n = 44) of the participants did not take any form of antioxidant supplementation. In the supplemented group 61% (n = 19) reported supplementing for a period > 12 months, 19.5% (n = 6) had been supplementing for a period < 6 months, and 19.5% (n = 6) had used supplements for a period > 6 months but < 12 months (Figure 2.5).

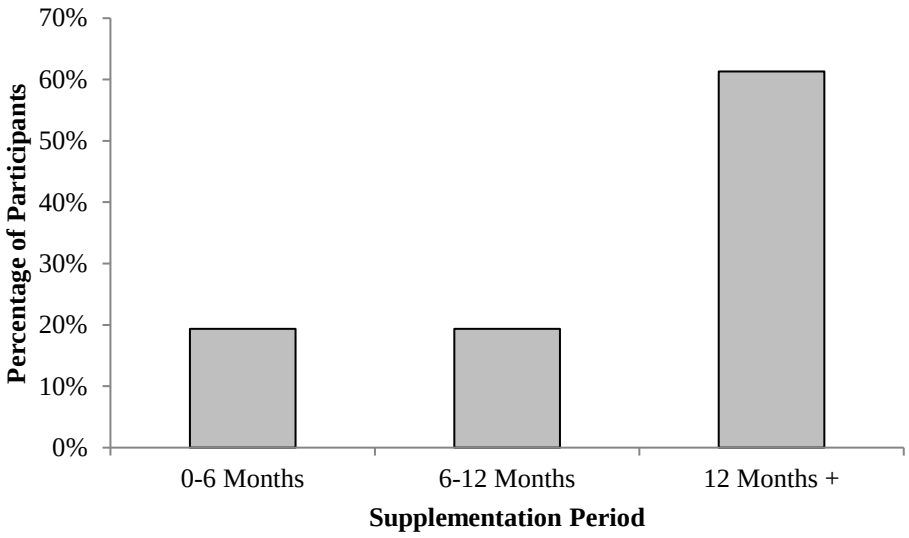


Figure 2.5 Duration of antioxidant supplementation for the supplemented group. All values are expressed as percentages.

The type of antioxidant supplements reported by the participants were: multivitamins (47%), vitamin C (32%), vitamin E (17%), and other (4%) (Figure 2.6). The main reasons given for using an antioxidant supplement were: to improve immunity (43%, n = 25), reduce fatigue (17%, n = 10), improve performance (16%, n = 9), improve exercise adaptations (10%, n = 6), maintain a balanced diet (9%, n = 5), professional recommendations (3%, n = 2), and peer recommendations (2%, n = 1) (Figure 2.7).

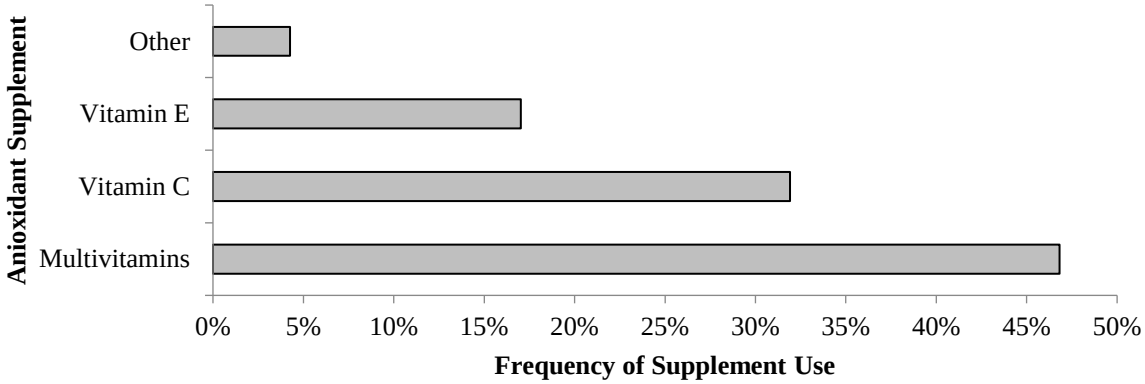


Figure 2.6. The frequency of each antioxidant supplement used within the supplemented group. All values are expressed as percentages.

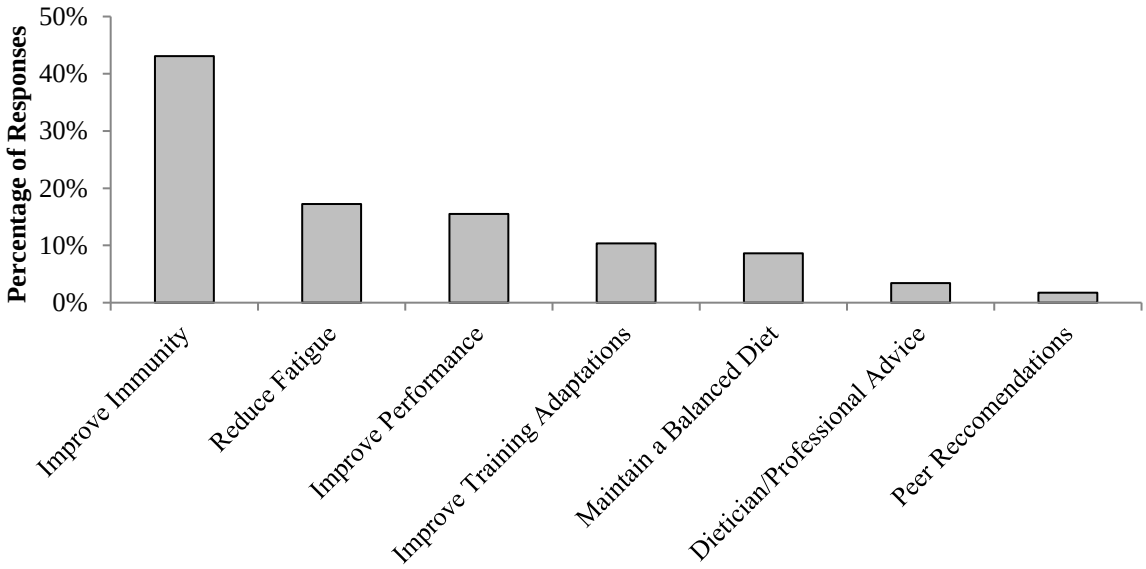


Figure 2.7 Rationale for the use of antioxidant supplements amongst the supplemented group, all values are expressed as percentages.

The consumption of antioxidant supplements was reported to be between more than once a day to only once per month (Figure 2.8). The frequency for consuming a multivitamin supplement was 5% (n = 1) for more than once a day, 68% (n = 15) for once a day, 9% (n = 2) for 4-6 days a week, 14% (n = 3) for 1-3 day per week, and 5% (n = 1) for less than once a month. The frequency for consuming a vitamin C supplement was 7% (n = 1) for more than once a day, 47% (n = 7) for once a day, 13% (n = 2) for 4-6 days a week, 13% (n = 2) for 1-3 day per week, and 20% (n = 3) for less than once a month. The frequency for consuming a vitamin E supplement was 12.5% (n = 1) for more than once a day, 50% (n = 4) for once a day, 12.5% (n = 1) for 4-6 days a week, 12.5% (n = 2) for 1-3 day per week, and 12.5% (n = 1) for less than once a month. The frequency of consumption for other antioxidant supplements was reported to be 100% (n = 2) for less than once a month (quercetin and grape seeds). The average number of antioxidant supplements used by the participants was 1 with 68% (n = 21). Nineteen percent (n = 6), reported using 2 antioxidant supplements, and 13% (n = 4) reported using 3 or more antioxidant supplements (Figure 2.9).

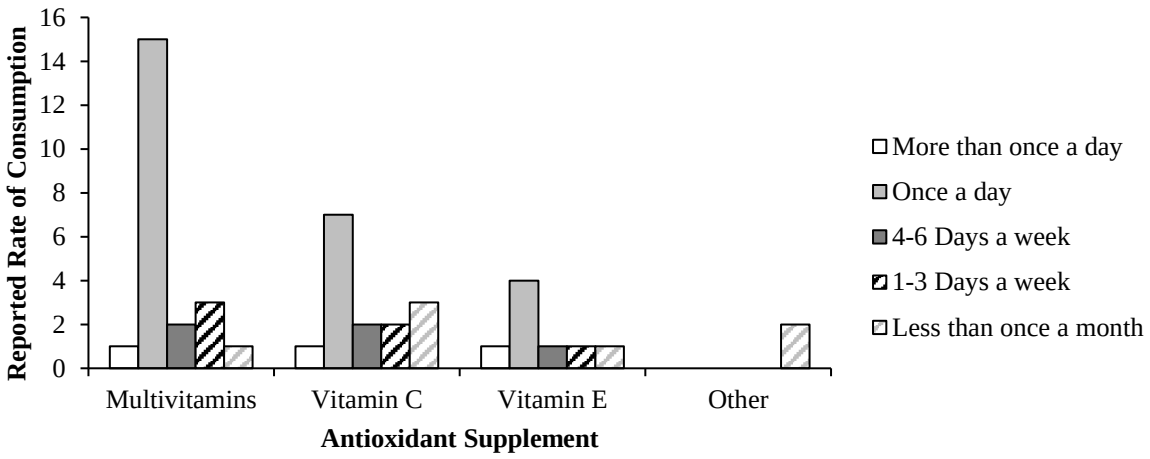


Figure 2.8 The reported rate of consumption for each antioxidant supplement on a daily to monthly basis. White bars = more than once a day, light grey bars = once a day, dark grey = 4 to 6 times a week, black and white stripe bars = 1 to 3 times a week, and grey and white stripe bars = less than once a month.

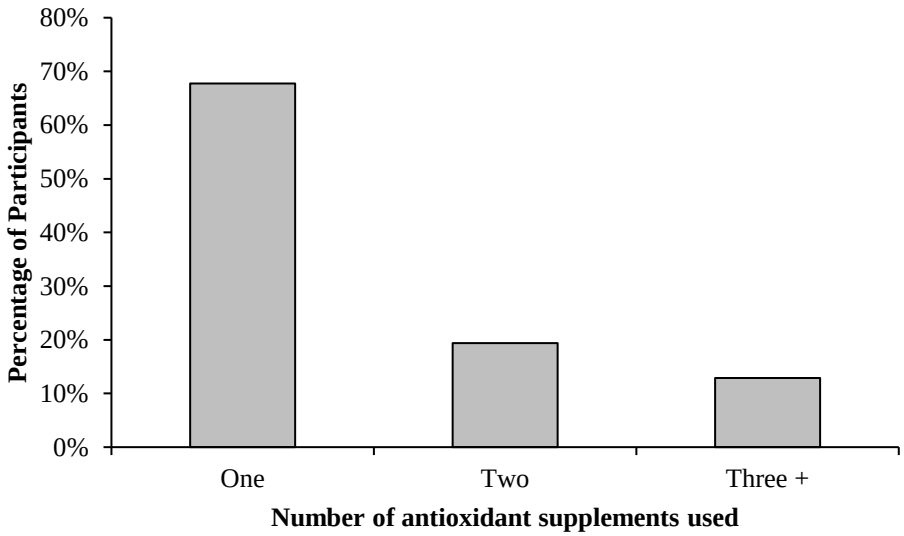


Figure 2.9 The number of antioxidant supplements consumed by the supplemented group. all values are expressed as percentages.

1.5 Discussion

Vitamin and antioxidant supplements are the most common dietary supplements used by elite, non-elite and recreational exercisers taking part in various sporting disciplines. Previous studies have reported supplementation frequency rates between 75% and 20% (Braun et al., 2009; Dascombe et al., 2010; Heikkinen et al., 2011; Lun et al., 2012). The present study focused only on antioxidant supplementation. Forty one percent of participants in the study reported taking at least one supplement. There were no statistical differences between the supplemented and non-supplemented groups when assessing for age, training hours per week, amount of years spent cycling, or the route undertaken by the participants.

The present study indicates training volume did not influence the frequency of antioxidant supplementation in recreational cyclists. Previous research has indicated that athletes undertaking $\geq$ 5 hours a week of training are more likely to use multivitamin, antioxidant, and dietary supplements (Giannopoulou et al., 2013; Tian et al., 2009). However, the findings from the present study demonstrate the volume of training did not influence incidence of supplementation, as equal numbers of participants in the present study reported supplementing and not using supplements (Figure 2.3). The reason for these discrepancies could be attributed to the population surveyed, both in terms of elite vs amateur and in terms of the type of sport being undertaken. The studies conducted by Tian et al., (2009) and Giannopoulou et al., (2009) assessed athletes from a variety of sporting backgrounds with endurance sports accounting for 9% and 0.4% of the total sample size, respectively. When examining factors likely to influence incidence of supplementation, if the results presented in the current study are taken together with previous studies, this may suggest the type of sport may influence the prevalence of antioxidant supplementation rather than the amount of time spent training on a weekly basis.

Furthermore, Lun et al., (2012) collected data pertaining to dietary supplementation within high performance athletes, which indicated the frequency of supplementation increased with the addition of weekly training hours. In this study, the incidence of supplementation amongst athletes within different training volume groups increased from 66% for those who trained between 0-5 hours a week, up to 95% in athletes training for > 25 hours a week. The same trend was observed with the consumption of multivitamin supplements with an increase in weekly training hours accounting for an increase in multivitamin supplementation from 33% to 63%. In contrast, the results of the current study found that amateur cyclists, who report above 10h of weekly training hours, do not habitually supplement more than those training 4-6hrs per week. Taken together it may be speculated that training volume is a more reliable predictor for supplementation habits in elite athletes compared to amateurs.

This study observed that there is a high prevalence of antioxidant supplementation in cyclists aged between 35 and 49. Many studies have focused on groups of athletes with mean ages between 20 and 30 (Braun et al., 2009; Burns et al., 2004; Dascombe et al., 2010; Giannopoulou et al., 2013; Lun et al., 2012; Ronsen et al., 1999; Tian et al., 2009), and only one previous study has assessed athletes' with a mean age above 30 (Knez and Peake, 2010). By studying elite athletes who have competed at the Olympic Games, World Championships, national level competitions, and athletes attending national sporting institutes; these studies have only been representative of elite cohorts and not representative of the recreational and/or ageing athlete. A study analysing UK high performance athletes indicated the age group with the highest frequency of supplementation was the 24 to 29 age group, with athletes over 40 representing 4.1% of the full sample and 1.7% of the supplement user group (Petroczi et al., 2008). These results were replicated in Finnish Olympic athletes, with the 'over 24'of years' age group consuming significantly higher amounts of vitamin supplements and overall

dietary supplements compared to the ‘under 21’ age group; unfortunately, the study did not provide any additional sub-groups beyond the ‘over 24’ group, so it is not possible to establish if there were athletes aged over 30 in their analysis (Heikkinen et al., 2011). The present study highlights the frequency of supplementation of antioxidant supplements in an age group older than 30 years of age and in a cohort of non-elite athletes, representing the wider population of cycling enthusiasts.

The current study sought to identify the rationale for using antioxidant supplements in a non-elite cycling cohort. The most frequent reason given was to improve immunity (43%). Other reasons given were to reduce fatigue (16%), improve performance (16%), improve exercise adaptations (11%), and maintain a balanced diet (9%). These findings are similar to previous research conducted in elite (Braun et al., 2009; Lun et al., 2012) and non-elite athletes (Knez and Peake, 2010; Tian et al., 2009; Tsitsimpikou et al., 2011) and support common misconceptions that supplementation will enhance immunity levels.

After prolonged or strenuous exercise, the immune system can become compromised, resulting in immunosuppression known as the “open window” where immunity levels are supressed for up to 72 hours (Nieman and Pedersen, 1999). It has previously been hypothesized that interventions using antioxidant supplements may prevent the occurrence of immunosuppression that occurs after prolonged and/or strenuous exercise. However, supplementation with vitamin C had no effect on reducing the incidences of URTI in marathon runners, when given a dose of 1000 mg/d (Himmelstein et al., 1999). These findings were replicated in a laboratory based experiment where vitamin C was given either acutely (Davison and Gleeson, 2007; Nieman et al., 1997) on the day of the experiment or supplemented chronically for a period of 2 weeks (Davison and Gleeson, 2006). The main factors associated with increased exposure to URTI were faster running speeds, training

volumes and the number of marathons competed (Himmelstein et al., 1999). The aforementioned studies found no differences between a placebo control group and a vitamin C supplemented group after 2.5 hours of exercise in markers related to immunosuppression, at the end of exercise bout or in the preceding hours after completing the exercise bout. Furthermore, a recent meta-analysis has indicated that well-nourished individuals should refrain from taking antioxidant supplements, as antioxidant supplements do not contain any compounds that would result in beneficial health effects (Bjelakovic et al., 2014).

Sixteen percent of participants in the current study reported consuming antioxidant supplements to either improve performance and/or reduce fatigue. Fatigue has been defined as any exercise-induced reduction in the ability to exert muscle force or power (Gandevia, 2001), that will subsequently effect one's performance. The cellular redox state of skeletal muscle can affect contractional force (Powers and Jackson, 2008; Reid, 2001) in that an oxidised cellular environment, characterised by excessive RONS, results in a reduction of contractional force. The mechanisms responsible for the reduction in muscle force during exercise are related to various stages of excitation-contraction coupling: 1) oxidation of the ryanodine receptor (RYR) affecting calcium (Ca^{2+}) release from the sarcoplasmic reticulum (SR) into the t-tubules; 2) decreased Ca^{2+} sensitivity of the myofibrillar proteins affecting cross-bridge formation; and 3) impairment of the SR Ca^{2+} - ATPase (SERCA) reducing the reuptake of Ca^{2+} into the SR (Allen et al., 2008; Hernández et al., 2012; Westerblad and Allen, 2011). These mechanisms influence prolonged low-frequency force depression (PLFFD) which is associated with delayed recovery following exercise (Hernández et al., 2012; Westerblad and Allen, 2011). Therefore, it seems rational to presume that the beneficial effects of antioxidant supplementation would enable muscle force/power output to be maintained throughout exercise and to improve recovery.

However, it should be noted that the muscle force maintenance and PLFFD is a multifaceted phenomenon that is affected by both central and peripheral factors. Central fatigue is associated with processes within motoneurons and the central nervous system (suboptimal cortical drive, reduced synaptic efficacy, reduced intrinsic motoneuron responsiveness, presynaptic inhibition, firing of group III/IV muscle afferents), whereas, peripheral fatigue encompasses processes distal to the neuromuscular junction (myofibrillar damage, cross-bridge formation, excitation-contraction coupling failure, ATP-availability) (Allen et al., 2008; Carroll et al., 2017; Gandevia, 2001). These various factors could explain why empirical evidence does not support the use of vitamin C and/or vitamin E supplements in reducing fatigue or improving recovery, with studies mainly showing no effect on reducing muscle damage (Theodorou et al., 2011), oxidative stress (Bloomer et al., 2006), or sporting performance (Braakhuis, 2012; Braakhuis and Hopkins, 2015).

Recent research has indicated that a subset of antioxidants called polyphenol compounds may be beneficial in reducing oxidative stress and skeletal muscle damage during exercise (Bowtell et al., 2011; Howatson et al., 2010; Malaguti et al., 2013), thus potentially facilitating sporting performance and/or recovery from exhaustive and/or prolonged exercise. However, further research is needed before evidence based recommendations can be given on the effects of antioxidant supplementation on reducing fatigue and/or improving sporting performance.

Eleven percent of the study sample reported using antioxidant supplements to enhance exercise adaptations. There is a growing number of studies which report negative effects of vitamin C and vitamin E supplementation on exercise adaptations, specifically in regards to mitochondrial biogenesis and endogenous antioxidant production (Gomez-Cabrera et al., 2008; Paulsen et al., 2014; Ristow et al., 2009; Strobel et al., 2011). RONS are integral

messengers involved in the intracellular signalling cascades responsible for mitochondrial biogenesis and endogenous antioxidant synthesis (Jackson, 2005; Ji, 2008), and by reducing RONS via antioxidant supplementation these important signalling pathways may be inhibited. However, conflicting studies have shown no inhibition of mitochondrial biogenesis or endogenous antioxidant expression after periods of supplementation with vitamin C and vitamin E (Higashida et al., 2011; Yfanti et al., 2010; Yfanti et al., 2012). Differences in exercise intensity and frequency, participant training status, and analytical techniques could explain the different outcomes in these studies. Thus, without identifying the intensity of the participants training sessions or their exact physical fitness levels, it would be unclear on whether antioxidant supplementation would have a detrimental effect on training induced adaptations.

A strength of the current study is its specificity in investigating only one form dietary supplement, antioxidants. Previous studies have not focused on a single subset of dietary supplements consumed by athletes, but have primarily focused on dietary supplements as a whole; including differing sub-categories such as carbohydrates, proteins, amino acids, minerals, and vitamins not classified as antioxidants such as the B – vitamins, and vitamin D. The main focus of the current study was to identify which antioxidant supplements were being consumed by amateur cyclists and the rationale for their use. It could be argued the current study does not provide a conclusive account of the true use of dietary supplements within amateur cyclists; however, with the main focus orientated on one form of supplementation it enables a more precise insight on why this subset of supplements may be consumed. Empirical research has identified the clear benefits of consuming carbohydrate and protein supplements, providing a clear rationale on why these supplements are used in an athletic population. When studies have been conducted on the use of dietary supplements as a whole, it is not certain why micronutrient supplements, such as antioxidants, may be used. At

present, within the scientific community, there is a consensus that additional antioxidant supplementation is not required in individuals who regularly consume a balance diet and undertaking regular exercise. There is no conclusive evidence to support their role in improving performance and/or recovery, or improving immunity in an athletic population. Therefore, it is important to ascertain why antioxidant supplements are being used within the current population and to identify interventions to educate non-elite athletes on the use of these supplements.

The main limitation of the study was that participants provided information through self-report responses. The use of self-report questionnaires can result in response bias from the participants using traits known as *social desirability* and *social approval bias* (Furnham, 1986). Social desirability is used more relatively as a defensive mechanism and is typically used to convey social norms to avoid criticism, rather than reflecting their own habits that they feel may be undesirable. For instance, reporting a greater intake of fruit and vegetables on a daily basis than they normally would consume. On the other hand, social approval bias can be effected by the knowledge a person has relating to the questions they are answering and generating an answer they believe is the "correct" response for the researcher (Furnham, 1986). A study investigating the effect of social approval bias on self-reported measures of fruit and vegetable consumption, identified that social approval bias was prominent in an intervention group that were supplied additional information regarding the health benefits of fruits and vegetables, and information regarding to the amount of fruit and vegetables that should be consumed on an average day. A control group received the same information pack as the intervention group without the additional information relating to the health benefits of fruits and vegetables and daily serving amounts. There were no significant differences in the groups when completing a 24-hour dietary recall for milk, doughnuts, cookies, cake, pastries, pies, or potatoes. However, when total fruit and vegetable intake was collated, the

intervention group had a significantly higher intake compared to the control group. The authors of the study identified this difference to the information given to the intervention group in the days prior to the 24-hour dietary recall, thus promoting social approval bias when reporting their fruit and vegetable consumption (Miller et al., 2008). In the present study, there is a potential for both social desirability and social approval bias to occur because the participants knew before completing the questionnaire the study was investigating the intake of antioxidant foods and supplements; resulting in an overestimation of the frequency of the foods and/or supplements they consumed to fit in what they believed to be the social norm or to provide answers they believed the researchers were trying to identify.

One of the aims of the thesis was to investigate the relevance of antioxidant supplements in real life cycling. The use of an online questionnaire within this study allowed the characterisation of use and type of antioxidant supplements in recreational cyclists. Previous research on supplement intake primarily focuses on either elite athletes or a range of sports and supplements grouped together, making it difficult to interpret why a specific dietary supplement may be used. The findings within this chapter provide information on the proportion and characteristics of amateur cyclists that use antioxidant supplements, and reasons for supplementation.

The findings from the present study identified habitual consumption of antioxidant supplements was not related to age, cycling experience, weekly training hours, or the route undertaken; implying that in this population there was not a specific ‘type’ of person who consumed antioxidant supplements. The main reasons given for consuming supplements were to maintain health, reduce fatigue, improve performance, maintain a balanced diet, and improve training adaptations. At present, there is no empirical evidence to support the use of

antioxidant supplements within this population for the reasons given by the participants. Therefore, participants may be consuming supplements for no beneficial reason.

To help identify the effects of habitual antioxidant supplementation on individuals undertaking recreational cycling events, more applied research should be conducted in this area. Future research could explore the effects of habitual antioxidant supplementation on the exercise-induced oxidative stress response to cycling in an applied, real-world setting.

Chapter Three

3. Habitual antioxidant supplementation does not affect the oxidative stress response after a 100 km cycling sportive

3.1 Abstract

Purpose: The purpose of the study was to investigate the effects of a non-competitive cycling event on markers of oxidative stress in recreationally active individuals. The effect of habitual antioxidant supplementation on these markers was compared to non-supplementation individuals in a "real world" setting.

Methods: Markers of oxidative stress and antioxidant capacity were assed in recreational active individuals who took part in the Tommy Godwin cycling sportive (100 km). Blood samples were taken from a supplemented (Supp) (n = 7, mean ± SD: age 53.4 ± 3.5 yrs; weekly training hours: 7.6 ± 2.4 hrs) and non-supplemented (NoSup) (n = 10, mean ± SD: age 49.9 ± 10.6 yrs; weekly training hours: 6.0 ± 3.1 hrs) group within 30 minutes prior to the start and within 10 minutes of finishing the sportive. Protein carbonylation (PC), malonaldialdehyde (MDA), and total antioxidant capacity (TAC) was assessed in plasma. Whole blood was used to assess reduced glutathione (GSH).

Results: Following completion of 100 km cycling sportive, TAC significantly increased compared to baseline in all cyclists (Supp: $p < 0.001$; NoSup: $p < 0.05$), there was a trend for a group reduction in GSH ($p = 0.054$). No change was observed in PC compared to baseline. PC was significantly higher in NoSup compared to Supp ($p < 0.05$) at the end of the sportive. MDA significantly increased in the NoSup compared to pre-values ($p < 0.05$), whereas no change was observed in the Supp.

Conclusions: A cycling sportive over a 100 km caused a change in antioxidant status regardless of supplementation. The effect of habitual antioxidant supplementation on other markers of oxidative stress could not be fully elucidated from the current results.

3.2 Introduction

The recent success of British cyclists in the Olympic Games, World Championships, and the Tour de France has increased the popularity of cycling within the United Kingdom. There has also been an increase in non-competitive cycling sportive events, as discussed in Chapter 2. The study presented in chapter 2 indicated the prevalence of antioxidant supplementation in amateur cyclists is around 40%. The most frequently consumed antioxidant supplements were identified as multivitamins that contain a combination of antioxidant compounds, and vitamin C and/or vitamin E.

Field studies that have investigated oxidative stress in response to exercise in the general population are under-represented (Knez et al., 2007). Exercise can provoke oxidative stress, especially in individuals unaccustomed to exercise. Sportive events are organised non-competitive cycling events for recreationally active individuals, unlike organised road-racing events they do not require a race license to participate in them. Due to the non-competitive nature of cycling sportives individuals who undertake them could be doing so for social or personal reasons. To our knowledge there are currently no studies investigating the effects of these events on markers of oxidative stress or information on why the general population choose to undertake these events.

The majority of field based studies that have investigated the effects of exercise-induced oxidative stress have focused on ultra-endurance events. These events provide a good model to explore the demands of extreme exercise on exercise-induced oxidative stress and subsequently evaluate the impact of extreme exercise on health. Studies have included assessment of oxidative stress following an ironman triathlon (Neubauer et al., 2008) the Grand Union Canal race, a 233 km running event (Turner et al., 2011) and the Spartathlon race, a 246 km running event (Skenderi et al., 2008). Elevations in oxidative stress markers

were observed in these studies, but markers returned to baseline within 5 days (Neubauer et al., 2008), 7 days (Turner et al., 2011), and 48 hours (Skenderi et al., 2008). These studies provide sufficient evidence to suggest that ultra-endurance events do not result in extended periods of oxidative stress in well-trained participants. However, the results may not be representative of less well-trained individuals who undertake sporting events on a more social level and/or to maintain/improve their current fitness levels.

Antioxidant supplementation in amateur athletes is common. The study presented in chapter 2 suggests people take antioxidant supplements to improve immunity, reduce fatigue, improve performance, training adaptations, and to maintain a balanced diet. Many randomised control studies have investigated the efficacy of antioxidant supplements in reducing exercise-induced oxidative stress. The supplementation period, the intensity of the exercise protocol, and the type and/or combination of antioxidant administered are all factors that can affect oxidative modifications post-exercise (Bloomer et al., 2006; Bunpo and Anthony, 2016; Gomez-Cabrera et al., 2006; Mastaloudis et al., 2004).

One possible explanation to explain why individuals choose to consume antioxidant supplements could be related to the health benefits reported from early epidemiological studies. These studies have reported that individuals who have sub-optimal levels of antioxidants in their diets are at higher risk of cardiovascular disease (Gey et al., 1993), lung disease (Miedema et al., 1993), age-related macular degeneration (Snodderly, 1995), coronary heart disease (Knekt et al., 1994), cancer (La Vecchia et al., 2001), and reduction in telomere length (Freitas-Simoes et al., 2016). Furthermore, a large epidemiological in elderly participants reported all-cause mortality and coronary heart disease mortality was reduced in those who consumed vitamin E supplements alone or in combination with vitamin C (Losonczy et al., 1996). Unfortunately, large scale randomised control trials have failed to support the epidemiology populations with risk factors associated with CVD or cancer, or

those already suffering from these conditions when supplemented with a variety of antioxidant supplements (Stanner et al., 2004; Willcox et al., 2008).

The data presented in Chapter 2 indicates those who consume antioxidant supplements have done for a period greater than 6 months. So, it is important to establish if supplementation has an effect. Unfortunately, the doses that have been administered in the majority of previous studies are well above the recommended levels for both vitamin C (90mg/d) and vitamin E (15mg/d or 22.4 IU/d), and thus may not reflect, or even be relevant to the amount being consumed by recreationally active individuals using over the counter supplements. Dose of supplement is important. Several training studies conducted in controlled laboratory settings have demonstrated an inhibitory effect on cellular signalling pathways (Gomez-Cabrera et al., 2008; Morrison et al., 2015; Paulsen et al., 2014; Ristow et al., 2009; Strobel et al., 2011). To our knowledge oxidative modifications within plasma do not directly relate to signalling processes occurring at a skeletal muscle level (Margaritelis et al., 2016); however, plasma oxidative modifications may shed some light on how habitual antioxidant supplementation could affect the response to exercise compared to a non-supplemented group.

The purpose of the present study was to assess oxidative stress in a group of cyclists completing a 100 km cycling sportive. An overarching aim of this thesis was to assess the effect of exercise on markers of oxidative stress in ‘real-life’ amateur cyclists, including those supplementing habitually with antioxidants, and thus there were no restrictions on participation in this study.

3.3 Methods

Participants

Eighteen participants, 15 male and 3 female, who undertook the Tommy Godwin cycling sportive in Solihull, (West Midlands, UK) took part in the current study. All the participants who registered to take part in the sportive received an email from the organisers informing them of the study. Those who registered an interest in the study contacted the researchers and were sent a participant information sheet (PIS) (Appendix 2), general health questionnaire (GHQ) (Appendix 9), and a consent form (Appendix 3). Participants were also recruited on the day of the sportive after reading the PIS and completing the GHQ and consent form. To enable the study to observe the effects of a recreational cycling event in weekend cycling enthusiasts' there were no exclusion criteria for the study. Those undertaking the event classed themselves as adequately prepared for the event, with no known medical conditions. Those with pre-existing medical conditions declared themselves fit and able to undertake the event. Ethical approval for the study was obtained through the University of Birmingham's Science, Technology, Engineering and Mathematics Ethical Review Committee.

Experimental design

The study assessed cyclists undertaking the Tommy Godwin cycling sportive over a 100 km course. Participants were asked to complete a 'Personal Information Sheet' (Appendix 4) which included the questions: how many years have you cycled for? On average how many hours do you cycle per week? How many cycle sportives have you completed, prior to the current one? What is the reason for participating in the Tommy

Godwin Challenge sportive? Do you currently consume any vitamin or antioxidant supplement? If yes, how long have you taken them for? What vitamin and/or antioxidant supplement do you take? Participants were also asked whether they felt their training had adequately prepared themselves for the event. At least 30 minutes before the start of the sportive, and within 10 minutes of completing the race, a blood sample was taken from an anti-cubital vein in the forearm. The participants' finishing time was also recorded. There were no restrictions placed on participants during the race with regard to riding speed, or consumption of food or drink. The event took place on Sunday 28th September 2014 and began at 9.00am from the Marie Curie Hospice, Solihull. The sportive took place on open countryside roads around the county of Warwickshire. The temperature fluctuated between 17^{o}C and 19^{o}C with a relative humidity between 50% and 60%.

Blood sampling

Plasma was obtained by centrifuging whole blood (5 mL) in a portable centrifuge (E8F Portafuge; LW Scientific, Lawrenceville, GA) at 1350 *g* for 15 minutes, then aliquoted and placed on dry ice. Whole blood (200μL) to allow assessment of GSH was immediately placed on dry ice. The samples were kept in dry ice until they were transferred to -80^{o}C freezers.

