

Exercise-Induced Oxidative Stress, Muscle Damage, and Inflammation in Prolonged High-Intensity Intermittent Exercise: Effect of Quercetin

Camilla Rebecca Holland

Submitted to the University of Hertfordshire in partial fulfillment of the requirement of the degree of Doctor of Philosophy

University of Hertfordshire

Submitted January 2020

Abstract

Soccer is characterised by prolonged periods of high-intensity intermittent exercise, the metabolic and mechanical demands of which can induce oxidative stress, skeletal muscle damage, and associated inflammation as part of the adaptive response to exercise. However, the negative effects of these responses include fatigue and muscle soreness, which can compromise recovery and impair performance. There is paucity in the literature regarding these phenomena following intensified periods of soccer, such as microcycles of a competitive in-season, where athletes undertake daily training sessions and competitive matches with minimal recovery periods.

Therefore, study one explored associations between oxidative stress (assessed via urinary MDA, a biomarker of lipid peroxidation) and high-intensity training load (GPS & HR derived variables) in a cohort of professional soccer players throughout microcycles of a competitive in-season. Results showed that urinary MDA decreased significantly over the season, but was not associated with high-intensity training load. Furthermore, lipid peroxidation was lower in professional soccer players when compared to recreational players. The observed progressive reduction in lipid peroxidation in professional soccer players may theoretically be explained as an adaptive response to regular participation in soccer training.

To further increase understanding of the physiological demands imposed on athletes within a training microcycle, study two profiled the short-term response of biomarkers indicative of (i) oxidative stress, (ii) muscle damage, and (iii) inflammation, following a 3-day intensified period of simulated soccer in trained athletes; with results

compared to a control group. The 3-day exercise protocol did not alter oxidative stress but induced transient cellular damage, which was accompanied by an inflammatory response, evidenced by significant post-exercise increases in (i) CK, (ii) LDH, (iii) CRP, (iv) IL-6, (v) MCP-1, (vi) total leukocyte and neutrophil counts. These findings may have negative implications for performance, as recovery may be incomplete prior to subsequent sessions; strategies that facilitate recovery by counteracting muscle damage and inflammation may be beneficial during this time.

Quercetin supplementation has been proposed as a nutritional recovery strategy that could benefit athletes during intensified periods of exercise, as it is postulated that quercetin has antioxidant, anti-inflammatory, and analgesic properties. Studies investigating the use of quercetin in intermittent exercise have been limited; therefore, study three expanded on study two, by examining the efficacy of prolonged quercetin supplementation to attenuate exercise-induced cellular damage and associated inflammation. For practical application, measures of perceptual recovery were also determined. Results revealed quercetin supplementation to be ineffective in alleviating (i) cellular damage, (ii) inflammation, (iii) perceived fatigue, and (iv) muscle soreness, in comparison to a placebo. Thus, the use of quercetin to improve recovery by counteracting cellular damage and inflammation during intensified periods of exercise characteristic of soccer, appears not to be supported. The NF- κ B classical pathway was studied to elucidate underlying mechanisms of exercise-induced inflammation but findings revealed no effect of the 3-day exercise protocol, or quercetin supplementation, on NF- κ B activity.

In conclusion, results showed a progressive reduction in a biomarker of lipid peroxidation throughout a competitive in-season in professional soccer players, but no association between lipid peroxidation and high-intensity training load. Interestingly, a 3-day intensified period of simulated soccer induced acute transient cellular damage and inflammation but no alterations in oxidative stress. Therefore, this thesis provides novel data regarding intensified periods of soccer and simulated soccer. Moreover, the results add to the current literature debating the use of antioxidant supplementation in athletes, providing no significant evidence to support the use of quercetin supplementation as a recovery strategy.

An overarching theme of this thesis was biomarker monitoring, which could be used to help sports scientists/coaches prescribe training loads and recovery strategies that negate the undesirable effects of exercise-induced muscle damage and associated inflammation in susceptible athletes when recovery time is limited.

Key words: oxidative stress, muscle damage, inflammation, soccer, quercetin

Acknowledgements

I would like to thank my supervisory team (Dr Lindsay Bottoms, Dr Lindsay Kass, & Dr Mike Roberts) and original supervisor, Dr Justin Roberts, for their continued support, advice, and encouragement. I would also like to thank my line manager Mr Jon Gillard and team who have been very supportive in helping me complete, and my colleagues across different departments for their help, advice, and loan of equipment. Especially my go to guys, my fellow techs and postgrads.

I also extend my thanks to all the participants who took part in my studies 'for the science', for their dedication and effort, and to the football club who were incredibly accommodating; without willing participants there really would be no sports science research.

Lastly, I would also like to thank my fiancé for entertaining himself with ultra-endurance events over the years whilst I study. I would also like to take this opportunity to thank my amazing mum, who has been very supportive of my studies and is my inspiration, academically and in the gym.

For those of you that said you cannot continue playing three team sports whilst studying for a PhD... I really should have listened sooner... I am now off to get fit again!

Table of Contents

Abstract	2
Acknowledgements	5
Table of Contents	6
List of Tables	10
List of Figures	12
List of Conference Proceedings	14
Commonly Used Abbreviations	15
Collaborations	21
Introduction	22
1.1 Research Aims & Hypotheses	25
1.1.1 Study one (experimental chapter 4)	26
1.1.2 Study two (experimental chapter 5)	27
1.1.3 Study three (experimental chapter 6)	28
Literature Review	30
2.1 Physiological Demands of Soccer	30
2.1.1 Quantifying training load.	33
2.1.2 Monitoring recovery.	35
2.2 Defining Exercise-Induced Oxidative Stress	40
2.2.1 Sources of exercise-induced RONS.	41
2.2.2 Negative & positive role of exercise-induced RONS.	44
2.3 Defining Antioxidants	47
2.4 Exercise-Induced Muscle Damage & Associated Inflammation	50

2.4.1 Primary & secondary muscle damage.	51
2.5 Assessment of EIOS, EIMD, & Associated Inflammation	55
2.5.1 Oxidative stress.	55
2.5.2 Muscle damage & inflammation.	56
2.5.3 Sampling time points.	58
2.6 EIOS, Muscle Damage, & Inflammation following Soccer-Specific Prolonged High-Intensity Intermittent Exercise	59
2.6.1 Single bouts.	59
2.6.2 Intensified periods.	61
2.6.3 Summary	65
2.7 Nuclear Factor Kappa-Light-chain-enhancer of activated B cells (NF- κ B)	71
2.7.1 Exercise-induced NF- κ B activation.	72
2.8 Recovery Strategies	81
2.8.1 Dietary antioxidant supplementation in athletes.	82
2.9 Summary & Rationale	108
General Methods	110
3.1. Ethics, Informed Consent, & Health Screening	111
3.2 Preliminary Physiological Testing	112
3.2.1 Anthropometric measures.	112
3.2.2 Determination of maximal oxygen uptake & maximum heart rate.	116
3.3 Main Experimental Testing	118
3.3.1 Instrumentation.	118
3.3.2 Three-day period of intensified exercise.	131
3.3.2.1 Familiarisation session.	133
3.3.2.2 Three-Day soccer-specific prolonged high-intensity intermittent	

exercise protocol.	133
3.3.3 Blood sampling & handling.	135
3.3.3.1 Capillary blood sampling, including lactate analysis.	135
3.3.3.2 Venous blood sampling.	136
3.3.3.3 Blood processing & storage.	137
3.3.4 Analytical methods.	138
3.3.4.1 Determination of MDA in serum.	139
3.3.4.2 Determination of Total Antioxidant Status.	141
3.3.4.3 Determination of Creatine Kinase.	145
3.3.4.4 Determination of Lactate Dehydrogenase.	149
3.3.4.5 Isolation of Nuclear Proteins & Quantification of NF- κ B p65 Subunit Activation.	152
3.3.4.6 Determination & quantification of inflammatory cytokines.	156
3.3.4.7 Determination of C-Reactive Protein.	158
3.3.4.8 Quantification of total leukocyte & neutrophil counts.	161
3.3.4.9 Haematocrit, Haemoglobin, & Plasma Volume Change.	162
3.4 Control of data.	164
3.4.1 Training load.	164
3.4.2 Dietary intake.	164
Chapter 4	168
4.1 Introduction	169
4.2 Methods	172
4.3 Results	188
4.4 Discussion	197
Chapter 5	209

Table of Contents	9
5.1 Introduction	210
5.2 Methods	214
5.3 Results	227
5.4 Discussion	241
Chapter 6	260
6.1 Introduction	261
6.2 Methods	263
6.3 Results	282
6.4 Discussion	292
General Discussion	304
References	335
Appendices	
APPENDIX A: Training Log	389
APPENDIX B: Estimated Food Record	391
APPENDIX C: Spectrophotometer Wavelength Accuracy	393
APPENDIX D: Dietary Antioxidant Questionnaire	394
APPENDIX E: DALDA	403
APPENDIX F: Intra- & Inter-individual Differences in Lipid Peroxidation	408
APPENDIX G: Intra- & Inter-Individual Differences in CK	410
APPENDIX H: Exemplar Perceptual Fatigue Questionnaire	411
APPENDIX I: Intra- & Inter-Individual Differences in Dependent Variables	412
APPENDIX J: Null Hypotheses	416
APPENDIX K: Cytokine & Growth Factor Responses	419