Methodology for assessing oxidative stress markers

Total antioxidant status assessed via the ferric reducing ability of plasma (FRAP) assay

The protocol has been adapted from the methodology previously used by Benzie and Strain., (1994). A FRAP reagent consisting of a 300 mM sodium acetate buffer (3 g/L sodium

acetate, 16 mL/L acetic acid), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl, and 20 mM ferric chloride; were added together in a 10:1:1 ratio immediately before use. Samples were diluted 1:1 with dH_2O and 10 µL was loaded into each well in triplicate. Standards were prepared using ascorbic acid (0 µM to 1000 µM) and 10 µL was loaded on to a 98 well plate in triplicate. The FRAP reagent, 300 µL, was then added to the plates and incubated for 8 minutes at room temperature. The absorbance was then read at a wavelength of 650 nm and a 6-point standard curve was generated providing a liner equation to convert the sample readings to their reducing power relative to ascorbic acid in µM. The intra- and inter-plate coefficients of variation were 4.1% and 11.1% respectively.

Lipid peroxidation assessed via the malonaldialdehyde (MDA) assay

The TBARs assay has been adapted from Kasielski & Nowak., 2001. A 100 µL of trichloroacetic (TCA) solution containing 0.05 M sulphuric acid and 1.23 M TCA (2:1) was added 1:1 to all samples in 2 mL tubes. All solutions were mixed thoroughly using a vortex. The colour/working reagent containing thiobarbituric acid (TBA = 0.067 g in 10 mL dH_2O, diluted 1:1 with acetic acid before use), deionised water, and 200 mM butylated hydroxytoluene in ethanol were added together in a ratio of 60:240:1 respectively. Eight hundred microliters (800 µL) was added to the TCA solution containing the samples and standards. All solutions were mixed thoroughly using a vortex. The lids were then tightly fastened and left in a water bath at 100 ^oC for 60 minutes. All tubes were then removed and immediately placed on ice for 10 minutes. Once cooled the samples and standards underwent centrifugation at 2,000 *g* 4 ^oC for 10 minutes. The samples and standards (200 µL) were loaded onto 98 well plates in triplicate with the absorbance read at 540 nm. Standards containing tetramethoxypropane (0 µM to 50 µM) were used to generate an 8-point standard

to allow the samples to be converted into μM of MDA. The intra- and inter-plate coefficients of variation were 4.9% and 8.5% respectively.

Protein carbonyl adducts measured via a protein carbonyl ELISA and the protein content of the plasma assessed by the bicinchoninic acid (BCA) assay

Samples and standards underwent a serial dilution in a sodium carbonate buffer (50 mM, pH 9.2) and 50 μL was then added to the wells of a Maxisorb plate and incubated for 1 hour at room temperature. The plate was then washed with a Tris buffered saline solution with 0.05% Tween (TBST (0.05%)) (50 mM Tris Base, 150 mM sodium chloride, 0.05% TWEEN 20) and then 50 μL of 1 mM of 2,4-dinitrophenylhydrazine (DNPH) was then added to the plate. The plate with gentle agitation was left for 1 hour at room temperature and washed with TBST (0.05%). TBS blocking buffer (50 mM Tris Base, 150 mM sodium chloride, 0.1% TWEEN 20) (200 μL) was then added to the plate and incubated overnight at 4 ^{o}C with gentle agitation. After overnight incubation, the plate was washed with TBST (0.05%) and 50 μL of anti-DNP antiserum (diluted 1:1000 in TBST blocking buffer) was added to the plate for 2 hours at room temperature. The plate was then washed with TBST (0.05%). A peroxidise labelled secondary antibody (diluted 1:5000 in TBST blocking buffer) was added to each plate (50 μL) and left to incubate for 1 hour at room temperature. The plate was then washed with TBST (0.05%) and 50 μL of citrate phosphate buffer (109 mM citric acid and 251 mM disodium phosphate 49:51, pH 5.0; add 8 μL of H_2O_2 and one 1-O-phenyliamine tablet to 10 mL of citric acid and disodium phosphate solution). The plate was then incubated in the dark for 30 minutes and a 2 M sulphuric acid stop solution was added to the plate. The absorbance was then read at a wavelength of 490 nm. A six-point standard curve was generated using know carbonyl concentrations ranging from 0.65 nM/mg to 3.75

nM/mg. All plasma PC values are expressed as nM/mg. The intra- and inter-plate coefficients of variation were 6.8% and 8.6% respectively.

To determine plasma protein concentrations of the samples a bicinchoninic acid assay (BCA) was used. Plasma samples were diluted 1:200 in ddH_2O and mixed using a vortex. Standards for the assay were comprised of bovine serum albumin (BSA) with a concentration range of 0 to 10 μg/μL. Plates were loaded with 10 μL of samples and standards in triplicate and stored at 4 ^oC until needed. The working solution contained BCA solution and copper sulphate solution in a ratio of 50:1 respectively. The working solution was then added to the plate, 200 μL, and incubated in the dark for 30 minutes at room temperature. The absorbance was read at a wavelength of 540 nm to determine the protein concentration of the plasma samples.

Reduced Glutathione

To determine the change of reduced glutathione (GSH) a commercial available glutathione assay kit was used (GSH-Glo™ Glutathione Assay, Promega, PN: V6911). GSH in each sample was determined through the conversion of a luciferin derivative to luciferin, which is catalysed by glutathione-S-transferase and is reliant on the presence of GSH for the reaction to occur. The amount of GSH present in the sample is determined by the reaction of a luciferase reagent, with the resulting luminescence being proportional to the total GSH pool in the sample. In brief, samples were diluted 1:5 with tricine (50 mM, pH 7.9), incubated on ice for 15 minutes then centrifuged at 10,000 g, 4 ^oC for 15 minutes. The supernatant was diluted 1:15 with ddH_2O and10μL was added to each well in duplicate (Corning® 96 Well Flat Clear Bottom White Polystyrene TC-Treated Microplates, PN: #3610). A GSH-Glo reagent, 100 μL, was added to each sample, mixed on a plate shaker and incubated for 30

minutes at room temperature. A luciferin detection agent, 100 μL, was added to each well and incubated for 15 minutes and the luminescence was then read. To determine the concentration of the samples, the average luminescence of a negative control was subtracted from the luminescence readings of each sample, and GSH concentrations were obtained through a linear-equation generated by an 8-point standard curve with values ranging from 0 μM to 5 μM GSH. The final values are reported as μM of GSH. The intra- and inter-plate coefficients of variation were 4.6% and 2.4% respectively.

Statistical Analysis

All data was analysed using the statistical analysis software package for social sciences (SPSS Version 22 for Windows, Chicago, IL). Group differences in baseline characteristics were analysed using independent t-tests, with equal variances assumed. Changes in oxidative stress markers were analysed using a group (Supplemented v Non-Supplemented) by time (Pre, Post) mixed-design analysis of variance (ANOVA), with Bonferroni correction. Within-subject (time) effects of equal variance was tested using Mauchley's Test of Sphericity, if the assumption of equal variance was not met, within-subject effects were subsequently taken from Greenhouse Geisser corrections. Between-subject (supplementation) effects of equal variance was tested using Levene's Test of Equal Variance, if the assumption of equal variances was not met, data was checked for normal distribution and data sets that were not normally distributed were log transformed before undertaking any statistical analysis. Post hoc analysis of the interaction effects was performed by a test of simple effects by pairwise comparisons (with Bonferroni correction). All values are means ± standard deviation (SD) unless otherwise stated, with the level of significance set at $p \leq 0.05$.

3.4 Results

Participant characteristics

The average age of the participants was 51.3 ± 8.6 (males = 51.4 ± 9.4, females 50.7 ± 3.8), the total amount of time spent cycling on a weekly basis was estimated at 6.6 ± 2.9 hrs (males = 6.9 ± 2.9, females 5.3 ± 3.1), the number of previous sportives undertaken was 4.2 ± 4 (males = 3.8 ± 4, females = 6 ± 4), and the overall time in minutes to complete the 100 km sportive was 287.1 ± 54.6 (males = 277.4 ± 54, females = 335.3 ± 27.6).

Reasons for undertaking the event

To develop an understanding on why the participants chose to participate in a non-competitive cycling event each participant was asked the question "What is the reason for participating in the Tommy Godwin Challenge sportive?" The responses given from the participants were: to raise money for charity, improve fitness, keep fit, pleasure, enjoyment, support a friend, undertake a longer distance ride, socialise, and complete first organised cycling event.

Supplemented and non-supplemented groups

The supplemented group consisted of 7 participants (males: n = 6, females: n = 1), and the non-supplemented group 11 participants (males: n = 9, females: n = 2). The average age of the supplemented groups was 53.4 ± 3.5 (males = 54.2 ± 3.2, female = 49 ± 0.0), and the non-supplemented group 49.9 ± 10.6 (males = 49.5 ± 11.9, females = 51.5 ± 4.9). The average number of hours cycled in a week for the supplemented group was 7.6 ± 2.4 (males =

7.8 ± 2.6, females = 6.0 ± 0), and the non-supplemented 6.0 ± 3.1 (males = 6.2 ± 3.1, females = 5.0 ± 4.2). The average number of sportives undertook by the supplemented groups was 6.1 ± 4.6 (males = 5.5 ± 4.7, females = 10 ± 0.0), and the non-supplemented group 2.9 ± 3.2 (males = 2.7 ± 3.3, females = 4.0 ± 2.8). The average finishing time for the supplemented group was 281 ± 35.4 minutes (males = 277.3 ± 36.5, females = 309.0 ± 0.0) and the non-supplemented group was 290.3 ± 65.5 (males = 277.4 ± 65.4, females = 348.5 ± 21.9). There were no significant differences between the groups for age, amount of hours cycles in a week, number of sportives undertook, or finishing times (Table 3.1).

Table 3.1 Data relating to the group differences between the supplemented and non-supplemented groups for age, cycling history, and finishing time on the day of the sportive.

	Supplemented Group (n = 7: m = 6, f = 1)	**Non-Supplemented Group (n = 11: m = 9, f = 2)**	***p*-value**
Age (yrs)	53.4 ± 3.5	49.9 ± 10.6	0.415
Weekly Cycling (hrs)	7.6 ± 2.4	6.0 ± 3.1	0.277
Finishing Time (mins)	281 ± 35.4	290.3 ± 65.5	0.758
Previous Sportives	6.1 ± 4.6	2.9 ± 3.2	0.095

Antioxidant supplementation type and frequency

The types of antioxidant supplements consumed by the participants in the supplemented group were: multivitamins and vitamin C. With duration of supplementation ranging from 1 month to 120 months (10 years) and the average duration of consumption being 27.6 ± 42.3 months. The frequency of antioxidant supplementation ranged from daily consumption to consumption once a week: daily consumption (n = 5), 3 times a week (n = 1), and on a weekly basis (n = 1).

Influence of exercise on markers of oxidative stress

There were no main effects for time ($F_{(1, 16)} = 2.45$, $p = 0.137$, $\eta^2 = 0.133$), group ($F_{(1, 16)} = 1.120$, $p = 0.306$, $\eta^2 = 0.065$), or interaction ($F_{(1, 16)} = 2.31$, $p = 0.148$, $\eta^2 = 0.126$), in MDA throughout the study (Figure.3.1). Furthermore, there were no main effects for time ($F_{(1, 16)} = 0.062$, $p = 0.806$, $\eta^2 = 0.004$), group ($F_{(1, 16)} = 2.893$, $p = 0.108$, $\eta^2 = 0.153$), or interaction ($F_{(1, 16)} = 1.98$, $p = 0.179$, $\eta^2 = 0.110$), in PC throughout the study (Figure 3.2).

Influence of exercise on antioxidant status

There was a significant effect for time in TAC ($F_{(1, 16)} = 42.70$, $p < 0.001$, $\eta^2 = 0.727$); but there were no group ($F_{(1, 16)} = 0.639$, p = 0.436, $\eta^2 = 0.038$) or interaction effects ($F_{(1, 16)} = 0.146$, $p = 0.708$, $\eta^2 = 0.009$). Post-hoc analysis revealed TAC significantly increased post-sportive in the supplemented (baseline: M = 532.4 ± 106.6 μM vs end of sportive: M = 648.2 ± 141.8 μM) and non-supplemented group (baseline: M = 483.6 ± 77.6 μM vs end of sportive: M = 614.9 ± 123.4 μM) (Figure 3.3).

There was a trend for an effect of time in GSH ($F_{(1, 16)} = 3.49$, $p = 0.08$, $\eta^2 = 0.179$), with no significant group ($F_{(1, 16)} = 0.796$, p = 0.385, $\eta^2 = 0.035$) or interaction effects ($F_{(1, 16)} = 0.160$, $p = 0.694$, $\eta^2 = 0.01$). Post-hoc analysis revealed GSH concentrations declined post-sportive in the supplemented (baseline: M = 30.2 ± 10.8 μM vs end of sportive: M = 26.1 ± 16.6 μM) and non-supplemented groups (baseline: M = 36.5 μM ± 13.5 vs end of sportive: M = 30.1 μM ± 12.3) (Figure 3.4).

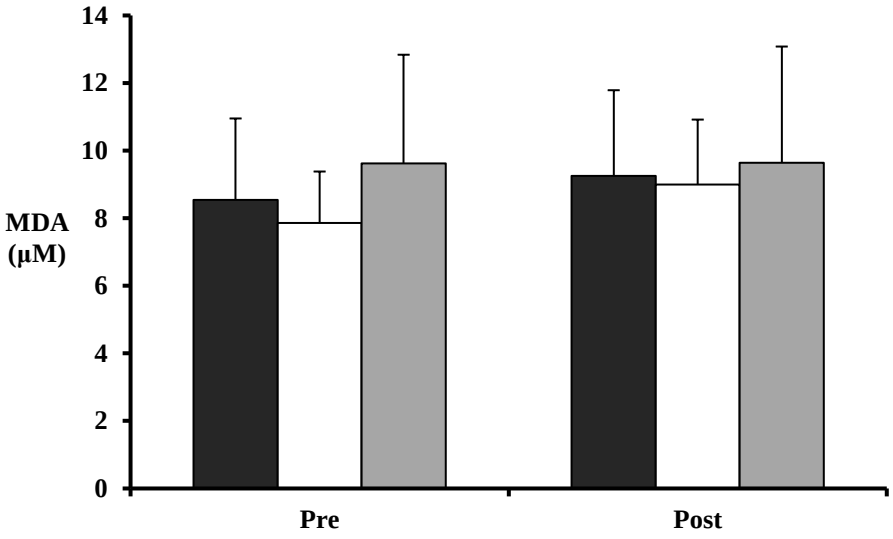


Figure 3.1 The change in MDA concentrations before and upon completion of the 100 km cycling sportive for all cyclists (black), non-supplemented cyclists (white) and supplemented cyclists (grey). Bars represent mean values with SD.

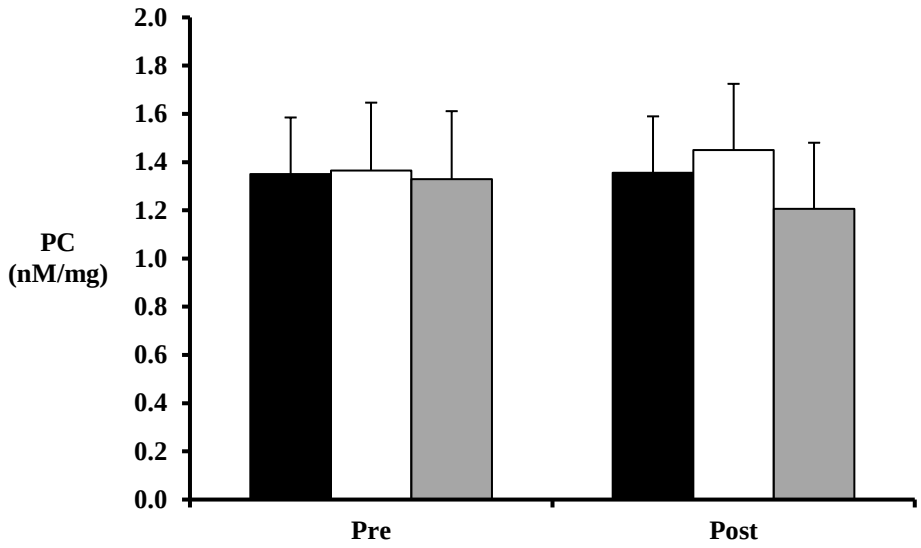


Figure 3.2 The change in PC concentrations before and at the end of the 100 km cycling sportive for all cyclists (black), non-supplemented cyclists (white) and supplemented cyclists (grey). Bars represent mean values with ± SD.

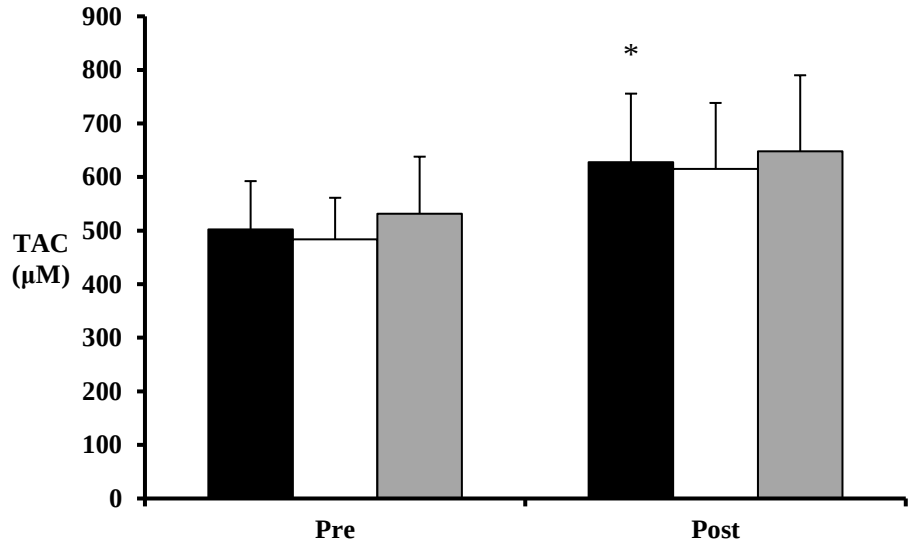


Figure 3.3 The change in total antioxidant capacity (TAC) concentrations before and at the end of the 100 km cycling sportive for all cyclists (black), non-supplemented cyclists (white) and supplemented cyclists (grey). Bars represent mean values ± SD. * indicates $p < 0.001$ compared to baseline concentrations.

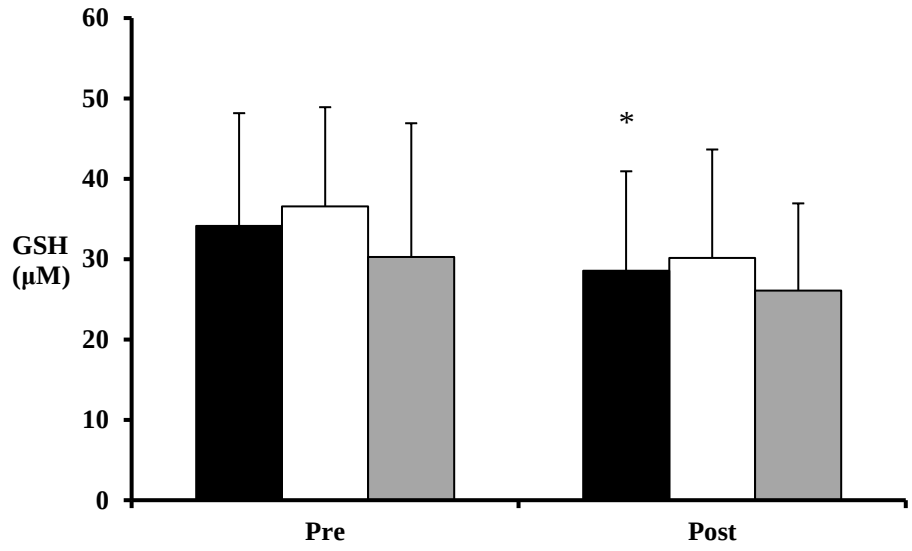


Figure 3.4 The change in GSH concentrations before and at the end of the 100 km cycling sportive for all cyclists (black), non-supplemented cyclists (white) and supplemented cyclists (grey). Bars represent mean values ± SD. * indicates $p = 0.08$ compared to baseline concentrations.

3.5 Discussion

To our knowledge, this is the first study to observe the oxidative stress response in non-competitive cyclists after completing a 100 km cycling sportive, and to assess the effects of habitual antioxidant supplementation on this response. TAC increased and GSH decreased in cyclists following the sportive, irrespective of supplementation. However, upon completion of the sportive there was a trend for the non-supplemented group to have higher concentration of PC compared to the supplemented group post-exercise, and the direction of change in PC was dependent on supplementation status. MDA increased from baseline in the non-supplemented group, but remained unchanged in the supplemented group. There were no significant differences between the groups at baseline or following the sportive for any of the measures assessed. Participants reported different reasons for participating in the event, primarily social (raise money for charity, support a friend, socialise) and emotional (pleasure, enjoyment) rationales.

Chapter 2 provided information on the type, frequency, and duration of antioxidant supplementation in cyclists undertaking a mass participation cycling event, the Wiggle Dragon Ride. The findings with the present study are in agreement with the findings from chapter 2. Both studies indicate that those who undertake sportive type events and consume antioxidant supplements have done so for a period ≥ 1 year, are likely to consume them daily, and the most common supplement is multivitamins containing antioxidants or specific individual supplements such as vitamin C.

The increase in TAC measured in both groups indicated that the cycling sportive was a sufficient stress to stimulate an antioxidant response, and is in agreement with experimental (Bloomer et al., 2006; Theodorou et al., 2011) and observational (Neubauer et al., 2008; Skenderi et al., 2008) studies. Studies supplementing participants with antioxidant

compounds generally indicate greater antioxidant concentrations in plasma compared to control groups (Bloomer et al., 2005; Cumming et al., 2014; Howatson et al., 2010), so it would be expected that a group of individuals who habitually consume an antioxidant supplement would have a higher baseline TAC than those who do not. The results presented herein show the supplemented group had a higher TAC than the non-supplemented group at baseline, but this was not significant. A possible explanation for this is the dose of the supplement. Research studies elevate the circulating levels of antioxidants above baseline concentrations by administering doses above the recommend intake values (Bloomer et al., 2005; Cumming et al., 2014). However, commercially available antioxidant supplements may not contain the higher doses administered in experimental research and, by definition, commercially available supplements contain antioxidants in a dose that is more in line with the daily recommended allowance. Thus, consumption of commercially available antioxidants may not replicate the plasma antioxidant concentrations found in experimental laboratory studies. Because of the observational nature of the study, and thus no dietary control measures put in place, the non-supplemented group may have consumed foods containing antioxidant compounds in the hours or days leading up to the sportive. A combination of these two factors could potentially explain the results for TAC in the present study.

As previously described, GSH is the most abundant endogenous antioxidant in the body. In previous studies, the concentration of GSH at the end of exercise has been reported to not change (Neubauer et al., 2008; Skenderi et al., 2008) or to initially increase post-exercise with reductions occurring in the hours (Bloomer et al., 2005) or days (Machefer et al., 2004; Turner et al., 2011) post-exercise. In contrast to these findings, the present study found a group trend ($p = 0.08$) for a reduction in GSH at the end of exercise. The conversion of oxidised glutathione (GSSG) back to GSH may be inhibited in the current study population

through a reduction in the enzyme glutathione reductase. Cobley et al., (2014) reported post-exercise decreases in the endogenous antioxidant proteins in old trained and untrained individuals after completing a high-intensity aerobic protocol. Considering the participants in the current study had an average age of 50 and undertaking the sportive for social related reasons, it could be possible that cellular/endogenous antioxidant senescence is present in these individuals, compromising the GSH recycling machinery.

Supplementation status did not affect the directional change in TAC and GSH at the end of the sportive between the two groups. However, the supplemented group displayed a reduction in PC and maintenance of MDA, whereas, the non-supplemented group displayed increased PC and MDA. Nevertheless, without being able to detect a difference in TAC it is difficult to comment on the effects of supplementation on the oxidative stress response caused by the sportive. Some caution should be used when interpreting the data for MDA. As discussed in section 1.4.6, the TBARS assay can artificially elevate levels of MDA through boiling the samples and the non-specific interactions of thiobarbituric acid (TBA) with aldehydes and proteins found in human plasma. However, there was no significant difference in MDA concentrations pre-or post-sportive between the groups to warrant further lipid peroxidation investigation. Previous research has indicated antioxidant supplementation can result in a lower concentration of PC at the end of an exercise bout compared to placebo (Bloomer et al., 2006; Goldfarb et al., 2011) but no studies have indicated antioxidant supplementation can reduce PC below baseline post-exercise. The mechanism responsible for the reduction in PC warrants further investigation. In this instance, there is no current evidence to support the role of antioxidant supplements reducing PC.

From the results, the supplemented group was better able to deal with the demand of the sportive, with no changes in MDA and a reduction in PC compared to baseline. Of course, other factors apart from supplementation may have caused this. The supplemented

group reported more cycling hours per week and reported completing a greater number of sportive events, potentially resulting in better adaptations to deal with the demands of the event. Although these factors were not significantly different between the groups it could be an indication as to why these differences existed.

In Chapter 2 habitual antioxidant supplementation was prevalent in recreational cyclists. One of the aims of this thesis was to explore the effect of habitual antioxidant supplementation on exercise-induced oxidative stress in a real-world setting. The results obtained in this chapter indicate antioxidant supplementation provided no greater attenuation in exercise-induced oxidative stress compared to a non-supplemented group in a cycling sportive event. The exercise-induced response to cycling was largely similar in supplemented and non-supplemented cyclists. This study was conducted in a real-world setting, and as such participants were a sample of free living individuals. This study did not seek to control any aspect of lifestyle in the participants, thus the results may not accurately reflect the true role of antioxidant supplements on exercise-induced oxidative stress in a controlled setting. Differences in: finishing times, cycling intensity, and fitness levels are some of the co-founding variables that could have affected the current results.

Moving forward, future research should focus on recruiting a subset of individuals who habitually consume antioxidant supplements, where co-founders such as exercise duration, exercise intensity, and participant fitness can be controlled. This would provide a more precise evaluation on the role of habitual antioxidant supplementation on exercise-induced oxidative stress.

Chapter Four

4. Habitual antioxidant supplementation does not affect resting levels of endogenous antioxidants or the oxidative stress response to steady-state cycling

4.1 Abstract

Introduction: Reactive oxygen and nitrogen species (RONS) are important exercise signalling molecules. Evidence suggests consumption of antioxidant supplements with exercise can attenuate RONS mediated signalling pathway, inhibiting endogenous antioxidant synthesis. The aim of the study was to investigate the effect of habitual antioxidant supplementation on resting endogenous antioxidants and oxidative stress during exercise.

Methods: Fourteen trained male cyclists either supplemented (Supp n = 4 mean ± SD: age 33 ± 2 yrs; $\dot{V}O_{2max}$ 56.2 ± 3.4 mL·kg^{-1}·min^{-1}) or non-supplemented (NoSup n = 10 mean ± SD: age 29.5 ± 1.8 yrs; $\dot{V}O_{2max}$ 59.6 ± 1.7 mL·kg^{-1}·min^{-1}) had a resting muscle biopsy and $\dot{V}O_{2max}$ test, then returned within 7 days to complete steady-state cycling for ~65 minutes (25 mins: 60% $\dot{V}O_{2max}$, 40 mins 80% $\dot{V}O_{2max}$, 95% $\dot{V}O_{2max}$ until fatigue (Post)). Blood samples were obtained pre, during, and post exercise to assess for changes in oxidative stress markers. Throughout the trial heart rate (HR), rate of perceived exertion (RPE), and $\dot{V}O_2$ were recorded at regular time points.

Results: There was no difference in HR, RPE, or $\dot{V}O_2$, between the groups at any time point. There was no difference in resting endogenous antioxidant protein concentrations of superoxide dismutase 1 or 2 (SOD1/2) or glutathione peroxidase-1 (GPx-1). Markers of oxidative stress assessed between the Supp and NoSup groups were not different at any individual time point during or following exercise. Total antioxidant capacity (TAC) increased significantly during exercise in NoSup ($p < 0.05$) and protein carbonylation decreased significantly during exercise in NoSup ($p < 0.05$). Malonaldialdehyde (MDA) decreased significantly post-exercise in both groups ($p < 0.05$).

Conclusion: Habitual antioxidant supplementation did not affect the resting endogenous antioxidant concentrations or compromise the exercise-induced oxidative stress in trained cyclists.

4.2 Introduction

In response to the transient increase in RONS that occurs in response to exercise, redox-sensitive transcription factors are activated resulting in increased gene transcription of endogenous antioxidants enzymes and their subsequent synthesis into functional proteins. Two redox-sensitive transcription factors have been associated with the *de novo* synthesis of endogenous antioxidants: nuclear factor – kappa β (NF-κβ) and activator protein – 1 (AP-1) (Hollander et al., 2001; Zhou et al., 2001). *In vitro* and *in vivo* studies have shown skeletal muscle exposed to pro-oxidants augments the binding of NF-κβ and AP-1 activation in the pursuing hours after exposure (Hollander et al., 2001; Zhou et al., 2001).

The induction of a pro-oxidant environment within skeletal muscle, as occurs following exercise, results in the activation of the redox-transcription factor NF-κβ through its upstream transcription factors extracellular-signal related kinase (ERK) and p38 mitogen activated protein kinase (p38-MAPK) (Ho et al., 2005); where AP-1 is activated through the combination of transcription factors c-jun and c-fos (Zhou et al., 2001). The majority of research investigating the transcriptional control of endogenous antioxidant has focused on the transcription factors regulating NF-κβ.

The activation of these signalling pathways is implicated in the synthesis of glutathione peroxidase-1 (GPx-1) (Zhou et al., 2001), Cu/Zn superoxide dismutase (SOD1) (Rojo et al 2001), and Mn superoxide dismutase (SOD2) (Hollander et al., 2001), in cell culture (Rojo et al., 2004; Zhou et al., 2001) and in animal models (Hollander et al., 2001), using a pro-oxidant stimulus or exercise, respectively. Human studies have supported these findings by demonstrating upregulation of the respective endogenous antioxidants during acute exercise completed until exhaustion and submaximal exercise (Berzosa et al., 2011;

Tauler et al., 2004), after consecutive days of exercise (Serrano et al., 2010), and in response to chronic aerobic (Shin et al., 2008) and resistance training (Parise et al., 2005).

The effect of using antioxidant supplements by individuals undertaking regular exercise has been debated for several years. Davies et al., (1982) was the first to demonstrate that exercise augmented markers of oxidative stress and this effect was enhanced in a group deficient in the antioxidant α-tocopherol. To date, the number of research studies reporting an effect on endogenous antioxidant enzymes, verses studies reporting no effect is equivocal. Several studies identified that antioxidant supplementation did not affect the upregulation of antioxidant enzymes in response to exercise training. These studies have been undertaken in animals and humans, indicating exercise combined with antioxidant supplementation did not have an inhibiting effect on the synthesis of SOD1, SOD2, and GPx-1 (Cumming et al., 2014; Higashida et al., 2011; Wadley and McConell, 2010; Yfanti et al., 2012). Furthermore, acute response studies conducted by Yfanti et al., (2012) and Cumming et al., (2014) indicated mRNA expression for SOD1 and SOD2 respectively, and GPx-1 was elevated compared to placebo after consuming vitamin C and vitamin E for a period of 16 (Yfanti et al., 2012) and 11 weeks (Cumming et al., 2014). However, this did not result in greater protein concentrations for the respective proteins at the end of the intervention period. Cumming et al., (2014) also indicated no differences in the family of proteins involved in the activation of the NF-κβ pathway: cytosolic NF-κβ p65, nuclear NF-κβ p65, and the inhibitory unit of NF-κβ cytosolic Iκ βα, responsible for the gene expression of SOD2 and GPx-1.

In contrast to these findings, a number of research studies have observed antioxidant supplements altered exercise-induced concentrations of SOD1, SOD2, and GPx-1 (Gomez-Cabrera et al., 2008; Morrison et al., 2015; Ristow et al., 2009). Several acute exercise studies indicate the administration of an antioxidant supplement, allopurinol, can reduce the activation of NF-κβ within lymphocytes (Gomez-Cabrera et al., 2006) and skeletal muscle

(Gomez-Cabrera et al., 2005), which resulted in a reduction in mRNA expression of SOD1 in skeletal muscle (Gomez-Cabrera et al., 2005). Although studies from Ristow et al., (2009) and Gomez-Cabrera et al., (2008) reported a reduction in mRNA expression of antioxidant enzymes, they did not indicate whether these differences affected the overall protein content of these enzymes. Morrison et al., (2015) found no differences in the mRNA expression of SOD1, SOD2, and GPx-1 after completing an acute bout of exercise, prior to and on completion of a 4-week high intensity training intervention. However, following an intervention period consisting of 8-weeks' supplementation with vitamin C (1000 mg/d) and vitamin E (400 IU/d), and 4 weeks high intensity interval training the protein level of SOD2, and total SOD activity were significantly higher in the placebo group.

All the aforementioned studies have used differing supplementation periods, from supplementation, for 1 day, on the day of the study (Gomez-Cabrera et al., 2006) to 16 weeks' supplementation (Yfanti et al., 2012). Although the latter study would provide useful information on longer term antioxidant supplementation, it is still only reflecting a small period of supplementation in an untrained cohort. The effects of chronic supplementation ($\geq$ 6 months) in trained individuals has not yet been addressed.