List of Tables

Table 2.1 <i>Common Biomarkers of Oxidative Stress, Muscle Damage, & Associated Inflammation in Exercise Studies</i>	39
Table 2.2 <i>Classes of Antioxidants</i>	49
Table 2.3 <i>Summary of Studies Investigating EIOS, EIMD, & EII in Soccer-Specific Exercise</i>	66
Table 2.4 <i>Summary of Studies Investigating NF-κB Activation in Response to Human Exercise</i>	77
Table 2.5 <i>Food Sources of Quercetin</i>	86
Table 2.6 <i>Summary of Studies Investigating Quercetin Supplementation in Human Exercise</i>	97
Table 3.1 <i>Laboratory Ambient Environmental Conditions</i>	112
Table 3.2 <i>Concurrent Validity of Measured Distance vs. SPI Pro Distance</i>	122
Table 3.3 <i>Intra-Unit Reliability of SPI Pro Distance and Speed</i>	124
Table 3.4 <i>Inter-Unit Reliability of SPI Pro Distance and Speed</i>	124
Table 3.5 <i>Exemplar of Treadmill Verification</i>	134
Table 3.6 <i>Randox Total Antioxidant Status Assay Kit Reagents</i>	143
Table 3.7 <i>Randox CK-NAC Assay Kit Reagents</i>	148
Table 3.8 <i>Randox LDH Assay Kit Reagents</i>	151
Table 3.9 <i>Nuclear Extraction Kit Reagent Preparations</i>	154
Table 3.10 <i>NF-κB p65 Subunit Activation Reagent Preparation</i>	155
Table 3.11 <i>Randox CRP Assay Kit Reagents</i>	159
Table 3.12 <i>Randox Full range CRP Assay Kit Reagents</i>	161
Table 3.14 <i>Ensure Plus® Nutritional content</i>	167
Table 4.1 <i>Descriptive Characteristics of Participants</i>	174
Table 4.2 <i>Ambient Conditions</i>	178
Table 4.3 <i>Training Variables & Body Mass</i>	190

Table 4.4 <i>Uncorrected Urinary Malondialdehyde Concentrations</i>	193
Table 4.5 <i>Correlations of High-Intensity Training Load & MDA</i>	196
Table 5.1 <i>Descriptive Characteristics of the Participants</i>	215
Table 5.2 <i>Dietary Intakes of the Experimental & Control Groups</i>	223
Table 5.3 <i>3-Day Performance Measures</i>	228
Table 5.4 <i>Relative Neutrophil Counts</i>	237
Table 5.5 <i>Oxidative Stress, Chemokine IL-8, Cytokine IL-10, & NF-κB p65 Responses to the 3-Day Exercise Protocol</i>	240
Table 6.1 <i>Descriptive Characteristics & Pre-Supplementation TAS Concentrations</i>	265
Table 6.2 <i>Quercetin Plus & Quercetin Placebo Ingredients List</i>	272
Table 6.3 <i>Dietary Intakes of the Quercetin & Placebo Groups</i>	275
Table 6.4 <i>3-Day Performance Measures</i>	283
Table 6.5 <i>Relative Neutrophil Counts</i>	288
Table 6.6 <i>TAS, MDA, CRP, & NF-κB p65 Responses to the 3-Day Exercise Protocol</i>	288
Table 6.7 <i>Localised Perceived Muscle Soreness</i>	291

List of Figures

<i>Figure 2.1.</i> Overview of lipid peroxidation.	45
<i>Figure 2.2.</i> The concept of hormesis.	47
<i>Figure 2.3.</i> Locations of primary antioxidants.	50
<i>Figure 2.4.</i> Schematic of NF- κ B classical pathway.	72
<i>Figure 2.5.</i> Simplified schematic of exercise-induced NF- κ B activation.	76
<i>Figure 2.6.</i> Polyphenol subclasses.	85
<i>Figure 2.7.</i> Quercetin supplementation may blunt NF- κ B activation.	89
<i>Figure 2.8.</i> Molecular structure of quercetin and quercetin metabolites.	91
<i>Figure 2.9.</i> “Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training?”	107
<i>Figure 2.10.</i> “Exercise-induced muscle damage: What is it, what causes it, what are the nutritional solutions?”	108
<i>Figure 3.1.</i> SPI Pro unit & vest.	119
<i>Figure 3.2.</i> Outline of the Walk-Jog-Run protocol & 30 m linear sprint course.	121
<i>Figure 3.3.</i> Bland-Altman plot of the difference in speed between the SPI Pro & criterion.	123
<i>Figure 3.4.</i> Exemplar of the modified Drust protocol.	132
<i>Figure 3.5.</i> Blood separation by density gradient centrifugation.	138
<i>Figure 3.6.</i> Reaction between MDA & TBA.	141
<i>Figure 3.7.</i> Exemplar scatterplot of standard MDA concentration curve.	141
<i>Figure 3.8.</i> Principle of the ABTS [®] method.	142
<i>Figure. 3.9.</i> Exemplar scatterplot of TAS standard concentration curve.	144
<i>Figure 3.10.</i> Antioxidant decolourisation of ABTS [®] in the TAS assay.	145
<i>Figure 3.11.</i> Principle of the Creatine Kinase assay.	146
<i>Figure 3.12.</i> Exemplar scatterplot of CK standard concentration curve.	147
<i>Figure 3.13.</i> Principle of Lactate Dehydrogenase.	150
<i>Figure 3.14.</i> Exemplar scatterplot of LDH standard concentration curve.	151
<i>Figure 3.15.</i> Exemplar protein concentration standard curve.	156
<i>Figure 3.16.</i> Randox Evidence Investigator [™] , biochip, & thermoshaker.	158

List of Figures	13
<i>Figure 3.17.</i> Scatterplot of CRP standard concentration curve.	160
<i>Figure 4.1.</i> Schematic representation of the study design.	176
<i>Figure 4.2.</i> Exemplar of MDA standard concentration curve.	183
<i>Figure 4.3.</i> Exemplar of Creatinine standard concentration curve.	185
<i>Figure 4.4.</i> High-intensity external & internal training load.	191
<i>Figure 4.5.</i> High-intensity accelerations & decelerations.	192
<i>Figure 4.6.</i> Malondialdehyde concentration in the experimental group.	194
<i>Figure 4.7.</i> Normalised Malondialdehyde to Creatinine concentrations.	195
<i>Figure 5.1.</i> Schematic of the overall study design.	218
<i>Figure 5.2.</i> The modified Drust protocol.	221
<i>Figure 5.3.</i> Daily heart rate response to the 3-day exercise protocol.	228
<i>Figure 5.4.</i> Serum CK and LDH responses.	230
<i>Figure 5.5.</i> Serum IL-6 responses.	233
<i>Figure 5.6.</i> Serum MCP-1 responses.	234
<i>Figure 5.7.</i> Serum CRP responses.	235
<i>Figure 5.8.</i> Whole blood leukocyte and neutrophil responses.	238
<i>Figure 6.1.</i> Schematic of the overall study design.	268
<i>Figure 6.2.</i> Mechanism of oxidised quercetin regeneration via vitamin C.	270
<i>Figure 6.3.</i> Quercetin supplementation.	272
<i>Figure 6.4.</i> Example of a 100 mm Visual Analogue Scale.	277
<i>Figure 6.5.</i> Mean serum LDH concentrations.	284
<i>Figure 6.6.</i> Mean serum IL-6 concentration.	285
<i>Figure 6.7.</i> Total leukocyte and neutrophil counts.	287
<i>Figure 6.8.</i> Overall well-being scores.	289
<i>Figure 6.9.</i> Visual Analogue Scale (VAS) scores.	291
<i>Figure 7.1.</i> Responses to the 3-day period of intensified simulated soccer.	329

List of Conference Proceedings

Posters:

Assessment of high-intensity training load and exercise-induced oxidative stress in professional soccer players.

Camilla R. Holland, Michael G. Roberts, Matthew W. Furber, Lindsay S. Kass, and Lindsay Bottoms

University of Hertfordshire, Hatfield, United Kingdom.

Accepted for ACSM Annual Meeting, 2020.

Effects of a Three-Day Period of Intense, Intermittent Exercise on Oxidative Stress and Inflammation.

Camilla R. Holland¹, Michael G. Roberts¹ and Justin D. Roberts²

¹University of Hertfordshire, Hatfield, UK; ²Anglia Ruskin University, Cambridge, UK.