It has been postulated that the ingestion of antioxidant supplements in conjunction with exercise may have a beneficial effect in reducing exercise-induced oxidative stress. A number of studies have identified a reduction in exercise-induced oxidative stress markers after receiving antioxidant supplementation (Bloomer et al., 2006; Goldfarb et al., 2011; Gomez-Cabrera et al., 2006; Mastaloudis et al., 2004). Collectively these studies have demonstrated a reduction in malondialdehydes (MDA) (Goldfarb et al., 2011; Gomez-Cabrera et al., 2006), F_2- isoprostanes (Mastaloudis et al., 2004) and protein carbonyls (PC)

(Goldfarb et al., 2011). However, it is not entirely clear whether the reduction in exercise-induced oxidative stress has a negative impact on redox-sensitive signalling cascades.

Therefore, the purpose of the present study was to investigate the effect of habitual antioxidant supplementation, for a period longer than 6 months, on baseline concentrations of endogenous antioxidant enzymes in the muscle of well-trained cyclists. Furthermore, the study also sought to characterise the redox response, in supplemented and non-supplemented cyclists, to a submaximal exercise protocol.

4.3 Methods

Participants

Fourteen trained male cyclists (mean ± SD age, height, mass, body mass index (BMI), maximal power output, $\dot{V}O_{2max}$ was 30.5 ± 5.7 years; 1.84 ± 0.05 m; 76.5 ± 9.6 kg; 22.4 ± 2.1 kg/m^2; 354.2 ± 42.8 W_{max}; 58.6 ± 6.0 mL·kg^{-1}·min^{-1} respectively) were recruited to the study from local cycling clubs and distribution of recruitment flyers at cycling events in the West Midlands. Individuals who had regularly cycled for a period greater than 12 months, undertook 5 or more hours of cycling a week, aged between 18-35 years, non-smokers, accustomed to exhaustive cycling and/or strenuous cycling, had no history of metabolic, cardiovascular or autoimmune disease, and not consuming non-steroidal anti-inflammatory drugs (NSAID's) or any prescription medications that could potentially interfere with redox homoeostasis were invited to participate in the study. Participants who reported consuming vitamin supplements for a period greater than 6 months containing vitamin C and E, and those who do not consume any form of vitamin supplements were both invited to participate in the study. Four males (mean ± SD age, height, mass, body mass index (BMI), maximal power output, $\dot{V}O_{2max}$ was 33 ± 5 years; 1.84 ± 0.06 m; 77.6 ± 8.4 kg; 22.9 ± 1.2 kg/m^2; 351.9 ± 19.4 W_{max}; 58.3 ± 6.8 mL·kg^{-1}·min^{-1} respectively) were recruited to the supplemented group and 10 males (mean ± SD age, height, mass, body mass index (BMI), maximal power output, $\dot{V}O_{2max}$ was 29.5 ± 5.6 years; 1.84 ± 0.05 m; 76 ± 9.2 kg; 22.3 ± 2 kg/m^2; 355.1 ± 19.4 W_{max}; 59.6 ± 6.8 mL·kg^{-1}·min^{-1} respectively) were recruited for the non-supplemented group. Because the study was interested in the effects of habitual vitamin C and E supplementation on the oxidative stress response to exercise and resting levels of endogenous antioxidants, there was no minimal or maximal dose that would be excluded from the study. Before attending the Human Performance Laboratory (HPL), each participant was sent a

participant information sheet (Appendix 5) outlining the study objectives and contacted by a member of the research team to ensure they met the inclusion criteria and understood the study protocol. All participants completed and signed a consent form and a general health questionnaire prior to undertaking the study (Appendix 6 and 9). Ethical approval for the study was obtained through the Solihull NHS National Research Ethics Committee (West Midlands, UK).

Study Design

The study utilised an independent group design and required participants to attend the HPL on two separate occasions over a period of 7-14 days. Participants were asked to attend both visits in a rested (no exercise 48 hours prior to each visit) and a fasted state (no food or drinks except water for $\geq$ 8 hours). Visit 1 consisted of a resting muscle biopsy from the vastus lateralis, followed by aerobic profiling ($\dot{V}O_{2max}$, W_{max}). Visit 2 was conducted 7-14 days after visit 1 and required participants to undertake the main exercise protocol described below. Between visits 1 and 2, participants were asked to continue daily training and food consumption *ad libitum*, but to refrain from exercise 48 hours before attending visit 2. The supplemented participants were asked to carry on consuming their vitamin and/or antioxidant supplement throughout the study. Blood samples were collected throughout visit 2 for the analysis of systemic oxidative stress at baseline, during the exercise protocol (60% $\dot{V}O_{2max}$, 80% $\dot{V}O_{2max}$, Post-Exercise), and in the recovery period (30 minutes post-exercise) (Figure 4.1. provides a schematic of the study design).

Determination of $\dot{V}O_{2max}$ and maximal power output

Maximal oxygen consumption ($\dot{V}O_{2max}$) was undertaken after an overnight fast (10 to 12 hours) and in the morning to factor for circadian rhythms. Participants undertook a graded exercise test on a stationary magnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands) until voluntary fatigue. All tests started at 95 W with an increase in 35 W every three minutes. Participants cycled at a self-selected cadence during the test, the cycle ergometer set in hyperbolic mode allowing an independent cadence to be performed. Maximal power output (W_{max}) was calculated by multiplying the amount of time, in seconds, completed in the last stage (a), by the amount of W's within each stage, 35, divided by the total time of the stage in seconds, 180, and adding this to the power output completed in the previous stage (b) (eq.1)

Eq.1 $W_{max} = a*(35/180) + (b)$

For a participant who had completed 45 seconds (a) of work in the final stage, and the last completed stage was at 340W (b), their Wmax would be calculated as 348.7 W (eq.2)

Eq. 2 $W_{max} = 45*(35/180) + (340)$

$W_{max} = 45*(.194) + (340)$

$W_{max} = 348.7$ W

Throughout the test, participants wore a face mask (7450 V2; Hans Rudolph) to allow breath-by-breath respiratory measurements [oxygen uptake ($\dot{V}O_2$) and carbon dioxide production (CO_2)] to be recorded throughout the test with the use of an automated gas-analysis system (Oxycon Pro; CareFusion UK Ltd). Before each test, gas analysers were calibrated using known concentrations of O_2 and CO_2 (O_2 14.79% and CO_2 5.07%; BOC Gasses), and the volume transducer was calibrated manually with a 3-L bidirectional syringe

(Jaegar) according to the manufacturer's recommendations. Heart rate (Polar S610i; Polar Electro Ltd.) and rate of perceived exertion were measured in the last 30 seconds of each stage. The cessation of the test occurred when the participant could no longer maintain a cadence over 50 rpm's or voluntary fatigue had been reached. Maximal oxygen uptake ($\dot{V}O_{2max}$) was calculated from the highest rolling 60 second mean if two of the following conditions were met 1) heart rate was within 10% of their age predicted maximum (220 beats/minute minus age); 2) a respiratory exchange-ratio $\geq$ 1.1; 3) a plateau in $\dot{V}O_2$ (< 2 $mL \cdot kg^{-1} \cdot min^{-1}$) with a further increase in workload.

Exercise Protocol

Participants completed a graded cycling protocol for 105-110 minutes on visit 2. The cycling protocol was performed on a stationary magnetically braked cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands) and was a prolonged cycling task combining two intensities [moderate (60% $\dot{V}O_{2max}$); high (80-95% $\dot{V}O_{2max}$)] that have been reported to induce changes in systemic oxidative stress markers (Wadley et al., 2016a). Workloads for each intensity of cycling were calculated from data obtained in visit 1. A linear equation was calculated from maximal oxygen uptake ($mL \cdot min^{-1}$) and maximal watts (W) to estimate the power output required to achieve a workload of 60%, 80%, and 95% $\dot{V}O_{2max}$. The cycling protocol consisted of cycling at 60%, 80%, and 95% $\dot{V}O_{2max}$ for 25, 40 minutes, and until fatigue, respectively, lasting approximately 110 minutes (Figure 4.1). The cycle ergometer was set in hyperbolic mode to allow for self-selected cycling cadences. In the 7th minute of each 10-minute interval (7-10, 17-20, 27-30, 37-40, 47-50, 57-60) breath-by-breath respiratory measures [oxygen uptake ($\dot{V}O_2$) and carbon dioxide production (CO_2)] were collected to ensure the estimated $\dot{V}O_2$ level was being achieved. If the estimated $\dot{V}O_2$ was not

met, the wattage was adjusted ± 5% of the estimated workload. Heart rate (Polar S610i; Polar Electro Ltd.) and rate of perceived exertion (Borg Scale), was recorded in the last 30 seconds of each 5-minute period.

Assessment of total antioxidant capacity, lipid peroxidation, and protein carbonyls were assessed using the methods previously stated in section 3.3.

Muscle Biopsy Procedure

A lateral section of the vastus lateralis was chosen for the muscle biopsy site and a lidocaine (1%, B.Braun, Melsungen, Germany) injection was applied to the skin and facia of the muscle. An incision was then made and a muscle biopsy (~50-100 mg) was obtained using the suction modified Bergstrom muscle biopsy technique (Tarnopolsky et al., 2011). Each muscle biopsy was then dissected to remove any connective tissue and fat then immediately snap frozen in liquid nitrogen before being stored at -80 ^{o}C until further analysis.

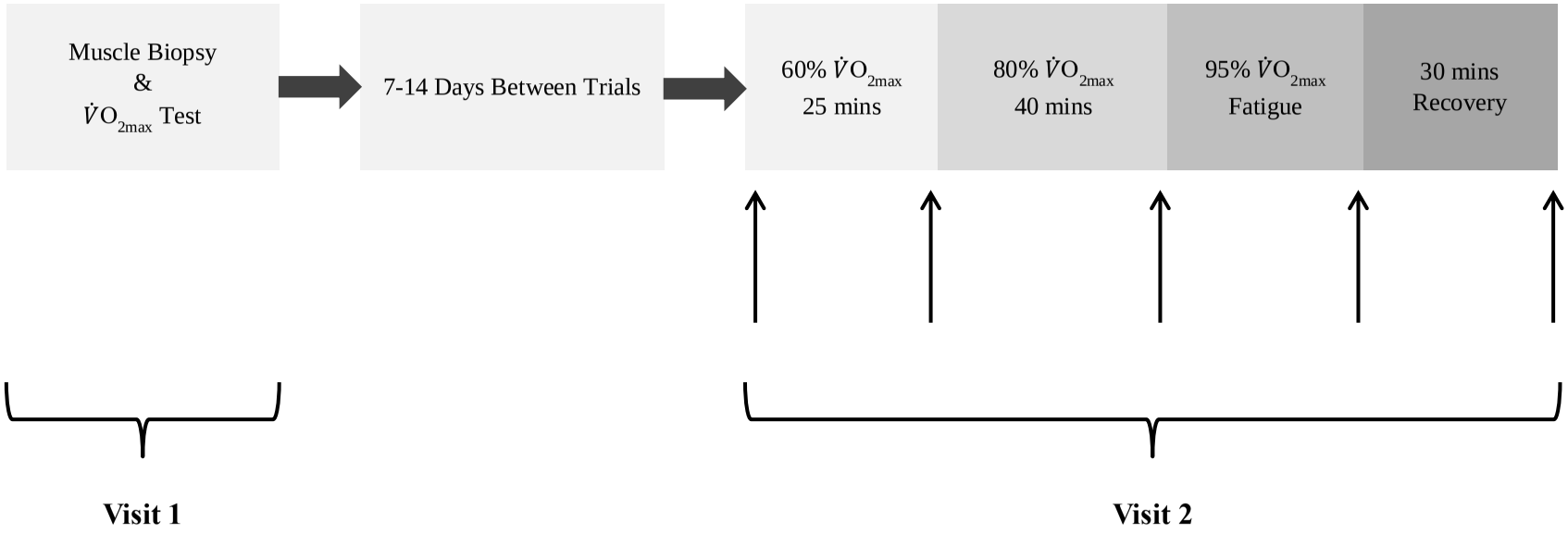


Figure 4.1 Participants attended the Human Performance Laboratory (HPL) to receive a muscle biopsy and undertake a cycling $\dot{V}O_{2max}$ test to determine workloads for the subsequent trial – visit 1. The participants returned to the HPL after 7-14 days to undertake steady state cycling at 60%, 80%, and 95% of $\dot{V}O_{2max}$ – with blood samples collected in the last minute of each stage – visit 2. Workloads were adjusted if $\dot{V}O_2$ drifted away from the calculated values. Arrows represent when blood was collected during the trials.

Muscle Homogenisation Procedure

Muscle tissue (~50 mg) was powdered on dry ice using a mortar and pestle. Approximately 50mg of crushed muscle was then homogenised using a Subcellular Protein Fractionation Kit (Thermo Scientific: 87790) to obtain extracellular and mitochondrial extracts. Tissue extracts were washed in ice-cold phosphate buffered saline (PBS: 0.1 M sodium phosphate, 0.15 M sodium chloride; pH 7.2) and placed in pre-chilled homogenization tubes. Thermo Scientific Halt Protease Inhibitor Cocktail (Pro No: 87786) was added to all buffers (1:100) before being used. To extract cytosolic muscle fractions ice-cold *Cytoplasmic Extraction Buffer* (CEB) (500 µL CEB for 50 mg of muscle extract) was added to chilled homogenization tubes and homogenised using a Douche handheld homogeniser for ~10-20 seconds. Upon homogenisation CEB solution was transferred into a Pierce Strainer placed in a pre-chilled 15 mL conical tube. The strainer was then centrifuged at 500 x *g* (4 ^{o}C) for 5 minutes. The supernatant, containing the cytosolic fraction, was immediately transferred into clean pre-chilled aliquots and stored at -80 ^{o}C. Ice-cold *Membrane Extraction Buffer* (MEB) (325 µL for 50 mg of muscle extract) was then added to the pellet to extract the membrane muscle fractions. MEB was mixed thoroughly with the pellet and then incubated for 10 minutes at 4 ^{o}C with gentle mixing. After the incubation period, the solution was centrifuged at 3000 x *g* (4 ^{o}C) for 5 minutes. The supernatant, containing the membrane extract, was immediately transferred into clean pre-chilled aliquots and stored at 80 ^{o}C.

Protein concentration of muscle fractions

Muscle fraction protein concentrations were determined using the RC DC Protein Assay (Bio-Rad, United Kingdom - Pro No: 5000122). In brief 25 µL of samples and standards were added to 125 µL of RC Reagent I (Bio Rad - Pro No: 5000117) and incubated for 1 minute at room temperature. 125 µL of RC Reagent II was then added to each tube and then centrifuged at 15,000 x *g* for 5 minutes. The supernatant was discarded after centrifugation and 127 µL of Reagent A (DC Reagent A and DC Reagent S 50:1) was added to each sample and standard until the precipitate had completely dissolved. DC Reagent B (1000 µL) was added to each sample and standard, and vortex immediately. After a 15-minute incubation period the absorbance was read at 750 nm using a spectrophotometer (BRAND - Model). An eight-point standard curve made up of protein concentrations from 0 mg/mL to 2 mg/mL of BSA was used to generate a linear regression equation to establish unknown protein concentrations of each sample.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Samples (20 mg/mL) containing laemili buffer with 5% mercaptoethanol (1:1) and molecular weight markers (20-30 µL) were loaded into stacking gels (0.5 M Tris Base, 0.4% w/v sodium dodecyl sulphate (SDS) pH 6.8, 30% w/v acrylamide 0.8% w/v bis-acrylamide, 10% ammonium persulphate solution (APS), N,N,N,N-tetramethyl-ethylenediamine (TEMED), ddH2O) on a 15% SDS resolving gel (15%) (1.5 M Trisma Base, 0.4% w/v SDS pH 8.4, 30% w/v acrylamide 0.8% w/v bis-acrylamide, 10% APS: 15 µL –TEMED, ddH_2O). Gels were then loaded into an electrophoresis rig with running buffer (25 mM Tris Base, 192 mM glycine, 0.1% SDS) and run for 60 minutes at 200 V. Gels were then transferred onto

nitrocellulose membranes (0.2 μm) and placed in a transfer unit with the addition of transfer buffer (25 mM Tris Base, 192 mM glycine, 20% methanol) for 60 minutes at 100 V. Ponceau S was added to membranes to test the transfer was effective and immediately washed with ddH_2O. Membranes were then washed 2 x 5 minutes with TBST (0.05%) (50 m M Tris Base, 150 mM sodium chloride, 0.05% TWEEN 20) and blocked for 60 minutes in TBST (0.05%) with 5% skimmed milk powder (Marvel Original dried skimmed milk powder – Republic of Ireland). Membranes were washed in TBST (0.05%) for 6 x 5 minutes and primary antibodies [anti-CuZn 1:1000 (millipore 07-403), MnSOD 1:1000(anti-SOD2 ab16956), and GPx-1 1:1000 (millipore ABN63)] were applied to the membranes and incubated at 4 ^{o}C overnight with gentle agitation. After the removal of the primary antibodies the membranes were washed 6 x 10 minutes with TBST (0.05%) and the secondary antibody [anti-Mouse IgG 1:7500 (Sigma A0168), and anti-rabbit 1:4000 (Sigma A6154)] was added for 60 minutes with gentle agitation. The membranes were then washed 6 x 10 minutes with TBST (0.05%) before a final wash with TBS (50 mM Tris Base, 150 mM sodium chloride). An enhanced chemiluminescence (ECL) (Optical ECL reagent, ab133406) was then applied to membranes with chemiluminesence signals detected using a G:BOX (Chemi-XR5, GeneSys, version 1.4.6.0) and quantified using G:BOX Syngene (Synopitics 5.0 MP) software.

Statistical Analysis

All data was analysed using the statistical analysis software package for social sciences (SPSS Version 22 for Windows, Chicago, IL). Group differences in bassline characteristics and physiological measures recorded during exercise were analysed using independent t-tests, with equal variances assumed. Changes in oxidative stress markers were

analysed using a group (Supplemented v Non-Supplemented) by time (Pre, 60% $\dot{V}O_{2max}$, 80% $\dot{V}O_{2max}$, 95% $\dot{V}O_{2max}$, Post) mixed-design analysis of variance (ANOVA), with Bonferroni correction. Within-subject (time) effects of equal variance was tested using Mauchley's Test of Sphericity, if the assumption of equal variance was not met, within-subject effects were subsequently taken from Greenhouse Geisser corrections. Between-subject (supplementation) effects of equal variance was tested using Levene's Test of Equal Variance, if the assumption of equal variances was not met, data was checked for normal distribution and data sets that were not normally distributed were log transformed before undertaking any statistical analysis. Post hoc analysis of the interaction effects was performed by a test of simple effects by pairwise comparisons (with Bonferroni correction). All values are means ± standard error (SEM) unless otherwise stated, with the level of significance set at $p \leq 0.05$.

4.4 Results

Participants

There were no significant differences between the supplemented and non-supplement group baseline characteristics for age, mass, height, body mass index, years cycled, weekly cycling hours, maximal power output, $\dot{V}O_{2max}$, or power to weight ratio (Table 4.1). The supplemented group on average consumed 153 mg/d (SD ± 72.7 mg/d) vitamin C and 73 IU/d (SD ± 47.2 IU/d) vitamin E, in the form of multi-vitamin supplement for a period of 3.1 years (SD ± 2.4 yrs).

Physiological parameters during steady state cycling at 60% $\dot{V}O_{2max}$ and 80% $\dot{V}O_{2max}$

The perturbations in heart rate, power output, rate of perceived exertion, and oxygen intake during steady-state cycling at 60% $\dot{V}O_{2max}$ and 80% $\dot{V}O_{2max}$ was not significantly different between the groups; furthermore, the predicted $\dot{V}O_2$ for each group during these periods was not statistically different (Table 4.2).

	Supplemented (n=4)	Non-Supplemented (n=10)	*p* - value
Age (years)	33 ± 4	29.5 ± 56	0.28
Height (m)	1.84 ± 0.06	1.84 ± 0.05	0.77
Weight (Kg)	77.6 ± 8.4	76.0 ± 9.2	0.92
BMI (kg/m^2)	22.9 ± 1.3	22.3 ± 2.1	0.59
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	56.2 ± 3.4	59.6 ± 1.7	0.34
Maximal Power Output (W_{max})	352 ± 19.4	355 ± 41.9	0.89
Watts per Kg (W/Kg)	4.5 ± 0.5	4.6 ± 0.5	0.85
Years Cycled (years)	5.2 ± 2.2	5.0 ± 2.1	0.84
Weekly Cycling (hours)	10.9 ± 3.6	10.7 ± 3.4	0.93

Table 4.1 Baseline demographic measurements of the supplemented and non-supplemented group for all baseline measurements. All means ± SD.

		Supplemented (n=4)	Non-Supplemented (n=10)	*p* – value
60% $\dot{V}O_{2max}$	Heart Rate (bpm)	132.5 ± 7.1	128.2 ± 1.5	0.59
	RPE	10.3 ± 0.9	10.8 ± 0.3	0.47
	Power Output (W)	182.2 ± 11.3	180.5 ± 7.0	0.89
	Actual $\dot{V}O_2$ (%)	61.7 ± 2.5	59.2 ± 0.9	0.25
	$\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	34.6 ± 1.7	35.4 ± 1.2	0.73
	$\dot{V}O_2$ ($mL \cdot min^{-1}$)	2666 ± 82.5	2680 ± 115	0.94
80% $\dot{V}O_{2max}$	Heart Rate (bpm)	163 ±11.5	162.2 ± 2.0	0.92
	RPE	15.7 ± 0.6	15.9 ± 0.4	0.84
	Power Output (W_{max})	251.5 ± 10.3	255.9 ± 9.2	0.79
	Actual $\dot{V}O_2$ (%)	81.5 ± 2.5	79.6 ± 1.0	0.41
	$\dot{V}O_2$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	45.5 ± 1.8	47.6 ± 1.7	0.49
	$\dot{V}O_2$ ($mL \cdot min^{-1}$)	3513 ± 71.9	3605 ± 158	0.73

Table 4.2 Exercise physiological measures during both steady-state cycling protocols at 60% $\dot{V}O_{2max}$ and 80% $\dot{V}O_{2max}$ for heart rate, power output, actual $\dot{V}O_2$ percentage, and $\dot{V}O_2$ $mL \cdot kg^{-1} \cdot min^{-1}$and $mL \cdot min^{-1}$.

Oxidative stress

Figure 4.2 shows the response of MDA, PC and Log10TAC at rest, during steady state exercise, and post-exercise in a supplemented and non-supplemented group. Throughout each time point and for each measurement there were no significant differences between the supplemented and non-supplemented group. During recovery MDA significantly reduced from 80% $\dot{V}O_{2max}$ ($p = 0.002$) and Post exercise ($p < 0.001$) to Post30 when both groups were combined. The supplemented group had a significant decrease in MDA from Post exercise to Post30 ($p = 0.035$) and a tendency for a reduction in MDA from 80% $\dot{V}O_{2max}$ to the same time point ($p = 0.058$). In the non-supplemented group, exercise significantly increased MDA from baseline to post exercise ($p = 0.034$). The non-supplemented group also had a reduction in MDA during recovery with significant reductions at Post30 compared to 80% $\dot{V}O_{2max}$ ($p = 0.002$) and Post exercise ($p < 0.001$). During exercise, there was a trend for PC to decrease during each exercise intensity and Post exercise, with a return towards baseline Post30. Exercise caused a significant decrease in PC from baseline to 80% $\dot{V}O_{2max}$ ($p = 0.015$) and there was a trend for a further reduction in PC post exercise ($p = 0.078$) compared to baseline when both groups were combined. In the non-supplemented group, exercise significantly reduced PC from baseline to 80% $\dot{V}O_{2max}$ ($p = 0.025$), but this was not replicated in the supplemented group (Figure.4.3.). An increase in exercise intensity caused a significant increase in TAC at 80% $\dot{V}O_{2max}$ ($p = 0.046$) and Post exercise ($p = 0.002$), which lasted into the recovery period, with TAC remaining significantly elevated at Post30 compared to baseline ($p = 0.009$) when the groups were combined. The same pattern emerged for the non-supplemented group with TAC significantly elevated at 80% $\dot{V}O_{2max}$ ($p = 0.031$), Post exercise ($p = 0.001$), and Post30 ($p = 0.008$). Although the same response was observed in the supplemented group, the large standard mean error at baseline may have prevented any significant differences being observed.

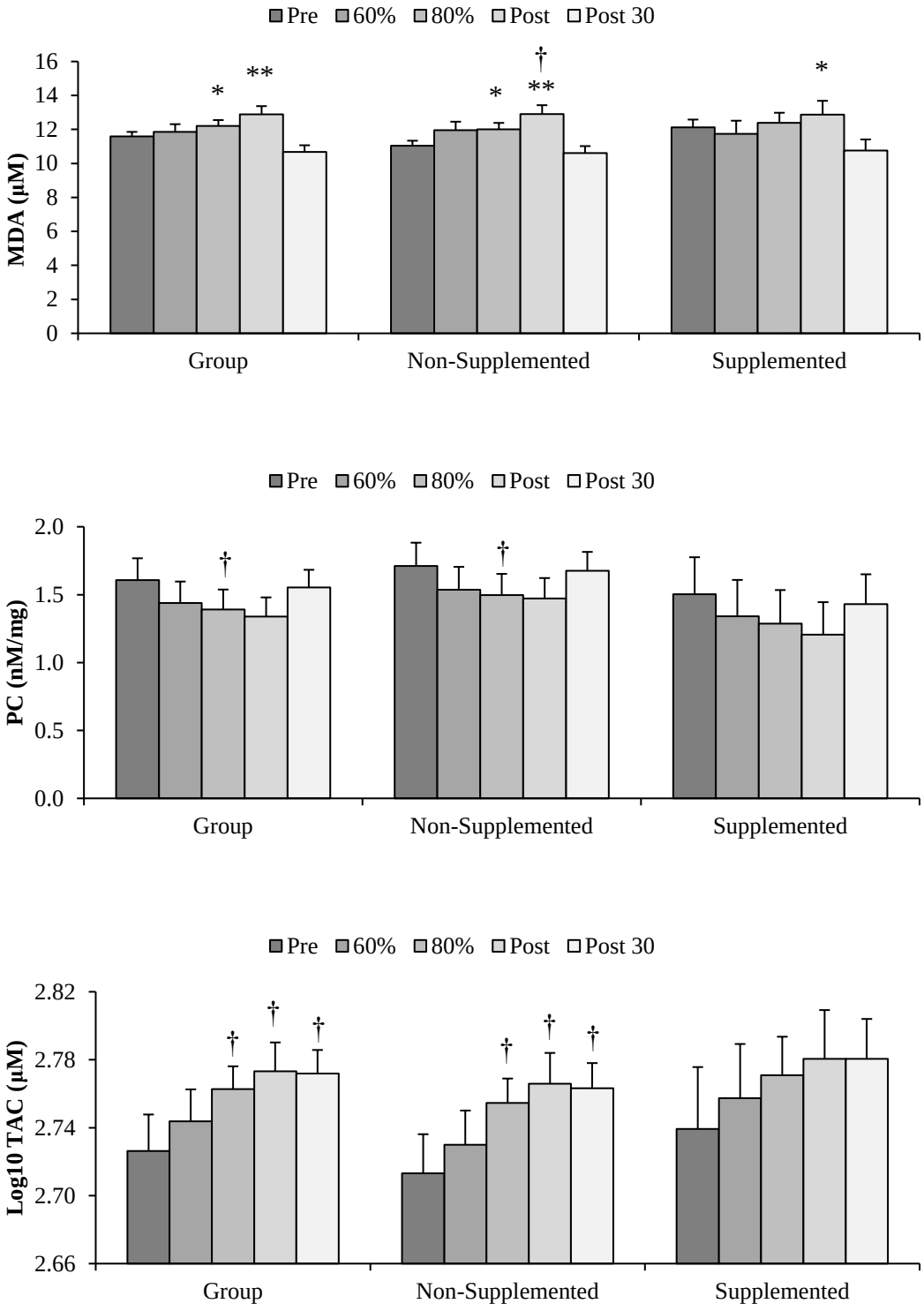


Figure 4.2 Changes in oxidative stress markers in response to steady-state exercise and recovery. Values are means ± SEM, * indicates $p < 0.05$ compared to Post30, ** indicates $p < 0.001$ compared to Post30, † indicates $p < 0.05$ compared to Pre

Muscle endogenous antioxidant concentrations

The skeletal muscle endogenous antioxidant concentration for SOD1 (p = 0.52), SOD2 (p = 0.728) and GPx-1 (p = 0.378) were not different between the non-supplemented and supplemented group (Figure 4.3).

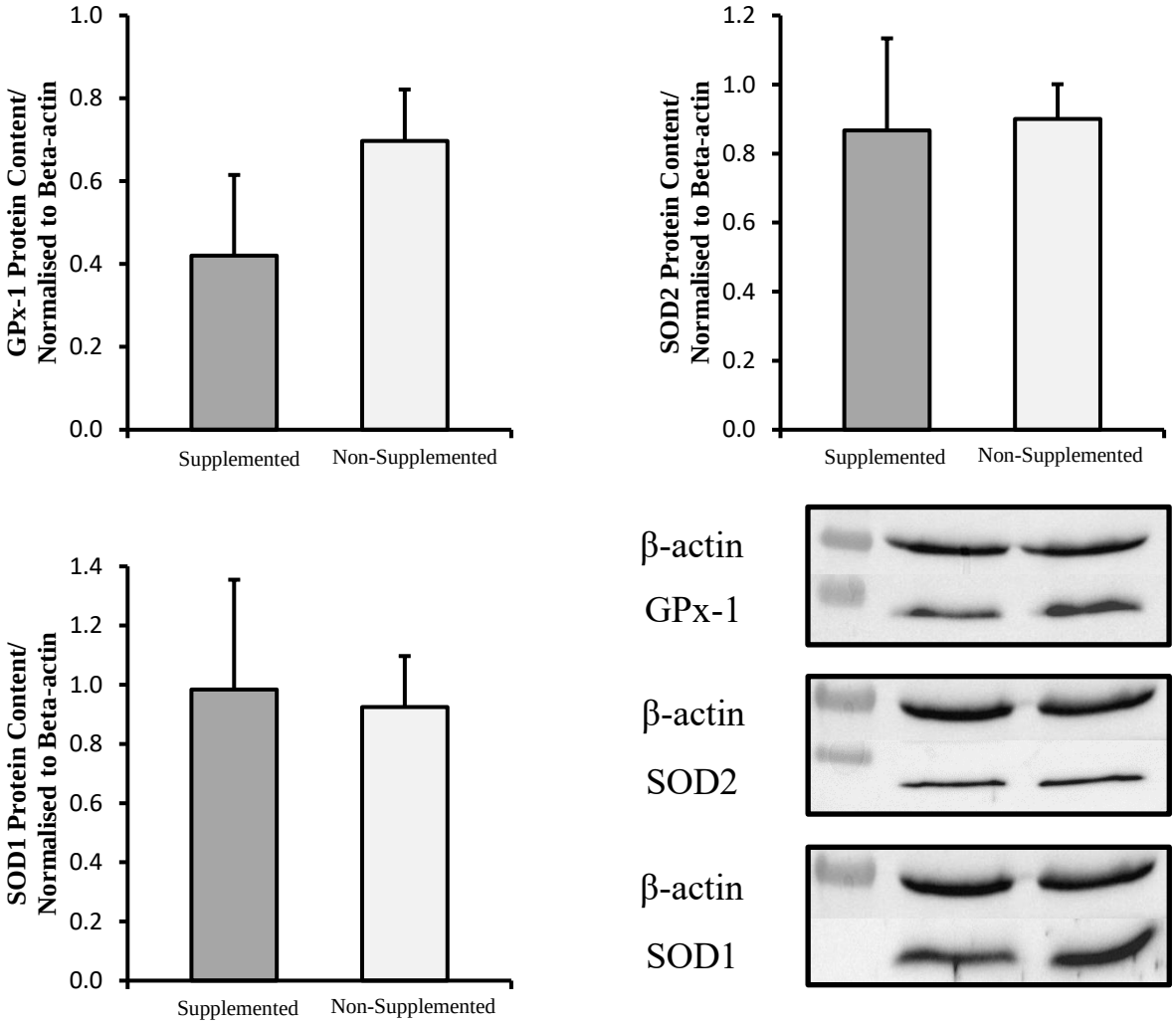


Figure 4.3 Mean averages (±SEM) for the superoxide dismutase-1 (SOD1), superoxide dismutase-2 (SOD2) and glutathione peroxidase-1 (GPx-1) in the supplemented (grey bars) and non-supplemented (light grey bars) groups at rest. Western blots depict pooled groups samples for both groups.

4.5 Discussion

The present study aimed to examine the effect of habitual antioxidant supplementation on the resting levels of the endogenous antioxidants SOD1, SOD2, and GPx-1 in trained cyclists. In addition, the study aimed to characterise the effect of supplementation on the oxidative stress response during steady state exercise. Resting levels of the endogenous antioxidants were not different (as assessed by protein content) between the supplemented and non-supplemented group. The oxidative stress response to steady state cycling at 60% $\dot{V}O_{2max}$, 80% $\dot{V}O_{2max}$, and Post-exercise was not different between the groups. MDA and TAC increased throughout exercise, with a tendency for a small reduction in TAC and a significant reduction in MDA during the recovery period, respectively. Exercise resulted in the reduction of PC compared to baseline values throughout each cycling bout, returning towards baseline values at the end of the recovery period.

The reduction in gene expression and protein content of endogenous antioxidants has been shown to be attenuated when exercise is combined with acute (Gomez-Cabrera et al., 2006) or prolonged (Gomez-Cabrera et al., 2008; Morrison et al., 2015; Ristow et al., 2009) antioxidant supplementation. Therefore, the current study wanted to examine the effects of habitual antioxidant supplementation on resting skeletal muscle endogenous antioxidant protein concentrations in trained cyclists. The current findings indicate there was no effect of habitual antioxidant supplementation on protein concentrations of the endogenous antioxidants GPx-1, SOD1, and SOD2 compared to a control group who did not consume antioxidant supplements. One main caveat within the current study is the low number of participants in the supplemented group. This may have impacted the statistical power of the results presented and therefore results are interpreted with caution. It was perhaps surprising that the current study was unable to recruit cyclists who habitually supplemented with antioxidants, given the results of the study presented in chapter 2. However, despite

recruitment being active for 2 years, the study was unable to recruit more cyclists who met the criteria and were supplementing antioxidants. Differences in TAC and PC may be particularly affected by participant number because of the large standard error mean in the supplementing group, indicating a high variation between individuals within this group.

The dose of supplements taken by the participants in the current study is also an important factor to consider when comparing the results presented herein with those of other studies. Previous studies have used either a dose of 1000 mg/d of vitamin C (Gomez-Cabrera et al., 2008) or a combination of 1000 mg/d of vitamin C and 400 IU of vitamin E (Ristow et al., 2009). The participants in the current study had an average daily intake of 153 mg/d of vitamin C and 74 IU of vitamin E, a tenth and a fifth respectively, lower than previous experimental studies. Although the average dose was considerably lower, the average duration of supplementation was greater than in all previous studies with an average supplementation intake of 3.1 years. Indicating, habitual low-dose antioxidant supplementation did not have a negative impact on resting endogenous antioxidant concentrations in individuals who undertake regular cycling in the current study. There is a need for more research in this area, employing lower doses of vitamin C and vitamin E to truly reflect the habitual intake of recreational athletes' and the general public to gain insight into the effect of habitual supplementation.

Habitual training volume and intensity is known to affect adaptation (Burgomaster et al., 2005; Holloszy, 2004; Jones and Carter, 2000). Yfanti et al., (2012) reported no significant changes in skeletal muscle content of SOD1, GPx-1, and catalase (CAT) in active men who were pre-supplemented for 4 weeks with vitamin C (500 mg/d) and vitamin E (400 IU/d), and continued this regime throughout a 12-week training protocol containing different cycling intensities. Cumming et al., (2014) reported similar results with no significant differences in GPx-1 and SOD2 in recreationally active males and females who consumed

vitamin C (1000 mg/d) and vitamin E (253 mg/d: 376 IU/d) during an 11-week training intervention that included a combination of different exercise intensities. It should be noted that even though both studies identified no differences between the groups in the respective interventions, one indicated GPx-1 decreased (Cumming et al., 2014) and the other an increase in GPx-1 (Yfanti et al., 2012). This may have been due to the differing mode of exercise used throughout training, i.e. running vs cycling.

In comparison, studies that have used a single exercise mode and intensity throughout an intervention period or not stipulated variations in the exercise session intensities have presented conflicting results. Ristow et al., (2009) who conducted a study combining resistance and aerobic training over a 4-week period in untrained and trained individuals reported decreased mRNA expression for SOD1, and GPx-1 in the trained group, and SOD2 in the untrained group when participants were supplemented with 1000 mg/d vitamin C and 400 IU/d vitamin E. Furthermore, significant changes were observed from baseline for SOD2 mRNA expression in the trained non-supplemented group and GPx-1 mRNA expression in the untrained non-supplemented group that were not observed in the supplemented group (Ristow et al., 2009). Morrison et al., (2015) reported similar findings when active males were supplemented for 8 weeks with 1000mg/d vitamin C and 400 IU/d vitamin E which included a high-intensity cycling intervention over the last 4 weeks. The protein content and activity of SOD1 was significantly increased after the intervention period in the non-supplemented group that was not replicated in the antioxidant group.

The findings of these studies taken together might imply that the variation in training mode and intensity may be an important factor when considering the effect of consuming antioxidant supplements on endogenous antioxidant capacity, and could explain why there was no difference in endogenous antioxidants in the current study. In the current study participants were trained cyclists, typically competing in time-trials, criterium races, and road

stages races; holding British Cycling race licenses. All participants had been involved with competitive cycling events for at least 12 months and undertook cycling as their main form of training. Thus, adaption to training would have arguably already occurred. When the training intensity is kept constant the addition of exogenous antioxidants to the endogenous antioxidant pool may be adequate to the deal with the repeated exercise stimuli. However, when training intensities are varied and provide differing exercise stimuli's the exogenous and endogenous systems may not be adequate to deal with such stimuli, resulting in adaptation in either condition.

The oxidative stress response between the two groups in the current study was not different, which is in agreement with previous exercise studies investigating the effects of antioxidant supplementation on markers of oxidative stress compared to control group (Bloomer et al., 2006; Theodorou et al., 2011). The response to steady-state exercise is in parallel to the results obtained in Chapter 5. The increasing exercise intensity and subsequent increase in the production of RONS is represented in the results by an increase in TAC and MDA. An interesting finding in the current study, that was also observed in Chapter 5 was the decrease in PC with exercise intensity. In the current study, PC decreased independent of supplementation that was also observed in Chapter 5, indicating either the exercise stimulus or training status of the participants may be responsible for these observed changes. Previous studies have also reported a reduction in PC upon completion of exercise (McGinnis et al., 2014; Wadley et al., 2015a), but the mechanism responsible for this is still unknown. It has been proposed by Wadley et al., (2016b) that it could potentially occur in response to increased PC clearance during exercise. However, further investigation is warranted to support this claim.

In addition to participant number, there are some notable limitations to the present study. Endogenous antioxidant enzyme content was only assessed at rest, and not following

an exercise bout. Thus, by not providing any information on redox-mediated signalling pathways post-exercise, it is difficult to interpret the effect of habitual antioxidant supplementation on these pathways. The supplementation dose was lower than that which has been used in previous studies investigating the effects of antioxidant supplementation on endogenous antioxidant adaptation. However, although the dose was lower, an aim of this thesis was to assess exercise and antioxidant supplementation in a "real world" setting, and thus habitual consumption at a dose selected by the cyclist was assessed.

Chapter 3 reported habitual supplementation did not attenuate exercise-induced oxidative stress compared to a non-supplemented group after a recreational cycling sportive. The work undertaken in the current chapter aimed to characterise the exercise-induced oxidative stress response to cycling in a controlled laboratory setting. In addition, the study addressed an aim of the thesis by establishing whether baseline concentrations of endogenous antioxidant enzymes were affected by habitual antioxidant supplementation. As discussed in Section 1.4.1, previous studies have primarily focused on short term supplementation (< 16 weeks) and supra-physiological doses (1000 mg/d vitamin C and 400 IU/d vitamin E), and it is not apparent whether habitual consumption of a commercially available antioxidant supplement can affect the concentration of endogenous antioxidant enzymes.

The results in the present study identified that self-reported habitual consumption of commercially available antioxidant supplements, for a period greater than 6 months, did not have a detrimental effect on resting skeletal muscle endogenous antioxidant enzyme concentrations in trained cyclists. Furthermore, supplementation did not attenuate markers of oxidative stress during steady-state cycling at 60% $\dot{V}O_{2max}$, 80% $\dot{V}O_{2max}$ or post-exercise compared to a non-supplemented group. These current findings indicate that habitual low-dose antioxidant supplementation did not provide additional antioxidant protection in well-trained cyclists who undertake 10 hours of cycling per week; and does not seem to negatively

affect the production of endogenous antioxidants, as has previously been observed. By recruiting free-living individuals who engage in regular exercise and choose to consume antioxidant supplements on a regular basis, this study provides additional information on the effects, or lack thereof, of antioxidant supplements in response to cycling.

Presently, the use of traditional antioxidants, such as vitamin C and E, have failed to alter the exercise-induced oxidative stress response in Chapters 3 and 4, or improve recovery or performance in other studies (Braakhuis, 2012; Braakhuis and Hopkins, 2015).There is a growing interest in the effects of polyphenol consumption (Bowtell et al., 2011; Connolly et al., 2006; Howatson et al., 2010). However, studies to date have not been conducted in an applied sport setting.

Chapter Five

5. Supplementation with a Montmorency Tart Cherry drink in trained cyclists does not reduce exercise-induced oxidative stress or improve subsequent days cycling time-trial performance: a placebo crossover design study

5.1 Abstract

Introduction: Montmorency Tart cherry (MC) supplementation can improve recovery after strenuous exercise by reducing exercise-induced inflammation, oxidative stress, and skeletal muscle damage. At present, it is not clear whether these changes can result in improved exercise performance in the days following strenuous exercise. The aim of this study was to investigate the effect of MC supplementation on recovery and performance 24 hours after strenuous excise in well-trained cyclists.

Methods: Eight male cyclists (mean ± SD: age 29.1 ± 5.3 yrs; $\dot{V}O_{2max}$ 57.7 ± 5.0 $mL \cdot kg^{-1} \cdot min^{-1}$) completed 2 cycling protocols separated by 24 hrs. Steady state cycling at 60% W_{max} , 80% W_{max} , and 95% W_{max} until fatigue, was followed by a time-trial (TT) after 24hrs. A placebo (PLA) and MC supplement was taken for 2 x day for 7 days before and on the day of the trials. In a cross-over design, participants undertook both arms of the trial separated by a 14-day washout. Blood samples were taken pre, during, and post exercise to assess oxidative stress and inflammatory markers.

Results: Throughout each exercise trial there were no differences between MC and PLA for protein carbonylation (PC), malonaldialdehyde (MDA), total antioxidant capacity (TAC) or interleukin-6 (IL-6). In response to steady-state cycling TAC ($p < 0.001$) and IL-6 ($p < 0.001$) significantly increased. During the TT, TAC ($p < 0.05$), IL-6 ($p < 0.05$), and MDA ($p < 0.05$) significantly increased throughout the trial. After 24 hrs recovery TAC ($p < 0.05$) and IL-6 ($p < 0.05$) decreased. MC supplementation did not improve TT performance compared to PLA (MC, 1605 secs ± 111; PLA 1609 secs ± 115).

Conclusion: These findings indicate 8 days of MC supplementation did not attenuate oxidative stress, nor augment antioxidant defences in trained cyclists compared to PLA. Furthermore, MC did not enhance recovery 24 hours after completing a strenuous cycling protocol or provide an ergogenic effect in subsequent TT performance.

5.2 Introduction

Many athletes consume antioxidants to allegedly alleviate the detrimental effects associated with strenuous exercise to potentially improve exercise performance and/or recovery. Thus, a great number of studies have investigated the effect of antioxidant supplementation on the physiological response to exercise. The consumption of traditional antioxidant compounds vitamin C and vitamin E has failed to improve exercise performance (Braakhuis, 2012; Braakhuis and Hopkins, 2015), or alleviate exercise-induced oxidative stress (Stepanyan et al., 2014; Theodorou et al., 2011) and inflammation (Davison and Gleeson, 2006; Davison and Gleeson, 2007). Therefore, attention has recently focused on polyphenol compounds, a sub-class of antioxidants that consist of several thousand bio-active compounds (Beecher, 2003; Manach et al., 2004) with the ability to inhibit oxidative stress (Mitjavila and Moreno, 2012; Graziani et al., 2005) and inflammation (Decendit et al., 2013; González et al., 2014). These compounds have been of interest to exercise scientists who are interested in potentially reducing exercise-induced oxidative stress and inflammation.

Cherries contain a combination of polyphenols consisting of phenolic acids (gallic acid, neochlorogenic acid, 3-coumaroylquinic acid, chlorogenic acid and ellagic acid), flavonols (quercetin, kaempferol, isoramnetin. and their glucosides), flavanols (catechin, epicatechin and procyanidins) and anthocyanins (malvidin, cyanidin, pelargonidin, peonidin, delphinidin, petunidin) (Blando et al., 2004; Howatson et al., 2010; Howatson et al., 2012; McCune et al., 2011). The compounds within cherries that have generated the greatest interest are the anthocyanin subclasses due to their anti-inflammatory and antioxidant effects (Decendit et al., 2013; González et al., 2014; McCune et al., 2011).

Exercise studies investigating the effectiveness of cherry supplementation have primarily used a Montmorecny cherry tart (MC) supplement to investigate its effectiveness in attenuating exercise-induced oxidative stress and inflammation in aerobic (Bell et al., 2014c; Bell et al., 2015; Howatson et al., 2010; Levers et al., 2016) and anaerobic (Bell et al., 2016; Bowtell et al., 2011; Levers et al., 2015) exercise. Findings from these studies are equivocal on the ability of MC to reduce exercise-induced oxidative stress, with some studies indicating a beneficial effect (Bell et al., 2014c; Bowtell et al., 2011; Howatson et al., 2010) and others reporting no-effect (Levers et al., 2015; Levers et al., 2016; McCormick et al., 2016). The positive interactions of MC initially seemed to be related to the attenuation in exercise-induced inflammation.

Several studies have reported a less-pronounced increase in the inflammatory marker interleukin-6 (IL-6) upon exercise cessation (Howatson et al., 2010) in the subsequent days (Bell et al., 2016; Bell et al., 2014c; Bell et al., 2015; Levers et al., 2016). However, contradictory findings have been reported with some studies indicating no-effect of supplementation on exercise-induced inflammation (Levers et al., 2015; McCormick et al., 2016). The positive effects of MC on exercise-induced oxidative stress and inflammation seem to be augmented in studies where low-phenolic diets are undertaken in the preceding 48 hours and in the recovery period of the trial (Bell et al., 2016; Bell et al., 2014c; Bell et al., 2015). However, these studies may not reflect the consumption habits of amateur or elite athletes'. There is a need for research which is perhaps more closely related to 'real'life'. Such research could focus on the effect of MC in free-living athletes where there are no dietary restrictions on polyphenol intake, and supplements are taken in addition to a balanced diet.

A well-established effect of consuming MC seems to be its ability to improve recovery and subsequent performance in the day(s) following strenuous resistance type or

weight bearing exercise. Numerous studies have identified a less pronounced attenuation in maximal voluntary contractions (MVC) (Bowtell et al., 2011; Connolly et al., 2006) and maximal voluntary isometric contractions (MVIC) (Bell et al., 2016; Bell et al., 2014c; Bell et al., 2015; Howatson et al., 2010) for periods up until 72 hours post-exercise (Bell et al., 2016; Bell et al., 2015; Connolly et al., 2006) after consumption of MC prior to strenuous exercise and in the recovery period. However, extrapolating these findings to applied sports settings could be erroneous at this present time. Bell et al., (2015) demonstrated that maintenance of MVIC in the recovery period did not subsequently improve performance in a 6-second maximal power output cycling bout compared to a control group who displayed reductions in MVIC. Furthermore, McCormick et al., (2016) reported no improvements in a battery of performance measures related to water-polo after a week of intensified training. In contrast to these findings, Bell et al., (2016) confirmed an effect of MC on subsequent days performance in soccer players, with less reduced declines in counter-movement jumps (CMJ) and 20 m sprints that was reflected by a less pronounced reduction in MVIC over the recovery period to the control group. To elucidate the effects of MC on improved recovery and the potential for this to allow for better performance on subsequent days, research should be conducted with applied sports performance measures, such as time-trials for runners and cyclists.

The aim of the present study is to explore the interaction of MC on exercise-induced oxidative stress and inflammation during a strenuous cycling bout and in the subsequent recovery period (24 hours) in free-living cyclists. The study aims to further determine whether improved recovery, indicated by an attenuation in markers of oxidative stress and inflammation, can improve subsequent days performance in a cycling time-trial.

5.3 Methods

Participants

Eight trained male cyclists were recruited to the study through advertisement at local cycling clubs and distribution of recruitment flyers at cycling events within the West Midlands area. Individuals who undertook 5 or more hours of cycling a week, had not consumed a vitamin and/or antioxidant supplement within the 30 days before undertaking the study, were non-smokers, and accustomed to exhaustive cycling were invited to participate in the study. Before being invited to the Human Performance Laboratory (HPL) to participate in the study, each participant was sent a participant information sheet (Appendix 7) outlining the study objectives and contacted by a member of the research team to ensure they met the inclusion criteria and understood the study protocol. All participants completed and signed a consent form and a general health questionnaire prior to undertaking the study (Appendix 8 and 9).

Experimental Design

The study was a counterbalanced, cross-over, single blind study. A counterbalance design was incorporated to allow for any learning effects of the study to be balanced and to control for any training improvements that may have occurred during the experimental period, a schematic diagram of the study design is shown in Figure 5.1. The initial visit to the HPL consisted of a fasted baseline blood sample being collected, with height and weight measurements recorded. The cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands) was set up to the participants required dimensions; saddle, handle bar height and

position were recorded to allow for identical bike set up for each trial. Thereafter, each participant undertook a $\dot{V}O_{2max}$ test to determine $\dot{V}O_2$ values and maximum power output (W_{max}). On completion of the $\dot{V}O_{2max}$ test each participant could recover *ad libitum* before undertaking a 30-minute time-trial familiarisation protocol, to control for any learning effects that could occur during subsequent time-trial (TT) performances.

On the 2nd visit to the HPL participants attended in a fasted state, except for consuming the experimental or placebo drink, and in a rested state – no exercise for a 48-hour period. Upon arrival a cannula (BD Vialon, 20GA, NJ, USA) was inserted in to an anti-cubital vein in the arm allowing for blood collections to be obtained at baseline (V1Pre, V2Pre, V3Pre), during the cycling trials (V260%, V280%, V360%), and post cycling (V2Post, V2Post30, V3Post). Participants then undertook an exhaustive cycling protocol, cycling for 60% W_{max} for 25 minutes, 80% W_{max} for 40 minutes, and 95% W_{max} until voluntary fatigue or unable to maintain a cadence of 50 revolutions per minute (RPM's). If the cadence dropped below 50 RPM's during the 80% W_{max} workload the watts were adjusted to allow the participant to complete this section of the protocol. An alteration in wattage was recorded and replicated in the subsequent trial. Blood samples were taken within the last 60 seconds of the 60% W_{max} (V260%) and 80% W_{max} (V280%) workloads, and at fatigue (V2Post). Participants were then asked to relax for 30 minutes and a post cycling blood sample was attained at 30 minutes (V2Post30).

Participants returned to the HPL for their 3rd visit the following morning in a fasted state, except for consuming the experimental or placebo drink. A cannula was inserted in to an anti-cubital vein in the arm to allow for a baseline blood sample to be collected and for further samples to be obtained during the trial. A 30-minute warm up at 60% W_{max} was completed by the participant before undertaking the time-trial protocol. Blood samples were

obtained at the end of the warm up period (V360%) and upon completion of the time trial (V3Post). Upon completing the 3rd visit a 14-day washout period was completed by each participant before returning to complete the 2nd and 3rd trials, 4th and 5th trials respectively after consumption of the opposing experimental drink. During each trial heart rate was recorded every 5 minutes for each workload except during the 95% W_{max}, and the TT where heart rate was recorded at predetermined time points related to total energy expenditure. Rate of perceived exertion (RPE) was recorded every 10 minutes during each workload, apart from during the 95% W_{max} workload and the TT. The time of the trials was between 7am and 9am. Each participant started their trials at the same time in the morning to avoid any circadian effects on cycling performance.

$\dot{V}O_{2max}$ test and time-trial

Maximal oxygen consumption ($\dot{V}O_{2max}$) and maximal power output (W_{max}) tests were undertaken after an overnight fast (10 to 12 hours) and in the morning to factor for circadian rhythms. Participants undertook a graded exercise test on a stationary cycle ergometer (Sport Excalibur, Lode, Groningen, Netherlands) until voluntary fatigue. All tests started at 95 W with an increase in 35 W every three minutes. Participants cycled at a self-selected cadence during the $\dot{V}O_{2max}$ and W_{max} test with the cycle ergometer set in hyperbolic mode allowing an independent cadence to be performed. W_{max} was calculated by multiplying the amount of time, in seconds, completed in the last stage (a), by the amount of W's within each stage, 35, divided by the total time of the stage in seconds, 180, and adding this to power output completed in the previous stage (b) (eq.1)

Eq.1 $W_{max} = a*(35/180) + (b)$

For a participant who had completed 45 seconds (a) of work in the final stage, and the last completed stage was at 340W (b), their W_{max} would be calculated as 348.7 W (eq.2)

Eq. 2 $W_{max} = 45*(35/180) + (340)$

$W_{max} = 45*(.194) + (340)$

$W_{max} = 348.7$ W

Throughout the test oxygen uptake ($\dot{V}O_2$), carbon dioxide production (CO_2) was continuously measured (Jaeger Oxycon Pro, Hoechberg, Germany); with heart rate (Polar S610i; Polar Electro Ltd.) and rate of perceived exertion measured in the last 30 seconds of each stage. The cessation of the test occurred when the participant could no longer maintain a cadence over 50 rpm's or voluntary fatigue had been reached. $\dot{V}O_{2max}$ was ascertained when $\dot{V}O_2$ did increase with increasing workload, and/or an RER of 1.05 had been achieved, and/or the participants' heart rate was within 10% of their age-predicted heart rate (220-Age).

The time-trial (TT) was designed for the participants to complete a set amount of work as fast as possible. The total amount of work to be carried out in kilojoules (KJ) during the TT was calculated through formulas used previously by Jeukendrup et al., (1996) and Currell and Jeukendrup, (2008), using desired estimated power output (75% W_{max}) and time of the TT (1800 secs) to determine the amount of KJ's required to simulate a 30-minute workload. The formula below was used to determine the workload for the TT:

Total Amount of Work (KJ) = (0.75 W_{max} x 1800 secs) / 1000

= ((0.75 x 348.7) x (1800)) / 1000

= (261.5 x 1800) / 1000

= (470745)/1000

= 470.75 KJ

The ergometer was set in the linear mode according to the formula:

$$Workload = L(\text{rpm})^2$$

where the rpm is the pedalling rate and the L is the linear factor. This factor was chosen in a way that would elicit a pedalling rate of 80 rpm's at 75% W_{max}. The alpha level to determine the linear factor was calculated using the formula below:

$$L = 75\%\ W_{max} / (80)^2$$

$$L = 261.5 / 6400$$

$$L = .041$$

During the TT participants could only see the amount of work completed on a computer screen connected to the cycle ergometer. All of the time trials were conducted in the same environment (19 ^{o}C/66.2 ^{o}F) without any verbal encouragement and in the absence of any factors that could affect the outcome of the TT e.g. music.

Drink consumption

Each participant was allocated the experimental (Cherry Active) and placebo (Cordial) drink in a counterbalanced approach. The drinks were consumed for 7 consecutive days to allow for a loading period of the experimental drink, taken in the morning (a.m.) and evening (p.m.) before returning to the HPL to undertake the 2nd and 4th visits. On the mornings of each trial the participants were asked to consume the drinks 30 to 60 minutes before undertaking the trial, and to consume the drink in the evening after the 2nd and 4th trials. The drinks were also consumed in the evening after completing the 2nd and 4th trials, and before the 3rd and 5th trials. This resulted in 9 consecutive days of supplementation for each experimental drink. In total 34 drinks were consumed throughout the study – 17 experimental drinks and 17 placebo drinks. To control for the effects of nutrition before each trial, participants were asked to record a 3-day food diary, 2 days leading up to the 2nd visit to the and for the remainder of the day after completing the 2nd visit. Participants were asked to eat an *ad libitum* diet and to consume the same foods and drinks recorded on their 3-day food diary when undertaking the cross-over section of the study.

Drink composition

The experimental drink contained 30 mL Montmorency tart cherry concentrate (Cherry Active, Hanworth, United Kingdom) (MC) and made to a final volume of 250 mL with water. The manufactures state every 30 mL contains between 90-110 Montmorency tart cherries and previous research has reported that CA contains 669 mg·mL^{-1} carbohydrates, < 1 mg·mL^{-1} fat, 31 mg·mL^{-1} protein, < 1 mg·mL^{-1} sodium and 9 mg·mL^{-1} anthocyanins (Bell et al., 2014c; Bell et al., 2015). The placebo (PLA) control drink was a commercially available fruit cordial (carbohydrates 3 mg·mL^{-1}, fat < 1 mg·mL^{-1}, protein < 1 mg·mL^{-1}, fibre < 1

$mg \cdot mL^{-1}$, and sodium 1 $mg \cdot mL^{-1}$, and trace of anthoycanins used for colouring) was combined with water and dextrose to make a final volume of 250 mL. A dextrose powder (MyProtein.com) was added to the placebo drink to ensure the carbohydrate values of both drinks were matched.

Assessment of total antioxidant capacity, lipid peroxidation, and protein carbonyls were assessed using the methods previously stated in section 3.3.

High sensitivity interleukin – 6 (IL-6) ELISA

Interleukin – 6 (IL-6) was measured using a commercially available high-sensitivity ELISA kit with a detectable range of 0.2 pg/mL to 10 pg/mL (Human IL-6 Quantikine HS ELISA Kit, Pro No: HS600B, R&D Systems, UK). In brief, 100 µL of assay diluent was added to each well and 100 µL of the sample and standard were then added and left to incubate for 2 hours at room temperature with gentle agitation. Plates were then washed and 200 µL of Human IL-6 HS Conjugate was added to each well and incubated for a further 2 hours with gentle agitation. The plates were then washed, 50 µL of substrate solution was then added and incubated for 1 hour at room temperature without agitation. After 1 hour 50 µL of amplifier solution was added and incubated for a further 30 minutes at room temperature. Finally, 50 µL of stop solution was added and the plates were analysed using a microplate reader set at a wavelength of 490 nm. An additional reading was taken at a wavelength of 650 nm and these readings were subtracted from the readings at 450nm to correct for optical imperfections within the plate. A linear regression equation was generated from an eight-point standard curve consisting of standards ranging from 0 pg/mL to 10 pg/mL. Plasma values are expressed as pg/mL of IL-6. The intra- and inter-plate coefficients of variation were 6.9% and 9.6% respectively.

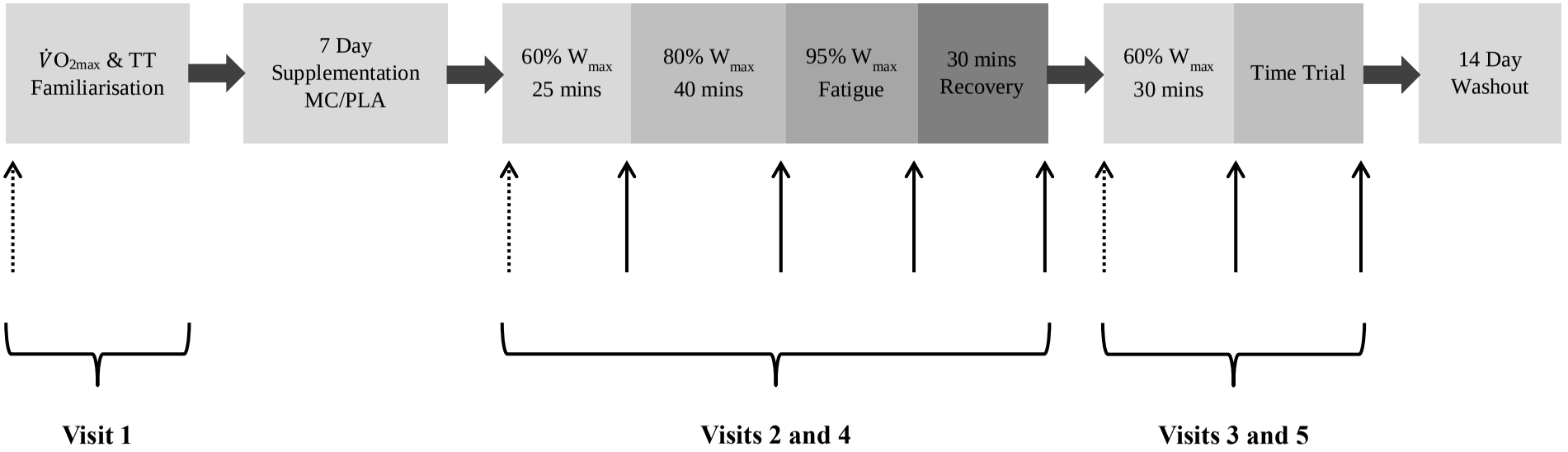


Figure 5.1 The study was a crossover design and counterbalanced with participants receiving either Montmorency Tart Cherry (MC) or placebo (PLA) for the first half off the intervention and opposing drink in the second half of the study. Participants attended the Human Performance Laboratory (HPL) on 5 separate occasions. Baseline measures and familiarisation to the time-trial (TT) were initially undertaken – visit 1. After 7 days supplementation participants returned to the HPL to undertake a fatiguing exercise protocol, followed by 30 minutes rest – visit 2. After 24 hours participants returned to the HPL to undertake a 30-minute warm up, followed by a cycling time-trial (TT) – visit 3. After a 14-day washout the participants consumed the opposing drink and completed visits 2 and 3, labelled visits 4 and 5 respectively. All arrows represent when blood was collected during the trial. Dashed arrows represent baseline samples.

Statistical Analysis

All data was analysed using the statistical analysis software package for social sciences (SPSS Version 22 for Windows, Chicago, IL). Outcome measures were assessed using repeated-measures analysis of variance (ANOVA), with Mauchay's Test of Sphericity used to test for equal variance, if the assumption of equal variance was not met within-subject effects were subsequently taken from Greenhouse Geisser corrections. Several statistical models were used to answer specific experimental questions for the effect of supplementation on the outcome variables at baseline 3(V1pre, V2pre, V3pre) x 2 (MC, placebo), during visit 2 5(V2pre, 60%, 80%, V2post, V2post30) x 2 (MC, placebo), supplementation effect on recovery 2(V2post30, V3pre) x 2(MC, placebo), and during visit 3 3(V3pre, 60%, V3post) x 2(MC, placebo). All values are means ± standard deviation (SD), with the level of significance set at $p \leq 0.05$.

5.4 Results

Participant demographics

All of the participant demographics are displayed in Table 5.1.

Power outputs and cycling times for steady state cycling and time-trial

The average watts maintained during the steady state trials during visit 2, irrespective of supplementation, were: at 60% W_{max} 208 W (± 17.5), 80% W_{max} 270 W (± 26.8), and 95% W_{max} 329.5 W (± 28.1). In comparison with predicted workloads: at 60% W_{max} all of the participants completed their predicted workloads; at 80% W_{max} the average workload for all participants was 78% W_{max} (± 2.4); and at 95% W_{max} participants cycled at their predicted workload until voluntary fatigue. The total trial time for MC was 4073 seconds (± 172) and for the PLA was 4061 seconds (± 206), there was no significant difference in total trial time between the groups, nor in the finishing time of the time-trial (MC group: 1605 seconds ± 111.2; PLA group:1610 seconds ± 114.6) (Table 5.2). Similarly, power output during the time-trial was not different between groups (MC group: 292 W ± 34.8; PLA group 292 W ± 40.1) (Table 5.2). The intra-individual differences of the participants finishing times and power outputs over the two trials are represented in figure 5.2. There was no effect of trial order, with the participants' first time-trial completed in 1609 seconds (± 103.9) and the subsequent trial in 1606 seconds (± 121.3).

Heart rate and perceived rate of exertion for steady state cycling and time-trial

During steady state cycling at 60% W_{max} and 80% W_{max} there were no significant differences in heart rate (60% $W_{max,}$ MC $p = 0.59$; 80% $W_{max,}$ $p = 0.148$) or rate of perceived exertion (60% $W_{max,}$ $p = 0.402$; 80% $W_{max,}$ $p = 0.142$) between the MC and PLA groups. There was also no significant difference in heart rate ($p = 0.358$) or rate of perceived exertion ($p = 0.451$) in the 30-minute steady state cycling at 60% W_{max} before the time-trial between groups. During the time-trial there was no significant difference in heart rate between the MC and PLA groups ($p = 0.317$) (Table 5.2).