Presented at ACSM Annual Meeting, Boston, May 2016.

Assessment of Training Load and Oxidative Stress in Professional Football Players.

Camilla R. Holland¹, Michael G. Roberts¹, and Justin D. Roberts²

¹University of Hertfordshire, Hatfield, UK; ²Anglia Ruskin University, Cambridge, UK.

Presented at University of Hertfordshire Life & Medical Sciences Annual Conference 2015.

Oral presentation:

Validity of a GPS Device to Assess Speed and Distance in Team Sports Movements.

Camilla R. Holland¹, Michael G. Roberts¹, James A. Johnstone², and Justin D. Roberts²

¹University of Hertfordshire, Hatfield, UK; ²Anglia Ruskin University, Cambridge, UK.

Presented at University of Hertfordshire Life & Medical Sciences Annual Conference 2014.

Publications: *in draft*

1. Assessment of Training Load and Lipid Peroxidation in Professional Football Players.
2. Effects of a Three-Day Period of Prolonged High-Intensity Intermittent Exercise on Biomarkers of Oxidative Stress, Muscle Damage, and Inflammation.

Commonly Used Abbreviations
(inc. Shortened Words, Symbols, & Units)

Abbreviation

~	Approximately
±	Plus, or minus
<	Less than
>	More than
≤	Less-than or equal to
≥	Greater-than or equal to
Σ	Sum of
Δ	Difference/Delta
%	Percentage
ε	Epsilon
η ²	Partial eta squared
♀	Female
♂	Male
x10 ⁹ L ⁻¹	Billion per litre
A	Absorbance
ABTS [®]	2, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate)
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
AU	Arbitrary Units
BASES	British Association of Sport and Exercise Sciences
BD	Body density
beats·min ⁻¹	Beats per minute
bf	Body fat
BM	Biomarker
Ca ²⁺	Calcium ions
CI	Confidence Interval
CK	Creatine Kinase
CK-MB	Creatine Kinase Brain isoform
CK-MM	Creatine Kinase Muscle isoform

cm	Centimetres
CMJ	Countermovement jump
COM	Comparison
CON	Control
COX	Cyclooxygenase
CO ₂	Carbon dioxide
Cr	Creatinine
CRP	C-Reactive Protein
CV	Coefficient of Variance
D	Day
DALDA	Daily Analysis of Life Demands
DAQ	Dietary Antioxidant Questionnaire
dH ₂ O	Distilled water
DNA	Deoxyribonucleic Acid
DOMS	Delayed Onset of Muscle Soreness
dp	Decimal Place
EGCG	Epigallocatechin Gallate
8-OH-2d-G	8-hydroxy-2'-deoxyguanosine
EII	Exercise-Induced Inflammation
EILP	Exercise-Induced Lipid Peroxidation
EIMD	Exercise-Induced Muscle Damage
EIOS	Exercise-Induced Oxidative Stress
ELISA	Enzyme-Linked Immunosorbent Assay
EXP	Experimental
FRAP	Ferric Reducing Ability of Plasma
g	Grams
<i>g</i>	Gravity
GSH:GSSG	Ratio oxidised to reduced Glutathione
GPS	Global Positioning System
GPX	Glutathione Peroxidase
<i>H</i>	Hypothesis
H ⁺	Hydrogen
Hb	Haemoglobin

Hct	Haematocrit
HETL	High-intensity External Training Load
HIACC	High-intensity Acceleration
HIDEC	High-intensity Deceleration
HIIE	High-Intensity Intermittent Exercise
HITL	High-intensity Internal Training Load
hr	Hour
HR	Heart rate
HRmax	Maximal heart rate
HRmean	Average heart rate
HRmin	Minimum heart rate
Hz	Hertz
ICC	Intra-class Correlation Coefficient
IKK	Inhibitor Kappa B Kinases
IL	Interleukin
Immed.	Immediately
IQR	Inter-Quartile Range
K2EDTA	Di-potassium Ethylenediaminetetraacetic Acid
kcal	Kilocalories
Kg	Kilograms
Km	Kilometres
Km·h ⁻¹	Kilometres per hour
kPa	Kilopascals
LoA	Limits of Agreement
LDH	Lactate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinase
max	Maximum
m	Metres
M or μ	Mean
MCP-1	Monocyte Chemoattractant Protein-1
MD	Muscle damage
MDA	Malondialdehyde
$\mu\text{g}\cdot\text{mL}^{-1}$	Micrograms per millilitre