Table 5.1 Mean demographic characteristics of the male cyclists ($n = 8$) who completed the study.

n = 8	Mean (SD)
Age (years)	29.1 ± 5.3
Height (m)	1.78 ± 0.05
Weight (Kg)	72.5 ± 7.4
BMI (kg/m^2)	22.7 ± 1.6
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	57.7 ± 5.0
$\dot{V}O_{2peak}$	59.4 ± 4.7
Maximal Power Output (W_{max})	348.3 ± 27.6
Watts per Kg (W/Kg)	4.8 ± 0.4

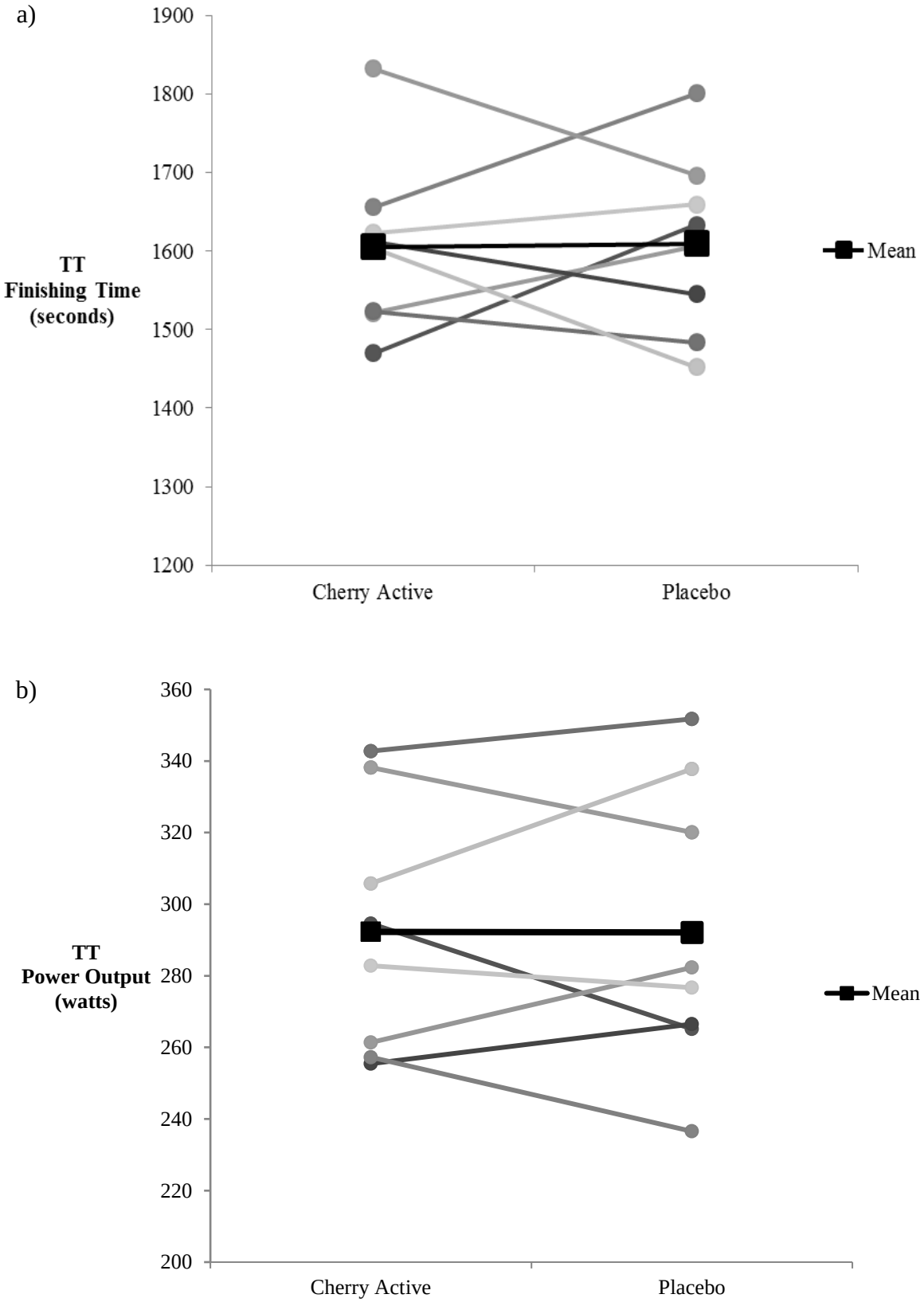


Figure 5.2 The mean and intra-individual differences upon completion of the time-trial for a) finishing time in seconds and b) power outputs in watts for each condition. There were no significant effects for trial order (p = 0.966)

Effect of supplementation on baseline oxidative stress and inflammation markers

To investigate the effectiveness of supplementation on baseline oxidative stress markers, baseline samples (preV1, V2pre, V3pre) for both conditions were compared. At baseline, there were no significant differences in FRAP, MDA, and PC concentrations between the conditions (FRAP: $F_{(1,7)} = 1.632$, $p = 0.242$, $\eta^2 = 0.189$, MDA: $F_{(1,7)} = 0.102$, $p = 0.759$, $\eta^2 = 0.014$), PC: $F_{(1,7)} = 3.303$, $p = 0.112$, $\eta^2 = 0.321$). Throughout the intervention period baseline concentrations of FRAP significantly increased, independent of supplementation ($F_{(2,14)} = 12.33$, $p = 0.001$, $\eta^2 = 0.638$) (Figure 5.3). There was a significant increase in FRAP from preV1 to V3pre (preV1: 513.4 ± 48 μM; V3pre: 556.7 ± 38.4 μM; $p = 0.037$) and V2pre to V3pre (V2pre: 507.8 ± 46.5 μM; V3pre: 556.7 ± 38.4 μM; $p = 0.002$). The inflammatory marker IL-6 was measured V2pre and V3pre to evaluate the effect of V2 on V3. There was no significant difference pre-V3 in IL-6 between the conditions ($F_{(1,7)} = 0.557$, $p = 0.277$, $\eta^2 = 0.074$) (Figure 5.6).

Effect of supplementation on oxidative stress and inflammation markers during and after steady state exercise (visit 2)

Supplementation did not stimulate any significant differences in oxidative markers (FRAP, MDA and PC) between the MC and PLA group during steady state exercise (FRAP: $F_{(1,7)} = 0.362$, $p = 0.566$, $\eta^2 = 0.049$; MDA: $F_{(1,7)} = 0.028$, $p = 0.871$, $\eta^2 = 0.04$), PC: $F_{(1,7)} = 0.105$, $p = 0.755$, $\eta^2 = 0.015$). Independent of supplementation exercise resulted in a significant increase in FRAP during the trial ($F_{(4,28)} = 23.05$, $p < 0.001$, $\eta^2 = 0.767$). FRAP was significantly increased compared to baseline at each time point in visit 2, V2pre to 60% W_{max} (V2pre: 507.8 ± 46.5 μM; 60% W_{max}: 549.2 ± 54.8 μM; $p = 0.015$), V2pre to 80% W_{max} (V2pre: 507.8 ± 46.5 μM; 80% W_{max}: 582.2 ± 37.5 μM; $p < 0.001$), V2pre to postV2 (V2pre:

507.8 ± 46.5 μM; postV2: 596.3 ± 46.9 μM; $p = 0.003$), V2pre to post30V2 (V2pre: 507.8 ± 46.5 μM; post30V2: 610.7 ± 55.2 μM; $p < 0.001$), and was significantly higher at V2post30 compared to concentrations at 60% W_{max} ($p = 0.04$) (Figure 5.3). MDA significantly changed during the trial independent of supplementation ($F_{(2.383,16.678)} = 9.08$, $p = 0.001$, $\eta^2 = 0.565$). MDA concentrations significantly increased from V2pre to 60% W_{max} (V2pre: 10.9 ± 1.4 μM; 60% W_{max}: 11.9 ± 1.7 μM; $p = 0.025$). MDA concentrations were also significantly reduced 30 minutes after cessation of cycling compared to concentrations at 60% W_{max} (V2post30: 10.5 ± 1.8 μM; 60% W_{max}: 11.9 ± 1.7 μM; $p = 0.004$) and immediately post-cycling V2post30: 10.5 ± 1.8 μM; V2post: 11.9 ± 1.7 μM; $p = 0.001$) (Figure 5.5). The inflammatory marker IL-6 was not significantly different between the two conditions during this branch of the trial ($F_{(1,7)} = 2.04$, $p = 0.196$, $\eta^2 = 0.226$). Independent of supplementation, exercise significantly increased IL-6 concentrations ($F_{(2,14)} = 33.57$, $p < 0.001$, $\eta^2 = 0.908$). There was a significant increase in IL-6 from V2pre to V2post (V2pre: 0.41 ± 0.31 pg/mL; V2post: 3.3 ± .1.1 pg/mL; $p < 0.001$) and V2pre to V2post30 (V2pre: 0.41 ± 0.31 pg/mL; V2post30: 2.3 ± 0.72 pg/mL; $p < 0.001$). Furthermore, IL-6 was significantly greater at postV2 compared to Post30V2 ($p = 0.018$) (Figure 5.6).

Table 5.2 Physiological differences between the two experimental conditions for heart rate (HR), rate of perceived exertion (RPE), trial completion time, and power output over the 2nd and 3rd trials. All values are means ± SD

	MC	PLA	*p* value
Total Trial Time (seconds)	4073 ± 172	4061 ± 206	0.64
TT Finishing Time (seconds)	1605 ± 111	1609 ± 115	0.92
TT Power Output (watts)	292 ± 34.8	292 ± 40.1	1.0
HR for TT (bpm)	169 ± 11.2	171 ± 9.5	0.32
HR 60%W_{max} 30 minutes (bpm)	136 ± 9.3	134 ± 9.2	0.36
RPE 60%W_{max} 30 minutes (bpm)	12.3 ± 1.2	11.9 ± 1.4	0.45
HR 60%W_{max} 25 minutes (bpm)	135 ± 10.2	137 ± 11.4	0.59
HR 80%W_{max} 40 minutes (bpm)	165 ± 9.7	169 ± 10.2	0.15
RPE 60%W_{max} 25 minutes	11.7 ± 1.0	12.1 ± 0.6	0.40
RPE 80%W_{max} 40 minutes	16.2 ± 1.2	17.1 ± 0.8	0.14

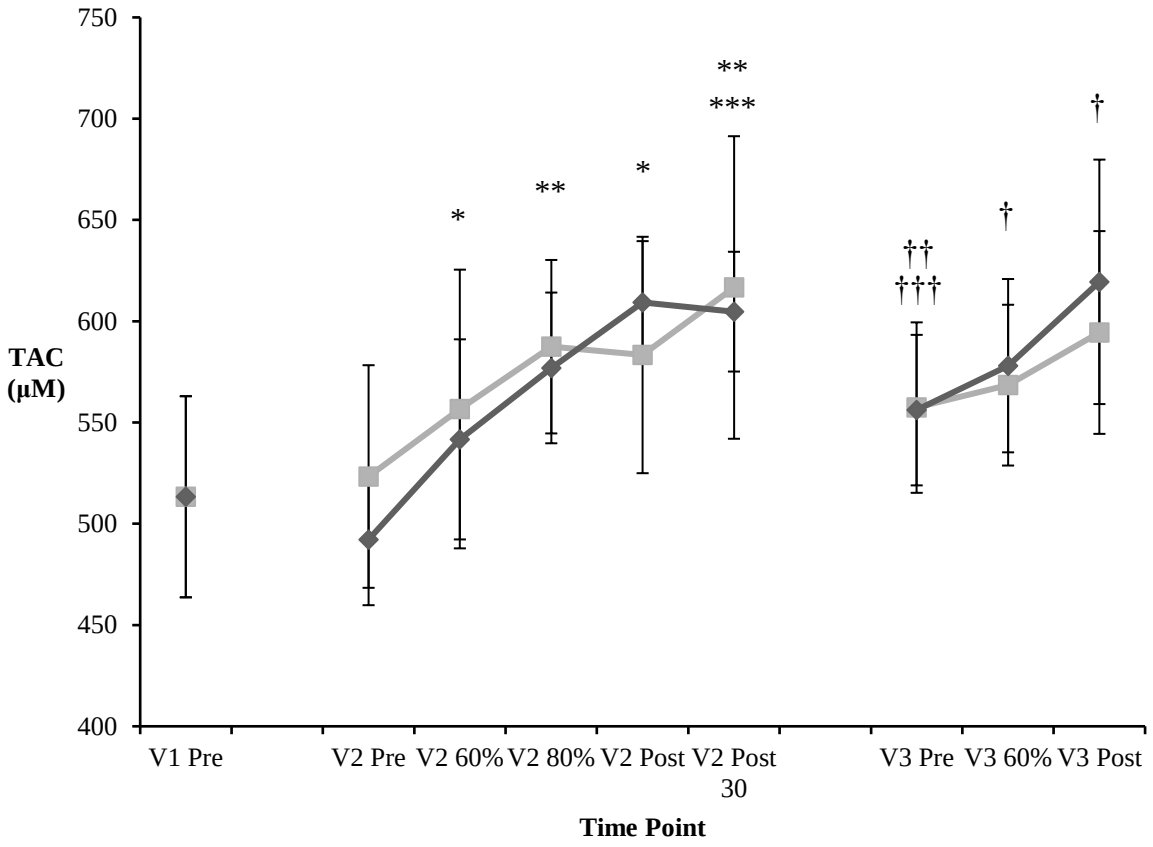


Figure 5.3 Changes in total antioxidant capacity (TAC) measured by the ferric reducing ability of plasma (FRAP) assay for the MC condition (light grey) and PLA condition (dark grey). All values are means ± SD. * indicates $p < 0.05$ and ** indicates $p < 0.001$ compared to V2pre, *** indicates $p < 0.05$ compared to V260%, † indicates $p < 0.05$ compared to V3pre, †† indicates $p < 0.05$ compared to V1pre and V2pre, ††† indicates $p < 0.05$ compared to V2post30.

The effect of supplementation on recovery 24 hours after completing a strenuous cycling bout

To determine the effectiveness of MC supplementation on recovery and performance, oxidative stress and inflammatory markers were compared between the two conditions after 24 hours (V2post30 vs V3pre) and during the 3rd trial. Supplementation did not stimulate any

significant differences between the MC and PLA group for FRAP ($F_{(1,7)} = 0.15$, $p = 0.710$, $\eta^2 = 0.021$), MDA ($F_{(1,7)} = 0.5$, $p = 0.507$, $\eta^2 = 0.067$), PC ($F_{(1,7)} = 1.179$, $p = 0.314$, $\eta^2 = 0.144$) or IL-6 ($F_{(1,7)} = 0.65$, $p = 0.447$, $\eta^2 = 0.085$) 24 hours after completing a strenuous bout of cycling. Independent of supplementation FRAP and IL-6 concentrations decreased from V2post30 to V3pre (FRAP: postV2: 610.6 ± 552 µM; V3pre: 556.7 ± 38.4 µM; $p = 0.005$: IL-6 V2post30: 2.3 ± 0.72 pg/mL; V3pre: 0.3 ± 0.17 pg/mL; $p < 0.001$). There was a trend for reduced PC in the PLA group compared to the MC at V3pre (PLA: 1.12 ± 0.33 nm/mg; MC: 1.33 ± 0.47 nM/mg; $p = 0.051$) (Figure.5.4).

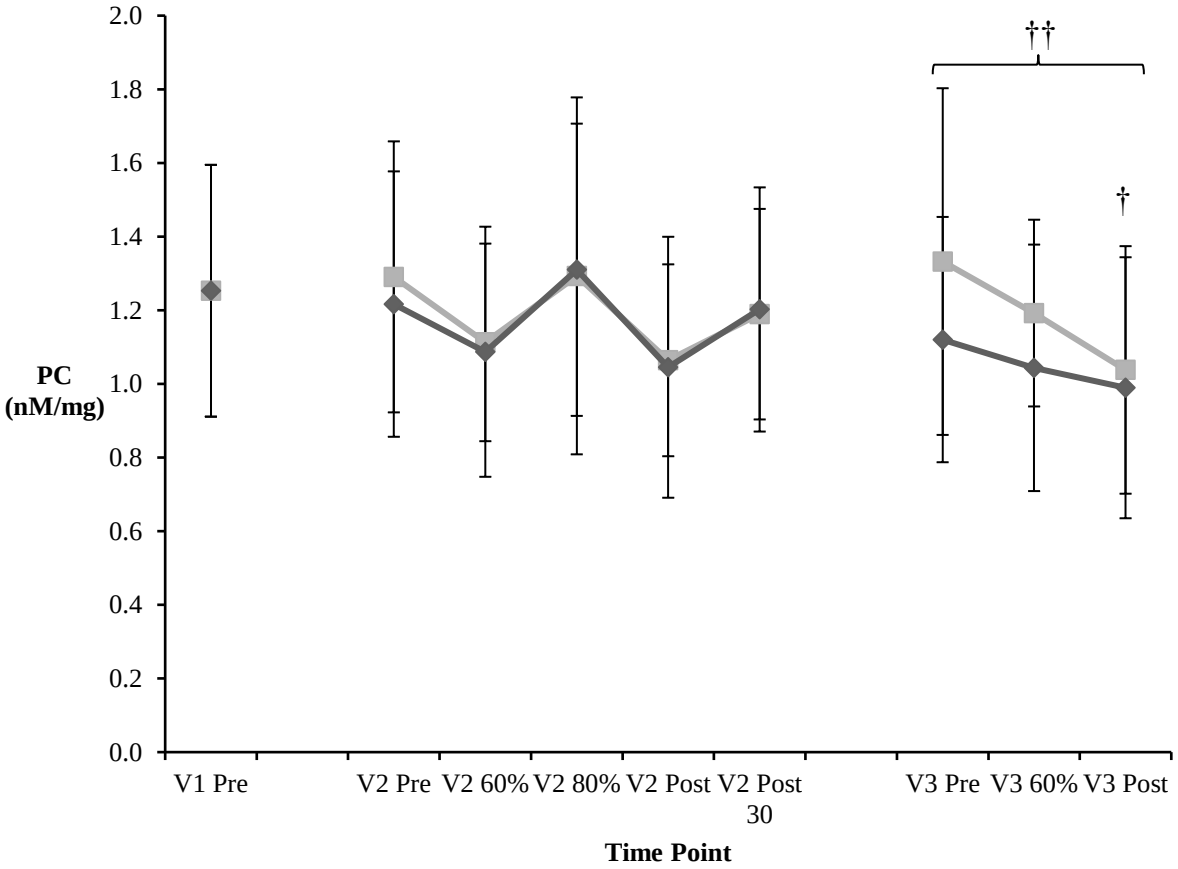


Figure 5.4 Changes in protein carbonyl (PC) content measured by a protein carbonyl ELISA for the MC condition (light grey) and PLA condition (dark grey). All values are means ± SD. † indicates $p < 0.05$ compared to V3pre, †† indicates $p < 0.05$ for an effect of supplementation during the 3rd trial.

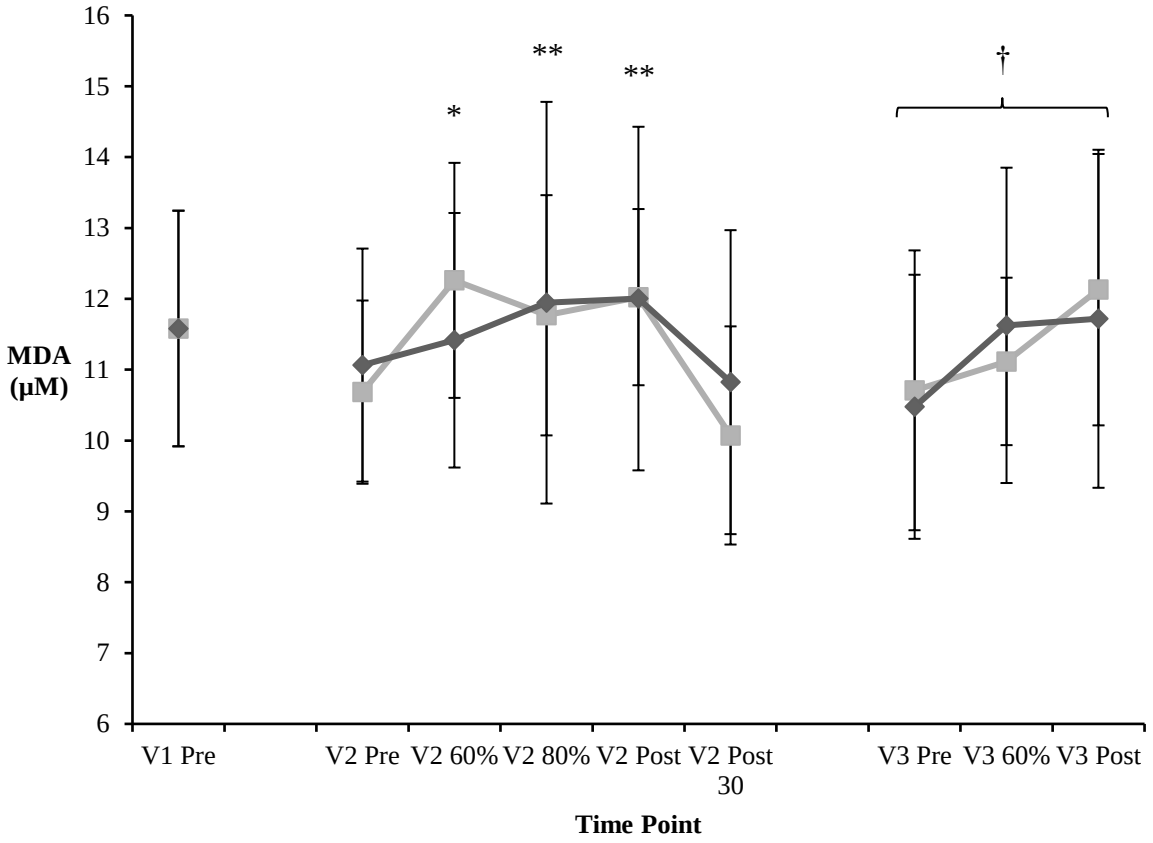


Figure 5.5 Changes in lipid peroxidation measured by the malondialdehyde (MDA) assay for the MC condition (light grey) and PLA condition (dark grey). All values are means ± SD. * indicates $p < 0.05$ compared to V2pre, ** indicates $p < 0.05$ compared to V2post30, † indicates $p < 0.05$ for an effect of time for the 3rd trial, pairwise comparisons failed to show any significant effects between the time points in the 3rd trial.

Supplementation did not stimulate any significant differences between the MC and PLA group for FRAP ($F_{(1,7)} = 0.471$, $p = 0.515$, $\eta^2 = .063$), MDA ($F_{(1,7)} = 0.02$, $p = 0.891$, $\eta^2 = 0.003$) or IL-6 ($F_{(1,7)} = 1.127$, $p = 0.324$, $\eta^2 = 0.139$) during the third trial. However, the MC condition (1.19 ± 0.15 nM/mg) had a significantly higher concentration of PC compared to the PLA condition (1.05 ± 0.07 nM/mg) during this branch of the trial ($F_{(1,7)} = 12.51$, $p = 0.01$, $\eta^2 = 0.641$) (Figure 5.4). Independent of supplementation, exercise significantly

increased FRAP ($F_{(2,14)} = 13.62$, $p = 0.001$, $\eta^2 = 0.661$), MDA ($F_{(2,14)} = 5.99$, $p = 0.013$, $\eta^2 = 0.461$), and IL-6 ($F_{(1,7)} = 32.77$, $p = 0.001$, $\eta^2 = 0.824$) but significantly reduced PC ($F_{(2,14)} = 8.92$, $p = 0.003$, $\eta^2 = 0.560$). FRAP significantly increased from V3pre to V3post (V3pre: 556.7 ± 38.4 μM; V3post: 606.9 ± 55.1 μM; $p = 0.011$) and 60% W_{max} to V3post (60% W_{max}: 573.3 ± 38.8 μM; V3post: 606.9 ± 18.6 μM; $p = 0.046$) (Figure 5.3). MDA significantly increased over the 3rd trial but pairwise comparisons did not detect any significant differences between the time points however there was a tendency for greater MDA concentration V3 post compared to V3pre ($p = 0.093$) (Figure 5.5). IL-6 significantly increased from V3pre to V3post (V3pre: 0.33 ± 0.17 pg/mL; V3post: 1.95 ± 0.89 pg/mL; $p = 0.001$) (Figure 5.5). PC significantly decreased from V3pre to V3post (V3pre: 1.2 ± 0.41 nM/mg; V3post: 1.01 ± 0.33 nM/mg; $p = 0.001$) (Figure 5.4).

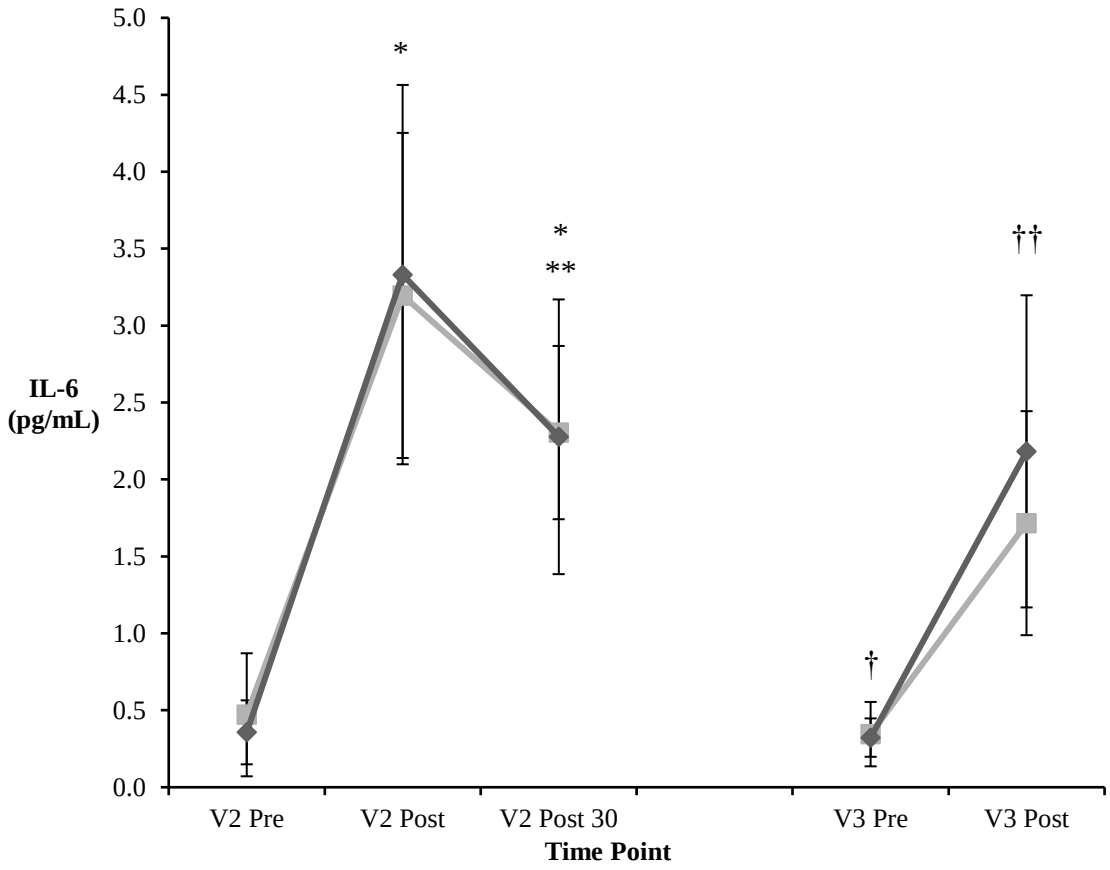


Figure 5.6 Changes in the inflammatory marker interleukin-6 (IL-6) measured by a high sensitivityIL-6 ELISA kit for the MC condition (light grey) and PLA condition (dark grey). All values are means ± SD. * indicates $p < 0.001$ compared to V2pre, ** indicates $p < 0.05$ compared to V2post, † indicates $p < 0.001$ compared to V2post, †† indicates $p < 0.05$ compared to V3pre.

5.5 Discussion

The aim of the present study was to assess the effects of supplementing MC on markers of oxidative stress and inflammation during a strenuous cycling protocol. Further, the study also aimed to investigate the potential for MC to improve recovery and subsequent days cycling performance in well-trained male cyclists. The results presented suggest that consumption of MC in the days preceding a strenuous cycling protocol did not negate exercise-induced changes in PC, MDA, or IL-6, nor did supplementation augment TAC during, or upon completion of the protocol, compared to consumption of a placebo controlled drink. Consumption of MC over a 24-hour period did not impact markers of oxidative stress or inflammation, during or following steady cycling at 60% W_{max}, or following a self-paced time-trial compared to placebo. Finally, subsequent days performance was not augmented through the consumption of MC.

In contrast to the results presented herein, previous studies have reported improved muscular recovery on subsequent days following strenuous or prolonged exercise bouts after the consumption of a MC supplement, indicating performance may be improved through the ingestion of MC before and upon completion of exercise. Although these studies identified significant differences in perceived muscle soreness, markers of oxidative stress, inflammation, and muscle recovery in the days following acute but strenuous exercise, none have included a direct performance measure to ascertain whether the observed changes had a practical performance outcome. However, maintenance of muscle force, as was observed by others over a 24 to 72-hour period when consuming a MC supplement (Bowtell et al., 2011; Connolly et al., 2006; Howatson et al., 2010), and would suggest increased power output, or quicker completion of a set workload compared to placebo. Furthermore, subjective measures of muscle soreness post-exercise are attenuated when consuming MC (Connolly et al., 2006;

Kuehl et al., 2010), suggesting that participants 'feel' less sore, and more able to perform at a greater intensity during the exercise bout.

Perhaps the most comparable work to that presented herein has been led by Bell et al., (2015). Bell's study assessed cycling performance following a simulated road race cycling protocol in trained cyclists over a 72-hour period. Pre-loading with MC concentrate for 4 days, and continual supplementation over the recovery period inhibited the decrease of MVIC observed in the control group. However, the decrease in MVIC seen in the control group did not affect 6 second maximal power output over the recovery period. An additional study from the same group investigated the effects of MC concentrate consumption on 3 consecutive days of simulated road cycling, employing the same protocol as the aforementioned study for the supplementation period. Performance measures *per se* were not directly measured in this study but the simulated road cycling protocol included 3 time-trial protocols lasting for a total of 9 minutes (2 x 2 minutes and 1 x 5 minutes) with the power outputs from each trial transformed into total work performed. Over the 3 trials there were no significant differences in the amount of work performed between the MC and placebo group, with an overall decrement in work performed for both groups over the 3 cycling trials. Although the study presented herein employed a different cycling protocol to induce fatigue, and a different measure of performance, it does concur with the findings that supplementation with MC does not augment performance compared to an isocaloric control drink (Bell et al., 2014c; Bell et al., 2015). The inclusion of a simulated time-trial places emphasis on a practical performance outcome.

The predominant polyphenol compounds that may act as an 'active ingredient' in MC are the subclasses of anthocyanins. Cyanidins are purported to constitute up to 93% of the anthocyanin's present in MC concentrate (Kirakosyan et al., 2009) with cyanidin 3-glucosylrutinoside being indicated as the main anthocyanin, followed by cyanidin 3-

rutinoside; with other anthocyanin compounds such as cyanidin 3-sophoroside, cyanidin 3-rutinoside 5 β-$_D$-glucoside, and peonidin 3-rutinoside contributing to the total anthocyanin content (Bell et al., 2014b; Blando et al., 2004; Kirakosyan et al., 2009; Kirakosyan et al., 2015; Seymour et al., 2014).

The dose administered during the study should have been adequate to augment the plasma levels of metabolites related to MC consumption. Dose-response studies have indicated that the consumption of MC was adequate to increase the concentration of secondary metabolites associated with the anthocyanin derivatives cyanidin (Bell et al., 2014b; Keane et al., 2016; Seymour et al., 2014) and peonidin (Keane et al., 2016). Studies have reported an increase in cyanidin-3-glucosylrutinoside, cyanidin-3-rutinoside, cyanidin-3-O-glucoside, protocatechuic acid (PCA), and vanillic acid (VA) after ingesting doses of MC equivalent to the consumption of 90 and 180 cherries (Bell et al., 2014b; Keane et al., 2016; Seymour et al., 2014). Furthermore, plasma concentrations of these secondary metabolites have been reported to be significantly elevated from baseline 30 to 60 minutes post-consumption and remain elevated for periods between 2 and 4 hours (Seymour et al., 2014; Keane et al., 2016). Therefore, the timing and the dose used in the current study should have been adequate to increase the plasma concentrations of secondary metabolites related to MC consumption.

The results presented here do not support those of previous studies (Bell et al., 2014c; Bell et al., 2015). The results show no significant change in MDA, PC or IL-6 in response to supplementation. In the two cycling studies conducted by Bell et al., (2014c;2015) supplementation resulted in significant differences in IL-6, CRP and lipid peroxidation. There are 2 probable explanations: 1) both supplemented and control groups were asked to follow a diet low in phenolic compounds; and 2) the difference in the design of the experiments, for example, within-subject design verses between-subject design. A low-phenolic diet would

potentially exaggerate the effect of supplementation by first reducing a participants' normal antioxidant intake and this may subsequently affect the response to exercise-induced oxidative stress. This effect has been seen in studies where diethyl maleate (DEM) has been administrated, to deplete skeletal muscle glutathione, and resulted in augmented oxidative stress upon completion of an acute exercise bout (Strobel et al., 2014). The present study did not restrict the consumption of polyphenols from the participants' diet because this may not reflect the habitual eating habits of an athlete in preparation for an event and/or competition.

Therefore, within the current study the participants were required to keep a food diary for 2 days leading up to and on the day of the trial, and asked to repeat this regime when returning to complete the other branch of the trial. By adopting this approach, it allowed the supplement to be assessed in an *ad libitum* setting, reflecting how the supplement responds when a participant consumes identical meals over the intervention period, without dietary restriction. The use of a within-subject design made it possible to employ this protocol because the participants would be returning to complete the second branch of the trial. The inclusion of a within-subject design may also account for the discrepancies between the previous studies that undertook a between-subject study design. To reduce the possibility of any learning effects affecting the results of the TT, participants were counterbalanced to each condition when recruited to the study.

Where a diet is not restricted during supplementation, addition of MC to the diet would be expected to increase antioxidant capacity. The results presented herein did not show an increase in antioxidant capacity (TAC) because of MC supplementation. However, TAC did increase in response to exercise in both supplemented and control groups. The present study used the ferric ion reducing antioxidant power (FRAP) assay to assess TAC, whilst other studies have used the Trolox equivalence antioxidant capacity (TEAC) assay. Both assays work on the principle of single electron transfer where a single oxidant substrate is

reduced through the addition of an antioxidant compound. The donation of an electron results in a colour change related to the antioxidant reducing capacity of the sample being investigated (equation. 1) (Huang et al., 2005). FRAP and TEAC use different oxidants to be subjected to reduction. Where FRAP uses a ferric salt (FeIII $(TPTZ)_2Cl_3$) as its oxidant and TEAC uses $ABST^{\bullet-}$ through the persulphate oxidation of 2,2-Azino-bi-(3-ethylbenzthiazoline sulphonate) (Huang et al., 2005). Even though the assays use differing substrates to detect antioxidant capacity the redox potential of both chemicals are very similar, ~0.70V for FeIII and ~68V for $ABST^{\bullet-}$, indicating there is very little difference between the assays (Huang et al., 2005). Therefore, in principle, comparing the results of the two different assays should not be judged as a limitation between the two studies.

Oxidant + antioxidant → reduced oxidant + oxidised antioxidant (1)

Howatson et al., (2010) demonstrated 5 days of supplementation with MC resulted in greater plasma total TAC compared to non-supplemented individuals, with significantly higher TAC on completion of a marathon. Furthermore, an additional 2 days of supplementation post-marathon resulted in TAC being maintained at baseline levels, whereas in the placebo group, TAC concentration decreased below baseline levels. In contrast to these findings and in agreement with the findings from the current study, Bowtell et al., (2011) found no differences in plasma TAC between supplemented and non-supplemented groups at any time point, either at the cessation of exercise or in the following days whilst still consuming the supplement. It should be noted that throughout the experiment Bowtell et al., (2011) did not report an increase in TAC in response to exercise, unlike the current study and the findings from Howatson et al., (2010). An explanation for these discrepancies could be the duration and mode of exercise. The present study consisted of a constant aerobic workload to be maintained for 105 minutes, in comparison to Howatson et al., (2010) where participants ran for an average of 240 minutes, which may have placed a higher demand on

the antioxidant system to cope with the sustained production of exercise-induced RONS. Bowtell et al., (2011) employed a resistance training model allowing for rest periods to be taken during each set, potentially leading to oscillating exercise-induced RONS production throughout the intervention period, thus reducing the demand of the antioxidant system compared to continual aerobic exercise. The time course of response between the two differing exercise modes could also explain this difference, with marked changes in resistance training occurring during the secondary inflammation phase, hours and days post-exercise.

It is now well established that the increase in metabolic activity during exercise results in the production of exercise-induced RONS from several sources within skeletal muscle and the endothelium upon exercise cessation (Mason and Wadley, 2014; Sakellariou et al., 2014). Endogenous antioxidants can neutralise these compounds. However, undertaking strenuous and/or unaccustomed exercise bouts produce an augmentation in RONS capable of overriding the endogenous antioxidant system, resulting in compromised muscle force production (Hernández et al., 2012; McKenna et al., 2006; Powers and Jackson, 2008; Westerblad and Allen, 2011), undesirable conformational changes to macromolecules such as proteins, lipids, and nucleic acids (Turner et al., 2011); and propagation of the inflammatory response post-exercise (Peake et al., 2007). A combination of these factors could therefore have a negative effect during the recovery period and on performance. To counteract these undesirable effects, the ingestion of exogenous antioxidants has been hypothesised to provide additional protection against RONS when undertaking strenuous and/or unaccustomed exercise

The present study did not distinguish any differences in markers associated with exercise-induced oxidative stress between the two experimental conditions (MC verses PLA). The elevation of lipid peroxidation adducts with increasing intensity and/or duration in this

study demonstrates all the exercise intensities and duration were adequate to augment exercise-induced oxidative stress. The significant decrease in MDA observed in the 30-minute post-exercise period could be related to its clearance, as MDA is converted to CO_2 and H_2O through a series of enzymatic reactions. The use of the thiobarbituric acid reactive substances (TBARS) assay has been criticised for its lack of specificity and sensitivity when measuring MDA adducts in plasma due to the reactivity of thiobarbituric acid (TBA) with sugars, amino acids, bilirubin, albumin, and non-related aldehydes resulting in overestimation of MDA (Gutteridge and Halliwell, 1990; Sachdev and Davies, 2008). However, it has been postulated that this assay can provide an insight into whether lipid peroxidation has occurred or if the samples being processed have remnants of lipid peroxidation within them, enabling a snapshot on lipid peroxidation *in vivo* (Esterbauer et al., 1991). Studies using the same assay to assess changes in lipid peroxidation post-exercise have also demonstrated an increased clearance of MDA/TBARS in the recovery phase and have postulated this occurs from increased catabolism, excretion, and redistribution to other tissues of MDA (Groussard et al., 2003; Leaf et al., 1997).

Protein carbonylation (PC) is arguably the most stable marker of ROS interaction with biomolecules. At present there are studies indicating PC concentrations increase (Lamprecht et al., 2008; Wadley et al., 2015b), decrease (McGinnis et al., 2014), or remain unchanged (Gaeini et al., 2006; Rahnama et al., 2007) in response to cycling in trained individuals. Most studies assess PC post-exercise following a single workload. The current study design enables a novel insight into how PC change in response to workload. Initially a workload at 60% W_{max} appeared to stimulate clearance of PC. During a steady state workload of 80% W_{max} for 40 minutes PC levels increased, when workload increased to 95% W_{max} until exhaustion, PC levels decreased. The 3rd trial further demonstrated a reduction in PC concentrations from pre-exercising concentrations with significant reductions occurring at the

end of both steady-state cycling and upon completion of the TT. The decrease in PC could be explained by the activity of the 20S proteasome, which has been reported to degrade up to 90% of oxidative modified proteins (Jung and Grune, 2008). However, as the cycling intensity increases and the magnitude of RONS produced may also increase, and the pool of oxidised proteins would then increase as a consequence. Nevertheless, it should be noted the concentration of PC's did not increase above baseline concentrations. Although this is purely speculation, production of new PC adducts vs clearance of existing adducts is the most probably explanation for the discrepancy in trend in this biomarker (Wadley et al., 2016b). The only significant effect of MC supplementation occurred during the 3rd visit, where mean values of PC were higher than the placebo group (MC: 1.25 nm/mg ± .103 vs PLA: 1.18 nm/mg ± .11). Considering there were no significant differences between the groups throughout the previous trial (trial 2) for PC or for any other marker assessed during the study, the significant *p* value could therefore represent a false-positive finding. The responses from both groups throughout the study were homogenous, indicating supplementation had little to no effect on the biological markers studied in the present study and the group differences occurred by chance, rather than as a direct result of the drink consumption.

The magnitude of the response of IL-6 during exercise depends on the exercise mode, duration, and intensity. When the exercise undertaken includes both metabolic and mechanical stimuli, such as undertaking a marathon running race over a distance of 42.2 km the increase in post-exercise IL-6 concentrations can reach ~80 (Howatson et al., 2010) to ~100 (Ostrowski et al., 1998) fold above baseline levels. However, exercise modalities that place a metabolic stress, without much mechanical stress, such as cycling, have found a less pronounced increase in IL-6. With cycling protocols performed at 60% $\dot{V}O_{2max}$ for 2 hours or until fatigue reporting a modest ~2.5 fold increase in IL-6 (Li and Gleeson, 2004), with more

intensive cycling protocols lasting an hour reporting a ~5 fold increase (Bell et al., 2014c; Bell et al., 2015). Our findings are consistent with the latter studies with a ~6.5 and ~4-fold increase observed at cessation of cycling after trial 2 and trial 3 respectively. Indicating the stress response to the cycling protocol used in the current study potentially occurred as a result of metabolic stress rather than mechanical stress due to the lower levels of IL-6 production. In contrast to previous studies there were no differences in IL-6 concentrations between the experimental and placebo conditions. The attenuation in the inflammatory markers IL-6 and CRP in the study conducted by Bell et al., (2014c) was hypothesised to occur due to the reduction of oxidative stress in the MC group post-exercise. The augmented oxidative stress observed in the control group was associated to increased cellular stress post-exercise, resulting in the elevated inflammatory response post-exercise. This rationale could be tentatively supported from the current results because both oxidative stress and inflammation markers increased during the trials. However, because the current study did not identify differences in the oxidative stress or inflammatory response between the two conditions it is hard to speculate whether the observed differences in the aforementioned studies occurred due to supplementation with Montmorency tart cherries or independent group responses to the cycling protocol.

An underlying principle of this thesis was to explore the effects of antioxidant supplementation in a real-world setting. With a lack of evidence for the beneficial effect of the more traditional antioxidants, athletes appear to be turning to more natural supplements. This study aimed to assess the effect of MC supplementation on physiological responses to exercise in free-living cyclists. The results presented largely agree with the findings in Chapter 4 where habitual low-dose supplementation failed to augment exercise-induced oxidative stress in steady-state cycling. Furthermore, the findings contradict the rationale for why people choose to consume antioxidant supplements (as reported in Chapter 2). The

current study was conducted in free living cyclists, and did not attempt to control or manipulate their diet, in order to best reflect common practice in recreational cyclists. However, future research may choose to explore the effects of polyphenols in participants consuming a high versus low-phenolic diets to explore the interactions between diet and polyphenol supplementation on exercise-induced oxidative stress and inflammation.

Chapter Six

6. General Discussion

6.1 Summary

The work undertaken in this thesis has investigated the habitual consumption of antioxidant supplements in a population of recreational cyclists and their effect on exercise-induced oxidative stress, baseline endogenous antioxidant concentrations, and performance. Chapter 2 and 3 investigated the habitual consumption of antioxidant supplements in recreational cyclists undertaking non-competitive cycling events. Chapter 3 aimed to investigate whether habitual antioxidant supplementation affected the oxidative stress response on completion of a cycling sportive, compared to a non-supplemented group. In chapters 4 and 5 the main focus was to assess how antioxidant supplementation may impact on cycling performance, recovery, and oxidative stress response in trained cyclists. An overarching hypothesis of this thesis was that habitual antioxidant supplementation with commercially available antioxidant supplements in recreational cyclists who undertake regular exercise would not affect exercise-induced oxidative stress. It was also hypothesised that the consumption of an antioxidant supplement in the preceding days and on the day of, demanding exercise would provide additional protection against exercise-induced oxidative stress and inflammation in well trained cyclist, resulting in improved recovery to allow for better performance on subsequent days.

6.2 Habitual consumption of antioxidant supplements

Chapters 1 and 2 investigated the habitual antioxidant supplementation in recreationally cyclists undertaking a cycling sportive. Previous research investigating the use of supplements in different exercise settings have predominantly focused on dietary supplements as a whole (Dascombe et al., 2010; Tian et al., 2009), incorporated differing modes of exercise (individual and team sports) (Heikkinen et al., 2011), focused on elite or

high performing athletes' (Lun et al., 2012) and not clearly defined why a specific dietary supplement was consumed (Braun et al., 2009; Dascombe et al., 2010; Tian et al., 2009). Therefore, chapter 1 investigated the rationale for individuals taking one particular supplement, in an amateur setting, and undertaking one mode of exercise.

The findings from chapter 1 indicated there was not one particular 'type' of person who consumed antioxidant supplements at a recreational cyclists' level. The study did not find any differences in age, years spent cycling, or hours spent cycling a week between supplemented and non-supplemented participants. The findings give a novel insight on the supplementation habits of individuals aged 35-49 that have not been identified previously. Forty percent of the participants reported antioxidant supplementation, which is lower than the reported rate of elite athletes' (Heikkinen et al., 2011; Lun et al., 2012) and younger athletes''(Dascombe et al., 2010; Tian et al., 2009) over 60% of the supplemented participants had supplemented for a period ≥ 12 months and this finding was replicated in chapter 2 with an average consumption period of 27 ± 42.3 months. The main form of antioxidant supplementation was multivitamins and individual vitamin supplements such as vitamin C and vitamin E, which has also previously been reported in other dietary supplementation studies (Lun et al., 2012; Tian et al., 2009). The reasons given for consuming antioxidant supplements were also in agreement with previous findings in elite (Braun et al., 2009; Dascombe et al., 2010; Lun et al., 2012) and non-elite athletes (Knez and Peake, 2010; Tian et al., 2009; Tsitsimpikou et al., 2011), with cyclists indicating their aim of improving immunity as the main reason for supplementation, along with exercise related and health factors also being stated as reasons for consumption by participants.

6.3 Habitual antioxidant supplementation on exercise-induced oxidative stress

It is well documented exercise is responsible for the augmentation in post-exercise oxidative stress markers (Bloomer et al., 2005; Tauler et al., 2005; Turner et al., 2011). Experimental studies investigating the effects of a variety of antioxidant compounds have found supplementation to be effective in reducing exercise-induced oxidative stress (Bloomer et al., 2006; Goldfarb et al., 2011; Gomez-Cabrera et al., 2006; Mastaloudis et al., 2004); but whether or not this attenuation in oxidative stress is detrimental to RONS mediated adaptations is still under debate (Carmen Gomez-Cabrera et al., 2012; Cobley et al., 2015; Peternelj and Coombes, 2011; Yfanti et al., 2012).

The effect of habitual antioxidant supplementation on exercise-induced oxidative stress was studied in chapters 3 and 4, in both an observational and controlled laboratory setting in recreational and well-trained cyclist, respectively. The findings from these studies indicated that habitual antioxidant supplementation, for an average period ≥ 2 years, did not have an effect on the oxidative stress markers malondialdehyde (MDA), protein carbonylation (PC), total antioxidant capacity (TAC), or reduced glutathione (GSH), compared to a non-supplemented control group. Identifying these findings in a field based setting where exercise intensity cannot be controlled, and in a laboratory setting in two differing populations may provide a novel insight into how habitual supplementation can affect the oxidative stress response to exercise. It must be noted that the dose of vitamin C (153 mg/d) and vitamin E (47.2 IU/d or 31.6 mg/d) consumed in chapter 4 is above the recommended daily allowance of 90 mg/d and 22.4 IU/d (15 mg/d), respectively. And although the dose is lower than that used in experimental control studies, it reflects a habitual intake rather than a preselected dose that may not reflect consumption of commercially available products.

The findings from chapter 4 in a population of well-trained cyclists indicates low-dose antioxidant supplementation does not negatively affect the baseline concentrations of SOD1, SOD2, and GPx-1. The inhibition of the synthesis of these proteins has been postulated to occur through the inhibition of the RONS mediated redox signalling post-exercise (Gomez-Cabrera et al., 2006; Morrison et al., 2015; Ristow et al., 2009). The study in chapter 4 did not directly measure changes in redox signalling post-exercise, so it would be inappropriate to comment on the effects of low-dose antioxidant supplementation on these pathways and subsequent adaptations. From available evidence and the activity levels of the participants in this study, it might be postulated that variation in exercise intensity and duration may be important factors for regulating baseline endogenous antioxidant proteins in this population, and that low-dose antioxidant supplementation does not inhibit the production of endogenous antioxidants. To fully elucidate the effects of low-dose antioxidant supplementation future studies should employ chronic supplementation (> 6 months) and investigate the post-exercise redox signalling pathways to support or contradict the findings from chapter 4.

6.4 Impact of Montmorency Tart cherry supplementation on cycling recovery and subsequent days performance

Montmorency Tart cherry supplementation is thought to have favourable effects on exercise-induced oxidative stress, inflammation, and an additional effect on these markers during recovery (Bowtell et al., 2011; Howatson et al., 2010). Results from chapter 5 add to the growing body of evidence regarding the use of MC pre-and post-exercise but are not in agreement with previous findings. Supplementation in the preceding days, on the day, and during the recovery period failed to illicit any differences in the oxidative stress markers

MDA, PC, TAC, and the inflammatory marker IL-6; either during exercise or 24 hours after undertaking exercise compared to placebo (PLA). Results from previous studies provided an indication that the beneficial changes observed whilst consuming MC could potentially improve exercise performance in the hours or days following strenuous exercise. A time-trial undertaken 24 hours after completing a strenuous cycling protocol failed to indicate an ergogenic effect of MC compared to a PLA. Some of the beneficial effects of MC have been observed in studies that have restricted dietary polyphenols, so it may be advised that athletes with a low polyphenol intake may benefit from MC consumption.

6.5 Conclusions

The research conducted within this thesis has aimed to investigate the role of habitual antioxidant supplementation in recreational and trained cyclist on exercise-induced oxidative stress and exercise mediated adaptations, as well as investigating the effect of a polyphenol supplementation on cycling recovery and performance. The work presented here adds to this evidence base with the following novel findings:

1. Antioxidant supplementation in amateur cyclists was not dependent on age, training volume, or cycling experience. Thus, incidence of supplementation in this population cannot be predicted.
2. Habitual antioxidant supplementation did not provide additional protection against exercise-induced oxidative stress during field based or laboratory settings in recreational or trained cyclists.
3. The endogenous antioxidant enzymes were not affected by habitual antioxidant supplementation in trained cyclists. This suggests that moderate to low antioxidant supplementation did not have a negative effect on RONS mediated signalling.

4. MC supplementation did not attenuate plasma oxidative stress or inflammation markers during or post exercise in trained cyclists compared to placebo.
5. Time trial performance after completion a strenuous cycling protocol in the preceding 24 hours was not enhanced by 8 days supplementation with MC.

Page 1

Participant Information Sheet and Consent Form

The questionnaire that follows is to form part of a study carried out by the University of Birmingham. When the term study is used, it refers to the questionnaire. As the results of this questionnaire may be published, you the participant, have to provide consent for us to use any of the material you provide. There are no commercial interests and any information supplied to the University of Birmingham will be handled in accordance with the Data Protection Act 1998.

PARTICIPANT INFORMATION SHEET

Investigating habitual intake of antioxidants through diet and supplementation in cyclists competing in the Wiggle Dragon Ride

What is the study about?
The study is investigating the habitual use of dietary vitamins/antioxidants, and vitamin/antioxidant supplements within cyclists of varying abilities competing in the Dragon Ride. At present there is no data on the use of vitamin/antioxidant dietary intake and/or use of vitamin/antioxidant supplements within cyclists. If supplements are consumed in addition to normal dietary intake, we also want to identify why these supplements are being used within this population.

What will you have to do?
If you decide to partake in the study you will be required to fill in an anonymous questionnaire. There will be an option for you to provide us with an email address so we can invite you to participate in future research projects. The questionnaire will collect data on sex, age, training history, dietary intake, supplement intake, and reasons for supplement intake.

What if I do not wish to continue at any stage?
You are free to withdraw from the study at any time. You can refuse to answer any question on the questionnaire. If you provide an email address on your completed questionnaire, you have the right to withdraw this information by contacting the principle investigator.

What are the benefits?
The study will help us identify the use of dietary and supplemented vitamins/antioxidants in cyclists. Your participation in this study will contribute to a wider knowledge about the use of vitamins and/or antioxidants in cyclists and the reasons why cyclists decide to consume vitamin and/or antioxidant supplements. If you are interested in the research you can decide to be sent a report of the findings when the study is completed by providing an email address at the end of the questionnaire. You can also contact the principle investigator to ask any further questions regarding the research.

What are the risks?
There are no perceived risks in this study.

What happens to the information?
All information is completely confidential to the researcher. All data will be collected and analysed by the researchers at the University of Birmingham, and will not be passed on to any third parties.

Do I have to take part?
No. It is up to you to decide whether or not to participate. If you decide you would like to be part of this research, you will need to complete the questionnaire below and indicate in the appropriate box that you give your consent for us to use the data you have provided. You are still free to withdraw from the study at any time and without giving a reason.

Further information
If you have any further questions about participating in the study, please feel free to contact the principle investigator below.

Principle investigator:
Matthew Soden B.Sc.
School of Sports and Exercise Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT
mxs955@bham.ac.uk

1. I have read the information regarding the study and I am aware that I can ask any further questions regarding the study by contacting the principle investigator.

I also understand that I can decline to answer any particular questions in the study, and can decline to complete any task requested of me. I agree to provide information to the researchers on the understanding that it is completely confidential. I understand that the information will be stored in manual and electronic files and is subject to the provisions of the Data Protection Act. I acknowledge that the information provided is being used by the University in accordance with the Act.

I confirm that I wish to participate in this study under the conditions set out here and in the Information Sheet. (tick "yes" to confirm, or "no" to decline)

Yes		No	

If the participant answers NO to question 1 they are taken to the end of the questionnaire.

If they answer YES they move onto the 2nd page of the questionnaire (Q.2).

Page 2

Demographics

2. Are you male or female? Please tick…

Male		Female	

3. Which category below includes your age? Please tick…

18-25		40-44		60-64	
25-29		45-49		65-69	
30-34		50-54		70-74	
35-39		55-59		75 +	

4. How long have you been cycling for? Please tick…

3 Years or Less		4-7 Years		8-11 Years		12 Years or More	

5. On average, how long do you spend cycling a week? Please tick…

0 - 3 hours	
4 - 6 hours	
7 - 9 hours	
Over 10 hours	

6. Which route are you participating in at the Dragon Ride? Please tick…

Corto (37km)	
Medio Fondo (125km)	
Grand Fondo (206km)	

Page 3

Vegetables

7. Please estimate your consumption of these vegetables – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Aubergine, Okra							
Avocado							
Beetroot							
Broccoli, Spring Greens, Kale							
Cabbage							
Carrots							
Cauliflower							
Celery							

Courgettes, Marrow, Squash							
Cucumber							
Garlic							
Green Beans, Runner Beans, Broad Beans							
Leeks							
Lettuce							
Olives							
Parsnips							
Peas, Mushy Peas							
Peppers (any colour)							

Spinach							
Swedes							
Sweet corn							
Tomatoes (canned/raw/sauce)							
Watercress, Mustard & Cress							

Page 4

Fruit

8. Please estimate your consumption of these fruits – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Apples							
Apricots							
Bananas							
Grapes							
Grapefruit							
Kiwi Fruit							
Mangoes							
Melon							
Nectarines							
Oranges							

Papaya							
Peaches							
Pineapple							
Plums							
Raspberries							
Red Currents / Black Currents							
Rhubarb							
Strawberries							

Page 5

Meat

9. Please estimate your consumption of these meats – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A month	Less Than Once A Month	Never
Bacon							
Beef eg. Roast Steak, Mince, Stew, or Casserole							
Beef Burgers							
Corned Beef, Spam, Luncheon Meats							

Chicken or Other Poultry eg Turkey							
Ham							
Lamb eg Roast, Chops or Stew							
Liver, Liver Paté, Liver Sausage							
Offal eg Liver, Kidney, Giblets							
Pork eg Roast, Chops, Stew or Slices							

Sausages							
Savoury pies eg Meat, Pork, Pasties, Steak & Kidney, Sausage Rolls							

Page 6

Fish

10. Please estimate the consumption of these fish products – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Fried Fish in Batter, as in Fish & Chips							
Fish Fingers, Fish Cakes							
White Fish, Fresh, Canned or Frozen eg. Cod, Haddock, Plaice, Sole, Halibut							
Oily Fish, Fresh, Canned, or Frozen eg. Mackerel, Kippers, Tuna, Salmon, Sardines, Herring							
Shellfish, eg. Crab, Prawns, Mussels							
Fish Roe, Taramasalata							

Page 7

Bread and Cereal

11. Please estimate your consumption of these bread products – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Crisp Bread eg. Rivita							
Granary Bread and Rolls							
White Bread and Rolls							
Wholemeal Bread and Rolls							

12. Please estimate your consumption of these cereals – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Breakfast Cereals containing fortified Vitamins							

Page 8

Drinks

13. Please estimate your consumption of these drinks (one serving = one small glass or cup)

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Cocoa, Hot Chocolate							
Herbal Tea							
Orange Juice							
Apple Juice							
Cranberry Juice							
Grapefruit Juice							
Grape Juice							
Red Wine							

Page 9

Oils and Snacks

14. Please estimate your usage of the following oils – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Canola Oil							
Coconut Oil							
Mixed Nut Oil							
Olive Oil							
Peanut Oil							
Vegetable Oil							

15. Please estimate your consumption of the following snacks – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Cakes (Home or Ready-Made) eg. Fruit, Sponge							
Almonds (Handful)							
Mixed Dried Fruit eg. Apricots, Apples, Pears, Mangoes							

Sunflower Seeds (Small Handful)							
Fruit Bars eg Apricot, Date (One)							
Cereal Bars / Flapjacks (One)							
Peanuts or Other Nuts (Small Handful)							
Figs							
Prunes							
Mixed Nuts & Raisins (Small Handful)							
Dates							

Page 10

Antioxidant and Vitamin Supplements

16. Do you consume vitamin or antioxidant supplements?

Yes		No	

If YES, then the participant will carry onto Q.17. If NO, then participant will be taken to Q.20

17. How long have you been consuming vitamin or antioxidant supplements? Please tick…

Less Than 6 Months		Between 6 & 12 Months		Longer Than 12 Months	

18. Why do you consume vitamin or antioxidant? Please tick appropriate boxes…

Boost Immunity / Improve Health	
Peer Recommendations (Coach / Family / Friends)	
To Improve Performance	
Reduce Fatigue / Recovery Time	
To Improve Training Adaptations	
Recommendations from a Dietician / Sports Nutritionist	
To Maintain a Balanced Diet Due to Inadequate Intake	

Other, please state in the section below

19. Please indicate which vitamin or antioxidant supplements you use and how often they are consumed – please enter a tick for each item

	More Than Once A Day	Once A Day	4-6 Days A Week	1-3 Days A Week	At Least Once A Month	Less Than Once A Month	Never
Beta Carotene							
Iron							
Magnesium							
Minerals							
Multivitamins							
Grape-Seeds							
Quercetin							
Omega 3							
Vitamin A							
Vitamin B-1							
Vitamin B-2							
Vitamin B-6							

Vitamin B-12							
Vitamin C							
Vitamin D							
Vitamin E							
Zinc							

Please enter any vitamin or antioxidant supplements not on the above list and how often you take them.
(More Than Once a Day, Once a Day, 4-6 Times a Week, 1-3 Times a Week, At Least Once a Month)

Page 11

20. Would you like to be contacted by the investigation team with a summary of the present study?

Yes		No	

If YES, then the participant will move onto Q.21. If NO, then the participant will move on to Q.22

21. Please enter your email address

22. If you live within the Midlands area or a suitable travelling distance from the University of Birmingham would you like to be invited to future research projects? All participants are reimbursed for travel expenses and their time.

Yes		No	

If YES, then the participant will move onto Q.23. If NO, then the participant will be taken to the end page of the questionnaire

23. Please enter your email address

End of Questionnaire

UNIVERSITY OF BIRMINGHAM

Participant Information Sheet

Examining the effects of a cycling sportive on antioxidant status and oxidative stress markers in cyclists who do, and do not, take antioxidant supplements.

Location
The study will be conducted at the Tommy Godwin Challenge sportive on Sunday 28th September, 2014 in Solihull.

What is the study about?
Recent research has shown that consumption of antioxidant supplements, specifically vitamin C and vitamin E, might reduce some of the favourable adaptations that happen as a result of exercise. We want to investigate this further by measuring the changes that occur when we exercise in 2 groups of cyclists: those who **do,** and those who **do not** take antioxidant supplements. We want to assess indicators of exercise adaptation in these 2 groups of people before and after a cycling sportive.

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are free to withdraw at any time and without giving a reason. You can withdraw from the study before any measures have been taken, before or after the sportive has been completed, or when all the measures have been collected. After the samples have been collected you can withdraw from the study up until the 26th October 2014.

What will I have to do?
If you decide to take part in the study you will be asked to provide 2 blood samples. One before and one after the Tommy Godwin Challenge sportive. The first blood sample will be taken at least 30 minutes before the start of the sportive. We will contact you in the week leading up to the sportive to arrange a suitable time to collect the first blood sample You will also be asked to disclose some personal information, for example your date of birth and email address. We would want to record your finishing time of the sportive and your reason for undertaking the event.

What are the benefits?
The study will improve our understanding of how antioxidant supplements may affect exercise training adaptations. Your participation in the study will contribute to a wider knowledge in the exercise community on the use of antioxidant supplements and may help inform how best to use supplements such as these in the future. If you are interested in the research you can choose to be sent a report of the findings once the research has been completed. You can also ask the researcher any questions about the study, before or during your participation. You can also enquire about future research projects that may involve fitness tests, body measurements, or nutritional interventions.

What are the risks?
Two blood samples will be taken, one before, and one after the sportive. You may experience a little discomfort when the needle is inserted, however the researchers are experienced in this technique so the pain experienced will be minimal. You may also develop a small bruise on your arm which can be prevented by applying pressure on the arm when the needle has been taken out. When giving blood there is a small chance of feeling faint, if this does occur you will be monitored before the start of the

sportive to assess whether it will affect your participation in the event. If any complications occur as a result of giving blood and you are unable to take part in the sportive you will be entitled to a full reimbursement of your entry fee (30km £12, 100km £20).

What happens to the information?
All information is completely confidential to the researcher. All information will be identified by code number, and will be seen only by the researcher. Blood samples will be collected, stored and analysed by the researchers at the University of Birmingham.

What happens if something goes wrong on the day of the study?
This study and all of the procedures within it have been approved by the science, engineering and mathematics ethical review committee (ERN_14-0921). As such the study is included within the University of Birmingham Liability Insurances and if you are harmed in any way by taking part in this research project your normal rights apply and you may have grounds for legal action.

What do I do now?
If you are still interested in being part of this study you will be asked to complete an informed consent form to confirm that you are happy to participate in the research. You will be asked to keep a copy of this information sheet and the signed consent forms. If you change your mind you are free to withdraw from the study at any time and without giving any reason.

Further Information
If you have any further questions about participating in the study, please feel free to contact one of the principle investigators below.

Investigator

Matthew Soden
School of Sport, Exercise, and Rehabilitation Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Principal Investigator

Dr Sarah Aldred
School of Sport, Exercise, and Rehabilitation Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Appendix 3: Consent form Chapter 3

Study Number:_____

Subject Identification Number:_____

UNIVERSITY OF BIRMINGHAM

CONSENT FORM

Title of Project: **Examining the effects of a cycling sportive on antioxidant status and oxidative stress markers in cyclists who do, and do not, take antioxidant supplements.**

Name of Researcher: Mr Matthew Soden, Dr Sarah Aldred

Please initial all boxes

1. I confirm that I have read and understand the information sheet dated for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason up until the 26th October 2014, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my data collected during the study, may be looked at by individuals from the University of Birmingham. I give permission for these individuals to have access to my records. ☐

4. I understand that my participation in this study will involve me having two blood samples taken as stated in the participant information sheet. ☐

5. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent.	Date	Signature

Participant Personal Information
Tommy Godwin Challenge Sportive Sunday 28th September 2014

Pre-Sportive

Subject Identification Number: .....

Name: .....

Date of birth: .....

Age on 28th September 2014: .....

Email address: .....

Which distance are you undertaking? 29km 100km

How long have you cycled for (years)? .....

On average how many hours do you cycle per week? .....

How many cycle sportives have you completed prior to the current one? .....

.....

What is your reason for participating in the Tommy Godwin Challenge sportive? .....

.....

Do you currently consume any vitamin or antioxidant supplement? YES NO

If yes, how long have you taken them for? (Please state years or months).....

How often do you take these supplements? (eg. daily, once a week) .....

.....

What vitamin and/or antioxidant supplements do you take? (eg. vitamin C, multivitamins, etc) .....

.....

Post Sportive

What was your finishing time? .....

Do you feel your training prepared you for the event? YES NO

UNIVERSITY OF BIRMINGHAM

Participant Information Sheet

The effects of habitual antioxidant supplementation on endogenous antioxidants and oxidative damage in well trained cyclists

Location
The study will be held at the Human Performance Laboratory (HPL), at the department of Sport and Exercise Sciences within the University of Birmingham.

What is the study about?
The study is investigating whether there is a difference in antioxidants that are produced within the body, known as endogenous antioxidants, between cyclists who habitually consume antioxidant supplements and cyclists who do not. Antioxidants are present naturally in the human body and are also found in foods such as fruit and vegetables. Vitamins are a good example of antioxidants in diet. These compounds can act as defences against stressors in the body, and are critical for metabolism. Recent evidence has demonstrated that the use of antioxidant supplements reduce the body's ability to produce its own defence system against oxidants, which are usually increased after a bout of exercise. Studies identifying these effects typically use short term intervention periods over 4 to 8 weeks. Therefore, we want to identify if habitual antioxidant supplementation has an effect on the levels of endogenous antioxidants (the body's adapted form of defence against oxidants) at rest and in response to an exhaustive bout of exercise.

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason.

What will I have to do?
If you decide to take part in the study you will be required to attend the Sport and Exercise Science Laboratory at Birmingham University on 2 separate occasions. On the first visit a muscle biopsy will be obtained from the thigh and a fitness test (VO_{2max} test) will be carried out on a stationary cycle ergometer. You will be given a 3 day food diary that you will be asked to fill in before returning to the laboratory. The second visit will be arranged 7-14 days after the first visit at a convenient date for you. We will ask you not to exercise for the 48 hours before your return to the lab and you will be required to attend in a fasted state (you can drink as much as you wish). Upon arrival a cannula will be placed in your arm to allow blood collection pre-during-post exercise. The exercise protocol will last for approximately 70 minutes and will last until voluntary fatigue has occurred.

Arriving at the laboratory

Before every visit it is important to avoid foods containing high levels of nitrates in the 24 hour period leading up to your return to the laboratory. We will therefore ask you not to eat foods such as: beetroots, spinach, and lettuce. On the day of the test we would like you to abstain from eating 6 hours before returning, and to avoid any caffeinated or sugar based drinks until after the laboratory

visit is complete. You are free to drink as much water as you please. We will also ask you not to exercise for 48 hours prior to returning to the lab.

1st Laboratory Visit

On your 1st visit to the Human Performance Laboratory you will have a small incision made in your thigh and a sample of muscle just smaller than a pea, will be extracted. After this you will then be invited to undertake a fitness test known as a VO_{2max} test on a stationary cycle ergometer that will last no longer than 30 minutes. The VO_{2max} test assesses the maximum oxygen volume that an individual can achieve, and this is a representation of your maximal ability in exercise. This allows us, as researchers to identify the correct intensity for your exercise tests. For example, your 2nd exercise bout involves a moderate intensity workload that is the equivalent to 60% of your maximal ability or VO_{2max} and a vigorous intensity workload that is the equivalent to 80% of your maximal ability or VO_{2max}.

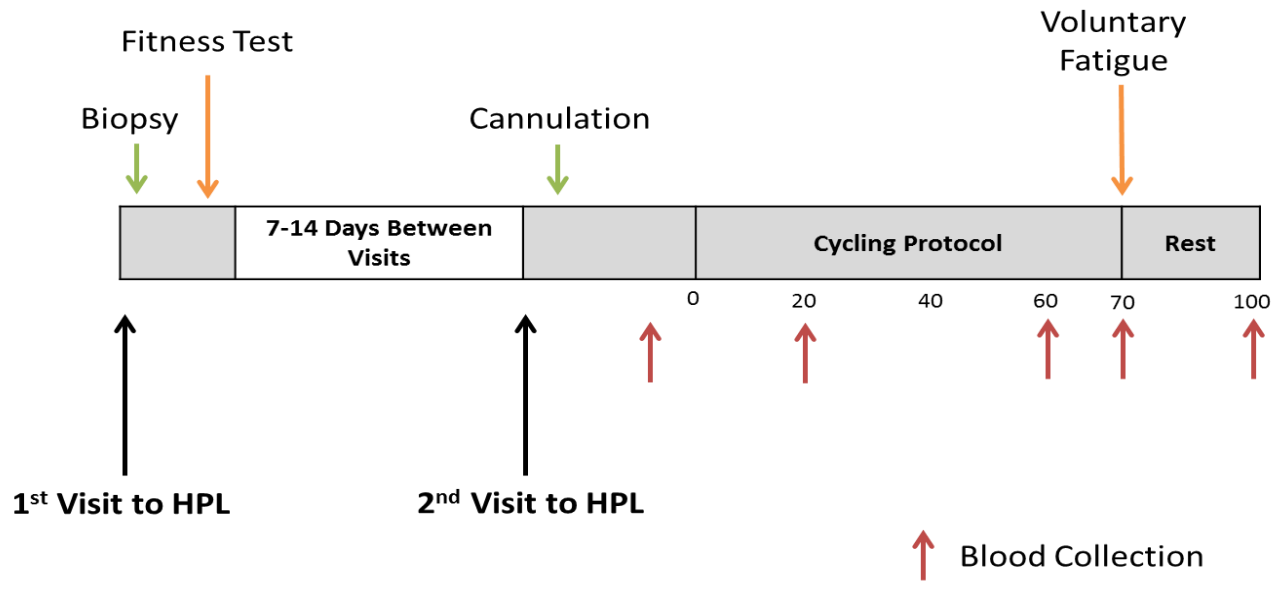
Before 2nd Laboratory Visit

We will ask you to keep a food diary for 3 days before you return for your 2nd visit to the laboratory. All meals, snacks, and drinks should be recorded and you will be asked to bring the food diary with you.

2nd Laboratory Visit

On your 2nd visit to the Human Performance Laboratory you will have a cannula placed in your arm by a trained researcher. This allows us to take several blood samples without the need for multiple needle pricks. The amount of blood taken for each sample is approximately the volume of a single tablespoon. A baseline blood sample will be taken before you commence any form of exercise. You will then perform 2 steady state cycling protocols, one for 25 minutes at a moderate intensity equal to 60% of your VO_{2max}, followed by an exhaustive cycling protocol equal to 80% of your VO_{2max} for 40 minutes on a stationary cycle ergometer. A blood sample will be collected at key stages during the test. Throughout the cycling protocol, a gas analyser will be used to determine you are working at the right intensity. As you attend the exercise session in the fasted state, upon completion of the trial you will be provided with a meal to consume, if you wish.

An outline of the study protocol



What are the benefits?

The study may help develop our understanding on how antioxidant supplements may affect training adaptations. Your participation in the study may help contribute to the wider knowledge on the use of antioxidant supplements and how best to use them in the future. If you are interested in the research

you can choose to be sent a report of the findings once the research has been completed. You can also ask the researcher any questions about the study, during your participation.

You will also receive training information from the fitness test. We can determine your fitness and give you information about appropriate training zones that could facilitate future training programs. You will be compensated up to the sum of £50 for travel, parking expenses etc.

What are the risks?
The aerobic fitness test and exhaustive cycling protocol may cause you to experience fatigue, which will be short lived and you should have fully recovered within two hours of the process. As you are healthy and active this risk is extremely small and the procedures are regularly conducted within the laboratory. All experimenters are first aid trained.

During the 2nd visit to the laboratory 5 blood samples will be taken over the course of the main experimental visit. You may experience a little discomfort when the cannula is inserted, however the researchers are experienced in this technique so the pain experienced will be minimal. You may also develop a small bruise on your arm which can be prevented by applying pressure on the arm when the needle has been taken out.

On your 1st visit to the HPL a muscle biopsy will be taken from the outside of the thigh. This procedure is a standard tool and has been used successfully on a regular basis by experienced staff in this laboratory. You may experience a small amount of pain while the local anaesthetic is administered. Thereafter the procedure may be associated with a feeling of pressure and/or mild discomfort, but only for a short time. After the experiment, your legs may still feel a bit stiff or sore but this should not last longer than 2-3 days. Sometimes a small scar, the width and half the length of a matchstick (5mm), where the incision was made will remain visible for a longer period of time. There is a minimal chance of infection of the wound, but we will use strict sterile techniques to minimize this risk. Complications such as bruising, infection and pain have been reported but this is rare (less than 2% chance). In the unlikely event that you experience any of the above symptoms, or any other symptoms related to the muscle biopsy following the study, we will contact the study physician who will be happy to discuss the problem with you. In the rare event that they are unavailable our advice would be to visit the Accident and Emergency Unit of your local hospital.

The National Health Service (NHS) provides a Patient Advice and Liaison Service (PALS) for any concerns that may arise after the muscle biopsy has taken place whilst the University department is not accessible due to it being closed. You, a family member, friend, or career can contact PALS and all matters will be addressed with confidentially. The nearest PALS to the University of Birmingham is listed below:

Patient Advice and Liaison Services (PALS): Queen Elizabeth Hospital
Birmingham
Edgbaston
Queen Elizabeth Medical Centre
West Midlands
B15 2TH

Contact Telephone Number: 0121 371 3280
Contact Email Address: PALS@uhb.nhs.uk

If you do not live near to the Queen Elizabeth Hospital visit their website to find the nearest PALS provider in your area: www.nhs.uk

All risks will be minimized by safe practice and conducted by trained members of the research team. If at any point during the protocol you feel uncomfortable or unable or unwilling to continue, testing will be ceased immediately.

What happens to the information?
All information is completely confidential to the researcher. All information will be identified by code number, and will be seen only by the researcher. Blood samples will be collected, stored and analysed by the researchers at the University of Birmingham.

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason.

What happens if something goes wrong on the day of the trials?
All procedures have been included within the University of Birmingham Liability Insurances and if you are harmed in any way by taking part in this research project your normal rights apply and you may have grounds for legal action.

What do I do now?
You will be asked to complete an informed consent form to confirm that you are happy to participate in this study. You will be asked to keep a copy of this information sheet and the signed consent forms. If you change your mind **YOU ARE FREE TO WITHDRAW FROM THE STUDY AT ANY TIME AND WITHOUT GIVING ANY REASON.**

Further Information
If you have any further questions about participating in the study, please feel free to contact one of the principle investigators below.

Matthew Soden
School of Sport and Exercise Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Dr Sarah Aldred
School of Sport and Exercise Science
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Appendix 6: Consent form Chapter 4

Study Number:_____

Subject Identification Number:_____

UNIVERSITY OF BIRMINGHAM

CONSENT FORM

Title of Project: **The effects of habitual antioxidant supplementation on endogenous antioxidants and oxidative damage in well trained cyclists**

Name of Researcher: Mr Matthew Soden, Dr Sarah Aldred

Please initial all boxes

6. I confirm that I have read and understand the information sheet dated for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

7. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

8. I understand that relevant sections of my data collected during the study, may be looked at by individuals from the University of Birmingham. I give permission for these individuals to have access to my records. ☐

9. I understand that my participation in this study will involve me having a muscle sample taken using the muscle biopsy procedure stated in the participant information sheet. ☐

10. I understand that my participation in this study will involve me having five blood samples taken as stated in the participant information sheet. ☐

11. I agree to take part in the above study. ☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

UNIVERSITY OF BIRMINGHAM

Participant Information Sheet

The Impact of Antioxidant Supplementation on Recovery and Level of Repeated Performance.

Location
The study will be held at the Human Performance Laboratory (HPL), at the department of Sport, Exercise and Rehabilitation Sciences within the University of Birmingham.

What is the study about?
Recent evidence suggests that cherry juice, a fruit-derived polyphenolic compound, can significantly reduce the muscle damage, which often occurs as a result of intense exercise. This study will investigate the effect of drinking cherry juice on cycling performance following a bout of intense exercise a day prior. We would like to investigate the effect of drinking cherry juice for 7 days on time-trial performance. We also wish to investigate the physiological changes, i.e. the changes that might occur in the blood stream and the muscle, as a result of drinking the cherry juice, which would explain any performance benefits we might see.

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason. You can withdraw from the study up until the 1st April 2015 for any reason, either before or after any of the trials you have undertaken or about to complete.

What will I have to do?
If you decide to take part in the study you will be invited to attend the Human Performance Laboratory (HPL) located at the School of Sport, Exercise and Rehabilitation Sciences at Birmingham University on 3 separate occasions over the course of 10 days. On the first visit a fitness test (VO_{2max} test) will be carried out on a stationary cycle ergometer, similar to an exercise bike you would find in the gym. This will help us to determine the workload for the subsequent exercise trials. The test enables us to work out your maximal power output (WattMax) by increasing the amount of energy (watts) you have to produce during the fitness test. Within this visit you will also undertake a 20km time-trial to familiarise yourself with the protocol that will be used later in the study. You will then be given a supply of Montmorency cherry juice or a placebo drink and instructions on when and how to consume the drinks. The second visit to the HPL will be arranged 7-10 days after the first visit at a convenient date for you. We will ask you not to exercise for the 48 hours before your return to the lab and you will be required to attend in a fasted state (you can drink as much water as you please). Upon arrival a cannula will be placed in your arm to allow blood collection pre-during-post exercise. Five blood samples will be taken in total, but because we will be using a cannula you probably won't notice we are taking a sample. The exercise protocol will last for approximately 70 minutes and will last until voluntary fatigue has occurred. The third laboratory visit will comprise a 30 minute steady state cycling period followed by a time-trial, where 20km is cycled as quickly as possible***. Before you undertake the study you can decide whether you would like to undertake both of the***

experimental conditions. This involves consuming both the Montmorency cherry juice and placebo drinks. This will allow us to determine whether either of the drinks has an effect on your personal time-trial performance, rather than your performance being part of a group comparison, which would be the case if you just undertake 1 part of the study. If you decide to undertake both experimental conditions there will be a 14 day washout period between the two conditions. Thereafter, you repeat the first part of the study; you will consume the opposing drink for the same period of time as during your first trial and the protocols undertaken for the 2nd and 3rd visit will be replicated. This is an addition to the study and you **are not required to complete this, it is completely optional to you as a volunteer**.

Arriving at the laboratory

For every visit it is important to avoid foods containing high levels of nitrates in the 24 hour period leading up to your return to the laboratory. We will therefore ask you not to eat foods such as: beetroots, spinach, and lettuce the day before you come in. On the day of the test we would like you to abstain from eating 6 hours before returning, and to avoid any caffeinated or sugar based drinks until after the laboratory visit is complete. You are free to drink as much water as you please. We will also ask you not to exercise for 48 hours prior to returning to the lab.

1st Laboratory Visit

On your 1st visit to the Human Performance Laboratory a baseline blood sample will be collected from your arm by a trained researcher. You will then be invited to undertake a fitness test known as a VO_{2max} test on a stationary cycle ergometer that will last no longer than 30 minutes. The VO_{2max} test assesses the maximum oxygen volume and power output that an individual can achieve, and this is a representation of your maximal ability in exercise. This allows us, as researchers, to identify the correct intensity for your exercise tests. Following this, you will be required to familiarise yourself with the time-trial protocol that will be used in your 3rd visit to the laboratory.

Before 2nd Laboratory Visit

For the 7 days prior to your 2nd visit you will need to consume the cherry juice, or placebo, as directed. We will ask you to keep a food diary for 3 days before you return for your 2nd visit to the laboratory. All meals, snacks, and drinks should be recorded and you will be asked to bring the food diary with you.

2nd Laboratory Visit

On your 2nd visit to the Human Performance Laboratory you will have a cannula inserted into your arm by a trained researcher. This is a very small flexible cannula that allows us to take several blood samples without the need for multiple needle pricks. The amount of blood taken for each sample is approximately the volume of a single teaspoon. A baseline blood sample will be taken before you commence any form of exercise. You will then be asked to perform 3 different steady state cycling intensities for a period of 70 minutes. For the first 25 minutes you will be asked to maintain a moderate workload equal to 60% of your WattMax. After this period you will be required to cycle for 40 minutes at an intense workload equal to 80% of your WattMax, followed by a final workload equal to 95% of your WattMax until voluntary fatigue; all completed on a stationary cycle ergometer. A blood sample will be collected at the end of each workload during the test. As you attend the exercise session in the fasted state, upon completion of the trial you will be provided with a meal to consume, if you wish.

3rd Laboratory Visit

On arrival a blood sample will be collected when you first arrive at the HPL. You will be asked to perform 30 minutes of steady-state cycling at 60% WattMax followed by a 20km time-trial where you should aim to complete the distance as quickly as possible. After completing the time trial a 2nd blood sample will be taken. Again, upon completion of the trial you will be provided with a meal to consume, if you wish.

On completion of this first section of the study, your total time in the study will be 8 days, and we will take 4 blood samples.

Cross Over Study Design (optional for participants who wish to undertake both experimental conditions)

If you decide to undertake both experimental conditions, as explained in the "what will I have to do?" section above, you will be consuming both the Montmorency cherry juice and the placebo drink. In between these 2 supplements, there will be a 14-day washout period where you will not be consuming any antioxidant supplement. After this period you will be invited back to the laboratory to repeat the first part of the study, this time consuming a different supplement. You will not be told which supplement you receive, to prevent any interference with your performance in the exercise tests. If you take part in both sections of our study your total time in the study will be 30 days, which includes the 14-day washout period. Eight blood samples will be taken in total over this 30-day period. As stated above, if you take part in both sections of the study it will allow us to determine whether either of the drinks has an effect on your ***personal*** time-trial performance, rather than your performance being part of a group comparison, which would be the case if you just undertake 1 part of the study.

What are the benefits?
The study may help develop our understanding on how antioxidant supplements may affect cycling performance. Your participation in the study may help contribute to the wider knowledge on the use of antioxidant supplements and how best to use them in the future. If you are interested in the research you can choose to be sent a report of the findings once the research has been completed. You can also ask the researcher any questions about the study, during your participation.

You will also receive training information from the fitness test. We can determine your fitness and give you information about appropriate training zones that could facilitate future training programs.

What are the risks?
The aerobic fitness test and exhaustive cycling protocol may cause you to experience fatigue, which will be short lived and you should have fully recovered within two hours of the process. As you are healthy and active this risk is extremely small and the procedures are regularly conducted within the laboratory. All experimenters are first aid trained.

During visits 2 and 3 to the laboratory blood samples will be taken over the course of the main exercise protocol. You may experience a little discomfort when the cannula is inserted, however the researchers are experienced in this technique so the pain experienced will be minimal. You may also develop a small bruise on your arm which can be prevented by applying pressure on the arm when the needle has been taken out.

What happens to the information?
All information is completely confidential to the researcher. All information will be identified by code number, and will be seen only by the researcher. Blood samples will be collected, stored and analysed by the researchers at the University of Birmingham. After the study has been completed all primary research data and evidence will be accessible in confidence to authorised researchers for verification purposes. The data will normally be preserved and accessible for 10 years.

Do I have to take part?
No. Taking part in this study is entirely voluntary. If you would like to participate, you will be given this information sheet to keep and be asked to sign a consent form, but you are still free to withdraw at any time and without giving a reason.

What happens if something goes wrong on the day of the trials?
All procedures have been included within the University of Birmingham Liability Insurances and if you are harmed in any way by taking part in this research project your normal rights apply and you may have grounds for legal action.
What do I do now?

If you meet the following inclusion/exclusion criteria (male, aged between 18-35, undertake 2 to 5 hours of moderate intensity exercise per week, non-smoker, do not suffer from chronic pulmonary, cardiovascular, autoimmune, metabolic, haemostatic, anaemic or malignant diseases; and have not taken any vitamin/antioxidant supplements, anti-inflammatory, or prophylactic medications in the 2 weeks before the study starts) you will be asked to complete an informed consent form to confirm that you are happy to participate in this study. You will be asked to keep a copy of this information sheet and the signed consent forms. If you change your mind you are free to withdraw at any time and without giving a reason. You can withdraw from the study up until the 1st April 2015 for any reason, either before or after any of the trials you have undertaken or about to complete.

Further Information
If you have any further questions about participating in the study, please feel free to contact one of the principle investigators below.

Co-Investigators

Chris Fagg
School of Sport, Exercise, and Rehabilitation Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Matthew Soden
School of Sport, Exercise, and Rehabilitation Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

Principal Investigator

Dr Sarah Aldred
School of Sport, Exercise, and Rehabilitation Sciences
University of Birmingham
Edgbaston
Birmingham
B15 2TT

UNIVERSITY OF BIRMINGHAM

Subject Identification Number: _____

CONSENT FORM

Project title: The Impact of Antioxidant Supplementation on Recovery and Level of Repeated Performance.

Please initial box:

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to ask questions and that I agree to take part in the above study. ☐
2. I understand that my participation is voluntary and that I am free to withdraw any time without my providing any reason up until the 1st April, 2015. ☐
3. I understand that my data and personal information will be saved on the work computer, and hard copies will stored in a locked filing cabinet accessible only by the lead researchers and will be kept for a period of 10 years, unless I ask to have this information deleted. ☐
4. I understand that my participation in this study will involve me having blood samples taken as stated in the Participant Information Sheet. ☐
5. I agree to take part in the above study. ☐

<u>If you have opted to volunteer for the cross-over study, please initial the boxes below:</u> ☐

1. I confirm that I have read the additional information on the participant information sheet entitled “Cross Over Study Design”.
2. I understand my participation is voluntary and that I am free to withdraw from the study during any portion of the “cross-over design” up until the 14th April, 2015. ☐

_____	_____	_____
Name of Participant	Date	Signature
_____	_____	_____
Name of Researcher	Date	Signature

Subject ID No……

The University of Birmingham

School of Sport and Exercise Sciences

General Health and Screening Questionnaire

Phone/Email: .....

Name of the responsible investigator for the study:

.....

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. Your answers will be kept strictly confidential.

1.	You are.....	Male	Female
2.	What is your exact date of birth? Day..... Month.....Year 19..... So your age is..... Years		
3.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
4.	Are you currently taking any medication?	YES	NO
5.	Have you taken any anti-inflammatory or prophylactic medications in the last 2 weeks?	YES	NO
6.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
7.	Do you currently smoke?	YES	NO
8.	Has your doctor ever diagnosed you with any of the following disease states? • Cardiovascular • Metabolic • Pulmonary • Haemostatic • Anaemic • Malignant	YES	NO
9.	Has your doctor ever said you have “heart trouble”?	YES	NO

10.	Has your doctor ever said you have high blood pressure?	YES	NO
11.	Have you ever taken medication for blood pressure or your heart?	YES	NO
12.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
13.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
14.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
15.	Have you had a cold or feverish illness in the last month?	YES	NO
16.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
17.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
18.	Do you suffer from asthma?	YES	NO
19.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
20.	Has your doctor ever said you have diabetes?	YES	NO
21.	Have you ever had viral hepatitis?	YES	NO
22.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
23.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO
24.	Do you take part in physical activity one or more times a week?	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed:..... **Date:**.....

References

Allen, D.G., Lamb, G.D. and Westerblad, H. (2008) Skeletal muscle fatigue: Cellular mechanisms. **Physiological Reviews,** 88 (287): 332.

Aoi, W., Naito, Y., Takanami, Y., et al. (2004) Oxidative stress and delayed-onset muscle damage after exercise. **Free Radical Biology & Medicine,** 37 (4): 480-487.

Arent, S.M., Pellegrino, J.K., Williams, C.A., et al. (2010) Nutritional supplementation, performance, and oxidative stress in college soccer players. **Journal of Strength & Conditioning Research,** 24 (4): 1117-1124.

Bailey, D.M., Williams, C., Betts, J.A., et al. (2011) Oxidative stress, inflammation and recovery of muscle function after damaging exercise: effect of 6-week mixed antioxidant supplementation. **European Journal of Applied Physiology,** 111 (6): 925-936.

Barbieri, E. and Sestili, P. (2012) Reactive oxygen species in skeletal muscle signaling. **Journal of Signal Transduction,** 2012 1-17.

Bedard, K. and Krause, K. (2007) The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. **Physiological Reviews,** 87 (1): 245-313.

Beecher, G.R. (2003) Overview of dietary flavonoids: nomenclature, occurrence and intake. **Journal of Nutrition,** 133 (10): 3248S-3254S.

Bell, P.G., McHugh, M.P., Stevenson, E., et al. (2014a) The role of cherries in exercise and health. **Scandinavian Journal of Medicine & Science in Sports,** 24 477-490.

Bell, P.G., Stevenson, E., Davison, G.W., et al. (2016) The effects of Montmorency Tart Cherry concentrate supplementation on recovery following prolonged, intermittent exercise. **Nutrients,** 8 441.

Bell, P.G., Gaze, D.C., Davison, G.W., et al. (2014b) Montmorency tart cherry (Prunus cerasus L.) concentrate lowers uric acid, independent of plasma cyanidin-3-O-glucosiderutinoside. **Journal of Functional Foods,** 11 82-90.

Bell, P.G., Walshe, I.H., Davison, G.W., et al. (2015) Recovery facilitation with Montmorency cherries following high-intensity, metabolically challenging exercise. **Applied Physiology, Nutrition, & Metabolism,** 40 (4): 414-423.

Bell, P.G., Walshe, I.H., Davison, G.W., et al. (2014c) Montmorency cherries reduce the oxidative stress and inflammatory responses to repeated days high-intensity stochastic cycling. **Nutrients,** 6 (2): 829-843.

Berlett, B.S. and Stadtman, E.R. (1997) Protein oxidation in aging, disease, and oxidative stress. **Journal of Biological Chemistry,** 272 (33): 20313-20316.

Berzosa, C., Cebrian, I., Fuentes-Broto, L., et al. (2011) Acute exercise increases plasma total antioxidant status and antioxidant enzyme activities in untrained men. **Journal of Biomedicine & Biotechnology,** 2011 540458.

Bjelakovic, G., Nikolova, D. and Gluud, C. (2014) Antioxidant supplements and mortality. **Current Opinion in Clinical Nutrition & Metabolic Care,** 17 (1): 40-44.

Blando, F., Gerardi, C. and Nicoletti, I. (2004) Sour cherry (Prunus cerasus L) anthocyanins as ingredients for functional foods. **Journal of Biomedicine & Biotechnology,** 2004 (5): 253-258.

Bloomer, R.J., Davis, P.G., Consitt, L.A., et al. (2007) Plasma protein carbonyl response to increasing exercise duration in aerobically trained men and women. **International Journal of Sports Medicine,** 28 (1): 21-25.

Bloomer, R.J., Goldfarb, A.H. and McKenzie, M.J. (2006) Oxidative stress response to aerobic exercise: comparison of antioxidant supplements. **Medicine & Science in Sports & Exercise,** 38 (6): 1098-1105.

Bloomer, R.J., Goldfarb, A.H., Wideman, L., et al. (2005) Effects of acute aerobic and anaerobic exercise on blood markers of oxidative stress. **Journal of Strength & Conditioning Research,** 19 (2): 276-285.

Bowtell, J.L., Sumners, D.P., Dyer, A., et al. (2011) Montmorency cherry juice reduces muscle damage caused by intensive strength exercise. **Medicine & Science in Sports & Exercise,** 43 (8): 1544-1551.

Braakhuis, A.J. (2012) Effect of vitamin C supplements on physical performance. **Current Sports Medicine Reports,** 11 (4): 180-184.

Braakhuis, A.J. and Hopkins, W.G. (2015) Impact of Dietary Antioxidants on Sport Performance: A Review. **Sports Medicine,** 45 (7): 939-955.

Braun, H., Koehler, K., Geyer, H., et al. (2009) Dietary Supplement use among Elite Young German Athletes. **International Journal of Sport Nutrition & Exercise Metabolism,** 19 97-109.

Breen, L., Philp, A., Witard, O.C., et al. (2011) The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. **Journal of Physiology,** 589 (Pt 16): 4011-4025.

Brickson, S., Hollander, J., Corr, D.T., et al. (2001) Oxidant production and immune response after stretch injury in skeletal muscle. **Medicine & Science in Sports & Exercise,** 33 (12): 2010-2015.

Brickson, S., Ji, L.L., Schell, K., et al. (2003) M1/70 attenuates blood-borne neutrophil oxidants, activation, and myofiber damage following stretch injury. **Journal of Applied Physiology,** 95 969-976.

British Cycling (2012) **After the gold rush: 200,000 rise in regular cyclists.** [Online]. Available from: http://www.britishcycling.org.uk/about/article/bc20121206-about-bc-news-After-the-gold-rush--200-000-rise-in-regular-cyclists-0 [Accessed September/20 2014].

Bunpo, P. and Anthony, T.G. (2016) Ascorbic acid supplementation does not alter oxidative stress markers in healthy volunteers engaged in a supervised exercise program. **Applied Physiology Nutrition & Metabolism,** 41 (2): 175-180.

Burgomaster, K.A., Hughes, S.C., Heigenhauser, G.J., et al. (2005) Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. **Journal of Applied Physiology,** 98 (6): 1985-1990.

Burke, L.M., Hawley, J.A., Wong, S.H., et al. (2011) Carbohydrates for training and competition. **Journal of Sports Sciences,** 29 (Suppl 1): S17-27.

Burns, R.D., Schiller, M.R., Merrick, M.A., et al. (2004) Intercollegiate student athlete use of nutritional supplements and the role of athletic trainers and dietitians in nutrition counseling. **Journal of the American Dietetic Association,** 104 (2): 246-249.

Butterfield, T.A., Best, T.M. and Merrick, M.A. (2006) The dual roles of neutrophils and macrophages in inflammation: A critical balance between tissue damage and repair. **Journal of Athletic Training,** 41 (4): 457-465.

Carmen Gomez-Cabrera, M., Ristow, M. and Vina, J. (2012) Antioxidant supplements in exercise: worse than useless? **American Journal of Physiology Endocrinology & Metabolism,** 302 (4): E476-E477.

Carr, A. and Frei, B. (1999) Does vitamin C act as a pro-oxidant under physiological conditions? **FASEB Journal,** 13 (9): 1007-1024.

Carroll, T.J., Taylor, J.L. and Gandevia, S.C. (2017) Recovery of central and peripheral neuromuscular fatigue after exercise. **Journal of Applied Physiology,** 122 (5): 1068-1076.

Chong, J., Poutaraud, A. and Hugueney, P. (2009) Metabolism and roles of stilbenes in plants. **Plant Science,** 177 143-155.

Cobley, J.N., Close, G.L., Bailey, D.M., et al. (2017) Exercise redox biochemistry: Conceptual, methodological and technical recommendations. **Redox Biology,** 12 540-548.

Cobley, J.N., Sakellariou, G.K., Owens, D.J., et al. (2014) Lifelong training preserves some redox-regulated adaptive responses after an acute exercise stimulus in aged human skeletal muscle. **Free Radical Biology & Medicine,** 70 23-32.

Cobley, J.N., McHardy, H., Morton, J.P., et al. (2015) Influence of vitamin C and vitamin E on redox signaling: Implications for exercise adaptations. **Free Radical Biology & Medicine,** 84 65-76.

Connolly, D.A.J., McHugh, M.P., Padilla-Zakour, O.I., et al. (2006) Efficacy of a tart cherry juice blend in preventing the symptoms of muscle damage. **British Journal of Sports Medicine,** 40 (8): 679-683.

Croft, K.D. (2016) Dietary polyphenols: Antioxidants or not? **Archives of Biochemistry and Biophysics,** 595 120-124.

Cumming, K.T., Raastad, T., Holden, G., et al. (2014) Effects of vitamin C and E supplementation on endogenous antioxidant systems and heat shock proteins in response to endurance training. **Physiological Reports,** 2 (10): e12142.

Currell, K. and Jeukendrup, A.E. (2008) Superior endurance performance with ingestion of multiple transportable carbohydrates. **Medicine & Science in Sports & Exercise,** 40 (2): 275-281.

Dahlgren, C. and Karlsson, A. (1999) Respiratory burst in human neutrophils. **Journal of Immunological Methods,** 232 (1–2): 3-14.

Dascombe, B.J., Karunaratna, M., Cartoon, J., et al. (2010) Nutritional supplementation habits and perceptions of elite athletes within a state-based sporting institute. **Journal of Science & Medicine in Sport,** 13 274-80.

Davies, K.J., Quintanilha, A.T., Brooks, G.A., et al. (1982) Free radicals and tissue damage produced by exercise. **Biochemical & Biophysical Research Communications,** 107 (4): 1198-1205.

Davison, G. and Gleeson, M. (2006) The effect of 2 weeks vitamin C supplementation on immunoendocrine responses to 2.5 h cycling exercise in man. **European Journal of Applied Physiology,** 97 (4): 454-461.

Davison, G. and Gleeson, M. (2007) The effects of acute vitamin C supplementation on cortisol, interleukin-6, and neutrophil responses to prolonged cycling exercise. **European Journal of Sport Science,** 7 (1): 15-25.

Decendit, A., Mamani-Matsuda, M., Aumont, V., et al. (2013) Malvidin-3-O-beta glucoside, major grape anthocyanin, inhibits human macrophage-derived inflammatory mediators and decreases clinical scores in arthritic rats. **Biochemical Pharmacology,** 86 (10): 1461-1467.

Dillard, C.J., Litov, R.E., Savin, W.M., et al. (1978) Effects of exercise, vitamin E, and ozone on pulmonary function and lipid peroxidation. **Journal of Applied Physiology: Respiratory, Environmental & Exercise Physiology,** 45 (6): 927-932.

Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. **Free Radical Biology & Medicine,** 11 (1): 81-128.

Fatouros, I.G., Jamurtas, A.Z., Villiotou, V., et al. (2004) Oxidative stress responses in older men during endurance training and detraining. **Medicine & Science in Sports & Exercise,** 36 (12): 2065-2072.

Finkel, T. (2011) Signal transduction by reactive oxygen species. **Journal of Cell Biology,** 194 (1): 7-15.

Forman, H.J., Augusto, O., Brigelius-Flohe, R., et al. (2015) Even free radicals should follow some rules: A Guide to free radical research terminology and methodology. **Free Radical Biology & Medicine,** 78 233-235.

Forman, H.J., Davies, K.J.A. and Ursini, F. (2014) How do nutritional antioxidants really work: Nucleophilic tone and para-hormesis versus free radical scavenging *in vivo*. **Free Radical Biology & Medicine,** 66 24-35.

Freitas-Simoes, T., Ros, E. and Sala-Vila, A. (2016) Nutrients, foods, dietary patterns and telomere length: Update of epidemiological studies and randomized trials. **Metabolism,** 65 (4): 406-415.

Furnham, A. (1986) Response bias, social desirability and dissimulation. **Personality and Individual Differences,** 7 (3): 385-400.

Gaeini, A.A., Rahnama, N. and Hamedinia, M.R. (2006) Effects of vitamin E supplementation on oxidative stress at rest and after exercise to exhaustion in athletic students. **Journal of Sports Medicine & Physical Fitness,** 46 (3): 458-461.

Gandevia, S.C. (2001) Spinal and supraspinal factors in human muscle fatigue. **Physiological Reviews,** 81 (4): 1725-1789.

Gey, K., Moser, U., Jordan, P., et al. (1993) Increased risk of cardiovascular-disease at suboptimal plasma concentrations of essential antioxidants - an epidemiologic update with special attention to carotene and vitamin-C. **American Journal of Clinical Nutrition,** 57 (5): 787-797.

Giannopoulou, I., Noutsos, K., Apostolidis, N., et al. (2013) Performance level affects the dietary supplement intake of both individual and team sports athletes. **Journal of Sports Science & Medicine,** 12 (1): 190-196.

Goldfarb, A.H., Garten, R.S., Cho, C., et al. (2011) Effects of a fruit/berry/vegetable supplement on muscle function and oxidative stress. **Medicine & Science in Sports & Exercise,** 43 (3): 501-508.

Gomez-Cabrera, M.C., Domenech, E., Romagnoli, M., et al. (2008) Oral administration of vitamin C decreases muscle mitochondrial biogenesis and hampers training-induced adaptations in endurance performance. **American Journal of Clinical Nutrition,** 87 (1): 142-149.

Gomez-Cabrera, M.C., Martinez, A., Santangelo, G., et al. (2006) Oxidative stress in marathon runners: interest of antioxidant supplementation. **British Journal of Nutrition,** 96 (Suppl 1): S31-3.

Gomez-Cabrera, M., Borras, C., Pallardo, F., et al. (2005) Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. **Journal of Physiology,** 567 (1): 113-120.

González, R., Ballester, I., López-Posadas, R., et al. (2014) Effects of flavonoids and other polyphenols on inflammation. **Critical Reviews in Food Science & Nutrition,** 51 (4): 331-362.

Graziani, G., D'Argenio, G., Tuccillo, C., et al. (2005) Apple polyphenol extracts prevent damage to human gastric epithelial cells in vitro and to rat gastric mucosa in vivo. **Gut,** 54 (2): 193-200.

Groussard, C., Rannou-Bekono, F., Machefer, G., et al. (2003) Changes in blood lipid peroxidation markers and antioxidants after a single sprint anaerobic exercise. **European Journal of Applied Physiology,** 89 (1): 14-20.

Gutteridge, J.M. and Halliwell, B. (1990) The measurement and mechanism of lipid peroxidation in biological systems. **Trends in Biochemical Sciences,** 15 (4): 129-135.

Halliwell, B. and Whiteman, M. (2004) Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? **British Journal of Pharmacology,** 142 (2): 231-255.

Halliwell, B. and Gutteridge, J.C.M. (2007) **Free Radicals in Biology & Medicine.** 4th ed. Oxford: Oxford University Press.

Hamilton, I.M., Gilmore, W.S., Benzie, I.F., et al. (2000) Interactions between vitamins C and E in human subjects. **British Journal of Nutrition,** 84 (3): 261-267.

Harman, D. (2006) Free radical theory of aging: an update: increasing the functional life span. **Annals of the New York Academy of Sciences,** 1067 10-21.

Hecht, F., Pessoa, C.F., Gentile, L.B., et al. (2016) The role of oxidative stress on breast cancer development and therapy. **Tumor Biology,** 37 (4): 4281-4291.

Heikkinen, A., Alaranta, A., Helenius, I., et al. (2011) Use of dietary supplements in Olympic athletes is decreasing: a follow-up study between 2002 and 2009. **Journal of the International Society of Sports Nutrition,** 8 (1): 1.

Hernández, A., Cheng, A. and Westerblad, H. (2012) Antioxidants and skeletal muscle performance: "Common knowledge" vs. experimental evidence. **Frontiers in Physiology,** 3 (46): 1-6.

Higashida, K., Kim, S.H., Higuchi, M., et al. (2011) Normal adaptations to exercise despite protection against oxidative stress. **American Journal of Physiology - Endocrinology & Metabolism,** 301 (5): E779-84.

Higuchi, M., Cartier, L.J., Chen, M., et al. (1985) Superoxide dismutase and catalase in skeletal muscle: adaptive response to exercise. **Journal of Gerontology,** 40 (3): 281-286.

Himmelstein, A.S., Robergs, R.A., Koehler, M.K., et al. (1999) Vitamin C supplementation and upper respiratory tract infections in marathon runners. **Journal of Exercise Physiology,** 1 (2): 170-174.

Ho, R.C., Hirshman, M.F., Li, Y., et al. (2005) Regulation of IkappaB kinase and NF-kappaB in contracting adult rat skeletal muscle. **American Journal of Physiology - Cell Physiology,** 289 (4): 794-801.

Hollander, J., Fiebig, R., Gore, M., et al. (2001) Superoxide dismutase gene expression is activated by a single bout of exercise in rat skeletal muscle. **European Journal of Physiology,** 442 (3): 426-434.

Holloszy, J.O., Higashida, K., Kim, S.H., et al. (2012) Response to letter to the editor by Gomez-Cabrera et al. **American Journal of Physiology - Endocrinology & Metabolism,** 302 E478-E479.

Holloszy, J. (2004) Adaptations of skeletal muscle mitochondria to endurance exercise: A personal perspective. **Exercise and Sport Sciences Reviews,** 32 (2): 41-43.

Howarth, K.R., Moreau, N.A., Phillips, S.M., et al. (2009) Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. **Journal of Applied Physiology,** 106 (4): 1394-1402.

Howatson, G., Bell, P.G., Tallent, J., et al. (2012) Effect of tart cherry juice (Prunus cerasus) on melatonin levels and enhanced sleep quality. **European Journal of Nutrition,** 51 909-916.

Howatson, G., McHugh, M.P., Hill, J.A., et al. (2010) Influence of tart cherry juice on indices of recovery following marathon running. **Scandinavian Journal of Medicine & Science in Sports,** 20 (6): 843-852.

Huang, D., Ou, B. and Prior, R. (2005) The chemistry behind antioxidant capacity assays. **Journal of Agricultural and Food Chemistry,** 53 (6): 1841-1856.

Huskisson, E., Maggini, S. and Ruf, M. (2007) The role of vitamins and minerals in energy metabolism and well-being. **Journal of International Medical Research,** 35 (3): 277-289.

Jackson, M.J., Edwards, R.H. and Symons, M.C. (1985) Electron spin resonance studies of intact mammalian skeletal muscle. **Biochimica et Biophysica Acta,** 847 (2): 185-190.

Jackson, M.J. (2005) Reactive oxygen species and redox-regulation of skeletal muscle adaptations to exercise. **Philosophical Transactions of the Royal Society of London - Series B: Biological Sciences,** 360 (1464): 2285-2291.

Jeandet, P., Delaunois, B., Conreux, A., et al. (2010) Biosynthesis, metabolism, molecular engineering, and biological functions of stilbene phytoalexins in plants. **BioFactors,** 36 (5): 331-341.

Jeukendrup, A., Saris, W.H., Brouns, F., et al. (1996) A new validated endurance performance test. **Medicine & Science in Sports & Exercise,** 28 (2): 266-270.

Jeukendrup, A.E. (2010) Carbohydrate and exercise performance: the role of multiple transportable carbohydrates. **Current Opinion in Clinical Nutrition & Metabolic Care,** 13 (4): 452-457.

Ji, L.L. (2008) Modulation of skeletal muscle antioxidant defense by exercise: Role of redox signaling. **Free Radical Biology & Medicine,** 44 (2): 142-152.

Jones, A. and Carter, H. (2000) The effect of endurance training on parameters of aerobic fitness. **Sports Medicine,** 29 (6): 373-386.

Jung, T. and Grune, T. (2008) The proteasome and its role in the degradation of oxidized proteins. **IUBMB Life,** 60 (11): 743-752.

Kang, C., O'Moore, K.M., Dickman, J.R., et al. (2009) Exercise activation of muscle peroxisome proliferator-activated receptor-γ coactivator-1α signaling is redox sensitive. **Free Radical Biology & Medicine,** 37 1394-1400.

Kawagishi, H. and Finkel, T. (2014) ROS and disease: finding the right balance. **Nature Medicine,** 20 (7): 211-213.

Keane, K.M., Bell, P.G., Lodge, J.K., et al. (2016) Phytochemical uptake following human consumption of Montmorency tart cherry (L. Prunus cerasus) and influence of phenolic acids on vascular smooth muscle cells in vitro. **European Journal of Nutrition,** 55 (4): 1695-1705.

Khassaf, M., McArdle, A., Esanu, C., et al. (2003) Effect of vitamin C supplements on antioxidant defence and stress proteins in human lymphocytes and skeletal muscle. **Journal of Physiology,** 549 (Pt 2): 645-652.

Kirakosyan, A., Seymour, E.M., Wolforth, J., et al. (2015) Tissue bioavailability of anthocyanins from whole tart cherry in healthy rats. **Food Chemistry,** 171 26-31.

Kirakosyan, A., Seymour, E.M., Llanes, D.E.U., et al. (2009) Chemical profile and antioxidant capacities of tart cherry products. **Food Chemistry,** 115 (1): 20-25.

Knekt, P., Reunanen, A., Jarvinen, R., et al. (1994) Antioxidant vitamin intake and coronary mortality in a longitudinal population study. **American Journal of Epidemiology,** 139 (12): 1180-1189.

Knez, W.L. and Peake, J.M. (2010) The prevalence of vitamin supplementation in ultraendurance triathletes. **International Journal of Sport Nutrition & Exercise Metabolism,** 20 (6): 507-514.

Knez, W.L., Jenkins, D.G. and Coombes, J.S. (2007) Oxidative stress in half and full Ironman triathletes. **Medicine & Science in Sports & Exercise,** 39 (2): 283-288.

Kosmidou, I., Vassilakopoulos, T., Xagorari, A., et al. (2002) Production of interleukin-6 by skeletal myotubes - Role of reactive oxygen species. **American Journal of Respiratory Cell & Molecular Biology,** 26 (5): 587-593.

Kuehl, K.S., Perrier, E.T., Elliot, D.L., et al. (2010) Efficacy of tart cherry juice in reducing muscle pain during running: a randomized controlled trial. **Journal of the International Society of Sports Nutrition,** 7 (17): 1-6.

Kuijsten, A., Arts, I.C.W., Vree, T.B., et al. (2005) Pharmacokinetics of enterolignans in healthy men and women consuming a single dose of secoisolariciresinol diglucoside. **The Journal of Nutrition,** 135 795-801.

La Vecchia, C., Altieri, A. and Tavani, A. (2001) Vegetables, fruit, antioxidants and cancer: a review of Italian studies. **European Journal of Nutrition,** 40 (6): 261-267.

Lamprecht, M., Greilberger, J.F., Schwaberger, G., et al. (2008) Single bouts of exercise affect albumin redox state and carbonyl groups on plasma protein of trained men in a workload-dependent manner. **Journal of Applied Physiology,** 104 (6): 1611-1617.

Leaf, D.A., Kleinman, M.T., Hamilton, M., et al. (1997) The effect of exercise intensity on lipid peroxidation. **Medicine & Science in Sports & Exercise,** 29 (8): 1036-1039.

Levers, K., Dalton, R., Galvan, E., et al. (2015) Effects of powdered Montmorency tart cherry supplementation on an acute bout of intense lower body strength exercise in resistance trained males. **Journal of the International Society of Sports Nutrition,** 12 (41):.

Levers, K., Dalton, R., Galvan, E., et al. (2016) Effects of powdered Montmorency tart cherry supplementation on acute endurance exercise performance in aerobically trained individuals. **Journal of the International Society of Sports Nutrition,** 13 (22):.

Li, T. and Gleeson, M. (2004) The effect of single and repeated bouts of prolonged cycling on leukocyte redistribution, neutrophil degranulation, IL-6, and plasma stress hormone responses. **International Journal of Sport Nutrition & Exercise Metabolism,** 14 (5): 501-516.

Losonczy, K., Harris, T. and Havlik, R. (1996) Vitamin E and vitamin C supplement use and risk of all-cause and coronary heart disease mortality in older persons: The established populations for epidemiologic studies of the elderly. **American Journal of Clinical Nutrition,** 64 (2): 190-196.

Lun, V., Erdman, K.A., Fung, T.S., et al. (2012) Dietary supplementation practices in Canadian high-performance athletes. **International Journal of Sport Nutrition & Exercise Metabolism,** 22 (1): 31-37.

Machefer, G., Groussard, C., Rannou-Bekono, F., et al. (2004) Extreme running competition decreases blood antioxidant defense capacity. **Journal of the American College of Nutrition,** 23 (4): 358-364.

Malaguti, M., Angeloni, C. and Hrelia, S. (2013) Polyphenols in exercise performance and prevention of exercise-induced muscle damage. **Oxidative Medicine & Cellular Longevity,** 2013 1-9.

Manach, C., Scalbert, A., Morand, C., et al. (2004) Polyphenols: food sources and bioavailability. **American Journal of Clinical Nutrition,** 79 (5): 727-747.

Margaritelis, N.V., Cobley, J.N., Paschalis, V., et al. (2016) Going Retro: Oxidative stress biomarkers in modern redox biology. **Free Radical Biology & Medicine,** 98 (2): 12.

Margaritelis, N.V., Kyparos, A., Paschalis, V., et al. (2014) Reductive stress after exercise: The issue of redox individuality. **Redox Biology,** 2 520-528.

Mason, S. and Wadley, G.D. (2014) Skeletal muscle reactive oxygen species: a target of good cop/bad cop for exercise and disease. **Redox Report,** 19 (3): 97-106.

Mastaloudis, A., Morrow, J.D., Hopkins, D.W., et al. (2004) Antioxidant supplementation prevents exercise-induced lipid peroxidation, but not inflammation, in ultramarathon runners. **Free radical Biology & Medicine,** 36 (10): 1329-1341.

Maughan, R.J. (1999) Role of micronutrients in sport and physical activity. **British Medical Bulletin,** 55 (3): 683-690.

McArdle, A., Pattwell, D., Vasilaki, A., et al. (2001) Contractile activity-induced oxidative stress: cellular origin and adaptive responses. **Cell Physiology,** 280 (3): 621-627.

McCormick, R., Peeling, P., Binnie, M., et al. (2016) Effect of tart cherry juice on recovery and next day performance in well-trained Water Polo players. **Journal of the International Society of Sports Nutrition,** 13 (41):.

McCune, L.M., Kubota, C., Stendell-Hollis, N.R., et al. (2011) Cherries and health: a review. **Critical Reviews in Food Science & Nutrition,** 51 (1): 1-12.

McDonagh, B., Sakellariou, G.K. and Jackson, M.J. (2014) Application of redox proteomics to skeletal muscle aging and exercise. **Biochemical Society Transactions,** 42 965-970.

McGinnis, G., Kliszczewiscz, B., Barberio, M., et al. (2014) Acute hypoxia and exercise-induced blood oxidative stress. **International Journal of Sport Nutrition & Exercise Metabolism,** 24 (6): 684-693.

McKenna, M.J., Medved, I., Goodman, C.A., et al. (2006) N-acetylcysteine attenuates the decline in muscle Na+,K+-pump activity and delays fatigue during prolonged exercise in humans. **Journal of Physiology,** 576 (Pt 1): 279-288.

Mecocci, P., Fano, G., Fulle, S., et al. (1999) Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. **Free Radical Biology & Medicine,** 26 (3-4): 303-308.

Michailidis, Y., Jamurtas, A.Z., Nikolaidis, M.G., et al. (2007) Sampling time is crucial for measurement of aerobic exercise–induced oxidative stress. **Medicine & Science in Sports & Exercise,** 39 (7): 1107-1113.

Miedema, I., Feskens, E.J.M., Heederik, D., et al. (1993) Dietary determinants of long-term Incidence of chronic nonspecific lung-diseases - the Zutphen Study. **American Journal of Epidemiology,** 138 (1): 37-45.

Miller, T.M., Abdel-Maksoud, M., Crane, L.A., et al. (2008) Effects of social approval bias on self-reported fruit and vegetable consumption: a randomized controlled trial. **Nutrition Journal,** 7 (18):.

Milne, G.L., Gao, B., Terry, E.S., et al. (2013) Measurement of F2- isoprostanes and isofurans using gas chromatography–mass spectrometry. **Free Radical Biology & Medicine,** 59 36-44.

Mitjavila, M.T. and Moreno, J.J. (2012) The effects of polyphenols on oxidative stress and the arachidonic acid cascade. Implications for the prevention/treatment of high prevalence diseases. **Biochemical Pharmacology,** 84 (9): 1113-1122.

Morrison, D., Hughes, J., Della Gatta, P.A., et al. (2015) Vitamin C and E supplementation prevents some of the cellular adaptations to endurance-training in humans. **Free Radical Biology & Medicine,** 89 852-862.

Muller, F.L., Liu, Y., Abdul-Ghani, M.A., et al. (2008) High rates of superoxide production in skeletal-muscle mitochondria respiring on both complex I- and complex II-linked substrates. **Biochemical Journal,** 409 491-499.

Munoz Marin, D., Olcina, G., Timon, R., et al. (2010) Effect of different exercise intensities on oxidative stress markers and antioxidant response in trained cyclists. **Journal of Sports Medicine & Physical Fitness,** 50 (1): 93-98.

Myburgh, K.H. (2014) Polyphenol supplementation: Benefits for exercise performance or oxidative stress? **Sports Medicine,** 44 S57-S70.

Nakhostin-Roohi, B., Babaei, P., Rahmani-Nia, F., et al. (2008) Effect of vitamin C supplementation on lipid peroxidation, muscle damage and inflammation after 30-min exercise at 75% VO2max. **Journal of Sports Medicine & Physical Fitness,** 48 217-224.

Naziroğlu, M., Kilinç, F., Uğuz, A.C., et al. (2010) Oral vitamin C and E combination modulates blood lipid peroxidation and antioxidant vitamin levels in maximal exercising basketball players. **Cell Biochemistry & Function,** 28 300-305.

Neubauer, O., Konig, D., Kern, N., et al. (2008) No indications of persistent oxidative stress in response to an ironman triathlon. **Medicine & Science in Sports & Exercise,** 40 (12): 2119-2128.

Nieman, D.C., Henson, D.A., Smith, L.L., et al. (2001) Cytokine changes after a marathon race. **Journal of Applied Physiology,** 91 109-114.

Nieman, D.C., Henson, D.A., Butterworth, D.E., et al. (1997) Vitamin C supplementation does not alter the immune response to 2.5 hours of running. **International Journal of Sport Nutrition,** 7 (3): 173-184.

Nieman, D.C. and Pedersen, B.K. (1999) Exercise and Immune Function. **Sports Medicine,** 27 (2): 73-80.

Niki, E. (2014) Role of vitamin E as a lipid-soluble peroxyl radical scavenger: in vitro and in vivo evidence. **Free Radical Biology & Medicine,** 66 3-12.

Nikolaidis, M.G., Kyparos, A., Dipla, K., et al. (2012a) Exercise as a model to study redox homeostasis in blood: the effect of protocol and sampling point. **Biomarkers,** 17 (1): 28-35.

Nikolaidis, M.G., Kyparos, A., Spanou, C., et al. (2012b) Redox biology of exercise: an integrative and comparative consideration of some overlooked issues. **The Journal of Experimental Biology,** 215 1615-1625.

Ostrowski, K., Rohde, T., Zacho, M., et al. (1998) Evidence that interleukin-6 is produced in human skeletal muscle during prolonged running. **Journal of Physiology,** 508 (Pt 3): 949-953.

Padayatty, S.J., Katz, J., Wang, Y., et al. (2003) Vitamin C as an antioxidant: Evaluation of Its role in disease prevention. **Journal of the American College of Nutrition,** 22 (1): 18-35.

Parise, G., Phillips, S., Kaczor, J., et al. (2005) Antioxidant enzyme activity is up-regulated after unilateral resistance exercise training in older adults. **Free Radical Biology & Medicine,** 39 (2): 289-295.

Paulsen, G., Cumming, K.T., Holden, G., et al. (2014) Vitamin C and E supplementation hampers cellular adaptation to endurance training in humans: a double-blind, randomised, controlled trial. **Journal of Physiology-London,** 592 (8): 1887-1901.

Peake, J.M., Suzuki, K. and Coombes, J.S. (2007) The influence of antioxidant supplementation on markers of inflammation and the relationship to oxidative stress after exercise. **Journal of Nutritional Biochemistry,** 18 (6): 357-371.

Pereira Panza, V.S., Diefenthaeler, F. and Luiz da Silva, E. (2015) Benefits of dietary phytochemical supplementation on eccentric exercise-induced muscle damage: Is including antioxidants enough? **Nutrition,** 31 1072-1082.

Perry, C.G.R., Heigenhauser, G.J.F., Bonen, A., et al. (2008) High-intensity aerobic interval training increases fat and carbohydrate metabolic capacities in human skeletal muscle. **Applied Physiology, Nutrition, & Metabolism,** 33 1112-1123.

Peternelj, T. and Coombes, J.S. (2011) Antioxidant supplementation during exercise training: beneficial or detrimental? **Sports Medicine,** 41 (12): 1043-1069.

Peterson, J., Dwyer, J., Adlercreutz, H., et al. (2010) Dietary lignans: physiology and potential for cardiovascular disease risk reduction. **Nutrition Reviews,** 68 (10): 571-603.

Petroczi, A., Naughton, D.P., Pearce, G., et al. (2008) Nutritional supplement use by elite young UK athletes: Fallacies of advice regarding efficacy. **Journal of the International Society of Sports Nutrition,** 5 22.

Pizza, F.X., McLoughlin, T.J., McGregor, S.J., et al. (2001) Neutrophils injure cultured skeletal myotubes. **American Journal of Physiology - Cell Physiology,** 281 335-341.

Pohanka, M., Pejchal, J., Snopkova, S., et al. (2012) Ascorbic acid: an old player with a broad impact on body physiology including oxidative stress suppression and immunomodulation: a review. **Mini-Reviews in Medicinal Chemistry,** 12 (1): 35-43.

Powers, S.K. and Jackson, M.J. (2008) Exercise-induced oxidative stress: cellular mechanisms and impact on muscle force production. **Physiological Reviews,** 88 (4): 1243-1276.

Powers, S.K., Ji, L.L., Kavazis, A.N., et al. (2011) Reactive Oxygen Species: Impact on Skeletal Muscle. **Comprehensive Physiology,** 1 (2): 941-969.

Quindry, J.C., Stone, W.L., King, J., et al. (2003) The effects of acute exercise on neutrophils and plasma oxidative stress. **Medicine & Science in Sports & Exercise,** 35 (7): 1139-1145.

Radak, Z., Ishihara, K., Tekus, E., et al. (2017) Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. **Redox Biology,** 12 285-290.

Radak, Z., Chung, H.Y., Koltai, E., et al. (2008) Exercise, oxidative stress and hormesis. **Ageing Research Reviews,** 7 (1): 34-42.

Radomski, M., Palmer, R. and Moncada, S. (1990) Characterization of the L-arginine-nitric oxide pathway in human platelets. **British Journal of Pharmacology,** 101 (2): 325-328.

Rahnama, N., Gaeini, A.A. and Hamedinia, M.R. (2007) Oxidative stress responses in physical education students during 8 weeks aerobic training. **Journal of Sports Medicine & Physical Fitness,** 47 (1): 119-123.

Reid, M. (2001) Plasticity in Skeletal, Cardiac, and Smooth Muscle Invited Review: Redox modulation of skeletal muscle contraction: what we know and what we don't. **Journal of Applied Physiology,** 90 724-731.

Ristow, M. (2014) Mitohormesis explains ROS-induced health benefits. **Nature Medicine,** 20 (7): 709-711.

Ristow, M., Zarse, K., Oberbach, A., et al. (2009) Antioxidants prevent health-promoting effects of physical exercise in humans. **Proceedings of the National Academy of Sciences of the United States of America,** 106 (21): 8665-8670.

Rojo, A., Salinas, M., Martin, D., et al. (2004) Regulation of Cu/Zn-superoxide dismutase expression via the phosphatidylinositol 3 kinase/Akt pathway and nuclear factor-kappa B. **Journal of Neuroscience,** 24 (33): 7324-7334.

Ronsen, O., Sundgot-Borgen, J. and Maehlum, S. (1999) Supplement use and nutritional habits in Norwegian elite athletes. **Scandinavian Journal of Medicine & Science in Sports,** 9 28-35.

Rutkowski, M. and Grzegorczyk, K. (2012) Adverse effects of antioxidative vitamins. **International Journal of Occupational Medicine & Environmental Health,** 25 (2): 105-121.

Sachdev, S. and Davies, K.J. (2008) Production, detection, and adaptive responses to free radicals in exercise. **Free Radical Biology & Medicine,** 44 (2): 215-223.

Sakellariou, G.K., Jackson, M.J. and Vasilaki, A. (2014) Redefining the major contributors to superoxide production in contracting skeletal muscle. The role of NAD(P)H oxidases. **Free Radical Research,** 48 (1): 12-29.

Santilli, F., D'Ardes, D. and Davi, G. (2015) Oxidative stress in chronic vascular disease: From prediction to prevention. **Vascular Pharmacology,** 74 23-37.

Serrano, E., Venegas, C., Escames, G., et al. (2010) Antioxidant defence and inflammatory response in professional road cyclists during a 4-day competition. **Journal of Sports Sciences,** 28 (10): 1047-1056.

Seymour, E.M., Warber, S.M., Kirakosyan, A., et al. (2014) Anthocyanin pharmacokinetics and dose-dependent plasma antioxidant pharmacodynamics following whole tart cherry intake in healthy humans. **Journal of Functional Foods,** 11 509-516.

Shin, Y., Lee, J., Song, W., et al. (2008) Exercise training improves the antioxidant enzyme activity with no changes of telomere length. **Mechanisms of Ageing & Development,** 129 (5): 254-260.

Sies, H. (2007) Total Antioxidant Capacity: Appraisal of a concept. **The Journal of Nutrition,** 137 1493-1495.

Sies, H. and Jones, D. (2007) Oxidative Stress. **Encyclopedia of Stress,** 25 (3): 45-48.

Silveira, L.R., Pilegaard, H., Kusuhara, K., et al. (2006) The contraction induced increase in gene expression of peroxisome proliferator-activated receptor (PPAR)-gamma coactivator 1alpha (PGC-1alpha), mitochondrial uncoupling protein 3 (UCP3) and hexokinase II (HKII) in primary rat skeletal muscle cells is dependent on reactive oxygen species. **Biochimica et Biophysica acta,** 1763 (9): 969-976.

Skenderi, K.P., Tsironi, M., Lazaropoulou, C., et al. (2008) Changes in free radical generation and antioxidant capacity during ultramarathon foot race. **European Journal of Clinical Investigation,** 38 (3): 159-165.

Smith, J.W., Pascoe, D.D., Passe, D.H., et al. (2013) Curvilinear dose-response relationship of carbohydrate (0-120 g-h(-1)) and performance. **Medicine & Science in Sports & Exercise,** 45 (2): 336-341.

Snodderly, D.M. (1995) Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins. **American Journal of Clinical Nutrition,** 62 (6): 1448-1461.

Sohal, R.S. and Orr, W.C. (2012) The redox stress hypothesis of aging. **Free Radical Biology & Medicine,** 52 (3): 539-555.

Sport England (Active People Survey 7 October 2012 - October 2013) **Once a week participation in funded sports amongst people aged 16 years and over (October 2012 – October 2013).** [Online]. Available from: http://www.sportengland.org/media/226699/1x30_sport_16-factsheet_APS7_final.pdf [Accessed February/10 2015].

Stanner, S., Hughes, J., Kelly, C., et al. (2004) A review of the epidemiological evidence for the 'antioxidant hypothesis'. **Public Health Nutrition,** 7 (3): 407-422.

Stepanyan, V., Crowe, M., Haleagrahara, N., et al. (2014) Effects of Vitamin E supplementation on exercise-induced oxidative stress: a meta-analysis. **Applied Physiology, Nutrition, & Metabolism,** 39 (9): 1029-1037.

Strobel, N.A., Matsumoto, A., Peake, J.M., et al. (2014) Altering the redox state of skeletal muscle by glutathione depletion increases the exercise-activation of PGC-1a. **Physiological Reports,** 2 (12): e12224.

Strobel, N.A., Peake, J.M., Matsumoto, A., et al. (2011) Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. **Medicine & Science in Sports & Exercise,** 43 (6): 1017-1024.

Suzuki, K., Totsuka, M., Nakaji, S., et al. (1999) Endurance exercise causes interaction among stress hormones, cytokines, neutrophil dynamics, and muscle damage. **Journal of Applied Physiology,** 87 (4): 1360-1367.

Talaulikar, V.S. and Manyonda, I.T. (2011) Vitamin C as an antioxidant supplement in women's health: a myth in need of urgent burial. **European Journal of Obstetrics, Gynecology, & Reproductive Biology,** 157 (1): 10-13.

Tanabe, K., Masuda, K., Hirayama, A., et al. (2006) Effect of spontaneous exercise on antioxidant capacity in rat muscles determined by electron spin resonance. **Acta Physiologica,** 186 (2): 119-125.

Tarnopolsky, M.A., Pearce, E., Smith, K., et al. (2011) Suction-modified Bergstrom muscle biopsy technique: Experience with 13,500 procedures. **Muscle & Nerve,** 43 (5): 717-725.

Tauler, P., Aguilo, A., Gimeno, I., et al. (2004) Different effects of exercise tests on the antioxidant enzyme activities in lymphocytes and neutrophils. **Journal of Nutritional Biochemistry,** 15 (8): 479-484.

Tauler, P., Sureda, A., Cases, N., et al. (2005) Increased lymphocyte antioxidant defences in response to exhaustive exercise do not prevent oxidative damage. **Free Radical Research,** 39 S80-S80.

Theodorou, A.A., Nikolaidis, M.G., Paschalis, V., et al. (2011) No effect of antioxidant supplementation on muscle performance and blood redox status adaptations to eccentric training. **American Journal of Clinical Nutrition,** 93 (6): 1373-1383.

Tian, H.H., Ong, W.S. and Tan, C.L. (2009) Nutritional supplement use among university athletes in Singapore. **Singapore Medical Journal,** 50 165-172.

Tidball, J.G. (2005) Inflammatory processes in muscle injury and repair. **American Journal of Physiology - Regulatory, Integrative and Comparative Physiology,** 288 345-353.

Tiidus, P.M. (1998) Radical species in inflammation and overtraining. **Canadian Journal of Physiology & Pharmacology,** 76 533-538.

Tsitsimpikou, C., Chrisostomou, N., Papalexis, P., et al. (2011) The use of nutritional supplements among recreational athletes in Athens, Greece. **International Journal of Sport Nutrition &Exercise Metabolism,** 21 377-384.

Turner, J.E., Hodges, N.J., Bosch, J.A., et al. (2011) Prolonged depletion of antioxidant capacity after ultraendurance exercise. **Medicine & Science in Sports & Exercise,** 43 (9): 1770-1776.

Turrens, J.F. (2003) Mitochondria formation of reactive oxygen species. **Journal of Physiology,** 552 (2): 335-344.

Urso, M.L. and Clarkson, P.M. (2003) Oxidative stress, exercise, and antioxidant supplementation. **Toxicology,** 189 (1-2): 41-54.

van't Erve, T.J., Lih, F.B., Kadiiska, M.B., et al. (2015) Reinterpreting the best biomarker of oxidative stress: The 8-iso-PGF2α/ PGF2α ratio distinguishes chemical from enzymatic lipid peroxidation. **Free Radical Biology & Medicine,** 83 245-251.

Vider, J., Laaksonen, D.E., Kilk, A., et al. (2001) Physical exercise induces activation of NF-kB in human peripheral blood lymphocytes. **Antioxidants & Redox Signaling,** 3 (6): 1131-1137.

Vina, J., Borras, C., Abdelaziz, K.M., et al. (2013) The free radical theory of aging revisited: the cell signaling disruption theory of aging. **Antioxidants & Redox Signaling,** 19 (8): 779-787.

Wadley, A.J., Chen, Y., Lip, G.Y.H., et al. (2016a) Low volume–high intensity interval exercise elicits antioxidant and anti-inflammatory effects in humans. **Journal of Sports Sciences,** 34 (1): 1-9.

Wadley, A.J., Chen, Y.W., Bennett, S.J., et al. (2015a) Monitoring changes in thioredoxin and over-oxidised peroxiredoxin in response to exercise in humans. **Free Radical Research,** 49 (3): 290-298.

Wadley, A.J., Killer, S.C., Svendsen, I.S., et al. (2015b) The impact of intensified training with a high or moderate carbohydrate feeding strategy on resting and exercise-induced oxidative stress. **European Journal of Applied Physiology,** 115 (8): 1757-1767.

Wadley, A.J., Turner, J.E. and Aldred, S. (2016b) Factors influencing post-exercise plasma protein carbonyl concentration. **Free Radical Research,** 50 (4): 375-384.

Wadley, G.D. and McConell, G.K. (2010) High-dose antioxidant vitamin C supplementation does not prevent acute exercise-induced increases in markers of skeletal muscle mitochondrial biogenesis in rats. **Journal of Applied Physiology,** 108 (6): 1719-1726.

Wallace, T.C. (2011) Anthocyanins in cardiovascular disease. **Advances in Nutrition,** 2 1-7.

Wang, J. and Arase, H. (2014) Regulation of immune responses by neutrophils. **Annals of the New York Academy of Sciences,** 1319 (1): 66-81.

Westerblad, H. and Allen, D.G. (2011) Emerging roles of ROS/RNS in muscle function and fatigue. **Antioxidants & Redox Signaling,** 15 (9): 2487-2499.

Willcox, B.J., Curb, J.D. and Rodriguez, B.L. (2008) Antioxidants in cardiovascular health and disease: Key lessons from epidemiologic studies. **American Journal of Cardiology,** 101 (10A): 75D-86D.

Wood, Z. (2012) **Olympics fever will help to make 2012 the year of the bike.** [Online]. Available from: http://www.theguardian.com/lifeandstyle/2012/jan/29/cycling-boom-olympics [Accessed September/20 2014].

Xu, Y., Gu, Y. and Qian, S.Y. (2012) An advanced Electron Spin Resonance (ESR) spin-trapping and LC/(ESR)/MS technique for the study of lipid peroxidation. **International Journal of Molecular Sciences,** 13 (11): 14648-14666.

Yfanti, C., Akerstrom, T., Nielsen, S., et al. (2010) Antioxidant supplementation does not alter endurance training adaptation. **Medicine & Science in Sports & Exercise,** 42 (7): 1388-1395.

Yfanti, C., Fischer, C.P., Nielsen, S., et al. (2012) Role of vitamin C and E supplementation on IL-6 in response to training. **Journal of applied physiology,** 112 (6): 990-1000.

Zhou, L.Z., Johnson, A.P. and Rando, T.A. (2001) NF kappa B and AP-1 mediate transcriptional responses to oxidative stress in skeletal muscle cells. **Free Radical Biology & Medicine,** 31 (11): 1405-1416.