μL	Microlitres
μM	Micromolars
mg	Milligrams
$\text{mg}\cdot\text{dL}^{-1}$	Milligrams per decilitre
$\text{mg}\cdot\text{d}^{-1}$	Milligrams per day
$\text{mg}\cdot\text{L}^{-1}$	Milligrams per litre
min	Minutes
mL	Millilitres
mm	Millimetres
$\text{mmol}\cdot\text{L}^{-1}$	Millimoles per litre
mRNA	Messenger Ribonucleic Acid
MS	Muscle Soreness
MVC	Maximal Voluntary Contraction
N/n	Number of participants/ per group
N	Newtons
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF- κB	Nuclear Factor kappa-light-chain-enhancer of Activated B cells
ng	Nanograms
Nm	Nanometres
NOX	NADPH oxidase
Nrf-2	Nuclear Factor Erythroid 2-Related Factor 2
O_2	Oxygen
ORAC	Oxygen Radical Absorbance Capacity
p	Probability
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Protein Carbonyls
PGC-1 α	Peroxisome Proliferator-Activated Receptor Gamma Co-activator 1-alpha
p65	Protein 65
pH	Potential of hydrogen
PLA	Placebo
PSS	Perceived Stress Scale

PUFA	Polyunsaturated Fatty Acid
PV	Plasma Volume
QC	Quality Control
QUE	Quercetin
<i>r</i>	Correlation Coefficient
rel	Relative to body mass
RBE	Repeated Bout Effect
RER	Respiratory Exchange Ratio
RNI	Reference Nutrient Intake
ROM	Range of Motion
RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
RPE	Rating of Perceived Exertion
RPE-L	Differential Rating of Perceived Exertion Legs
rpm	Revolutions per minute
s	Seconds
SD / σ	Standard Deviation
SI	Système Internationale
SIRT	Sirtuin
SSC	Stretch-Shortening Cycle
SOD	Superoxide Dismutase
sRPE	Session Rating of Perceived Exertion
SPSS	Statistical Package for the Social Sciences
STPD	Standardised Temperature Pressure Dry
T	Time point
TAC	Total Antioxidant Capacity
TAS	Total Antioxidant Status
TBARS	Thiobarbituric Acid Reactive Substances
TDC	Total Distance Covered
TEAC	Trolox Equivalent Antioxidant Capacity
TL	Training Load
TNF- α	Tumour Necrosis Factor alpha
TT	Time Trial

UI	International Units
U·L ⁻¹	International Units per litre
USA	The United States of America
VAS	Visual Analogue Scale
$\dot{V}CO_2$	Volume of Carbon Dioxide (rate)
$\dot{V}O_2$	Volume of Oxygen (rate)
$\dot{V}O_{2max}$	Maximal Oxygen uptake (rate)
XO	Xanthine Oxidase
W	Watts

Collaborations

I would like to thank Dr Jonathan Bloomfield who very kindly provided training and the use of the GPS system used in experimental chapter 4. I also extend thanks to Dr James Johnstone who assisted in coordinating the GPS validity and reliability study and Dr Matthew Furber who coordinated and assisted in $\dot{V}O_2$ max testing in chapter 4.

In experimental chapters 5 and 6, Mr Allen Huxley of Randox Laboratories provided training in the procedures and performed the quantitative determination of (i) inflammatory cytokines using a Randox Evidence Investigator™ and (ii) CRP using a Randox RX Imola™, to which I extend my thanks. I would also like to thank my fellow PhD students (Luke Oates & Dr Terun Desai) who assisted in monitoring participants during data collection in chapters 5 and 6, and Dr Lynsey Northeast, for acting as an additional first aider.

I would lastly like to thank Dr Justin Roberts and the company Biocare® for sponsorship of the quercetin supplement used in chapter 6.

Introduction

Exercise generates reactive oxygen and nitrogen species (RONS) in contracting skeletal muscle in a dose-dependent manner (Davies, Quintanilha, Brooks, & Packer, 1982; Kawamura & Muraoka, 2018; Powers, Radak, & Ji, 2016), the effects of which are complicated and unclear. Current understanding is that of the hormesis theory (Radak et al., 2017). Low to moderate levels of exercise-induced reactive species promotes force generation (Reid, Khawli, & Moody, 1993) and antioxidant training adaptations within skeletal muscle (Ji, Gomez-Cabrera, & Viña, 2006; Powers et al., 1994; Powers, 2017; Sen & Packer, 1996). However, strenuous, prolonged, or unaccustomed exercise can lead to a state of exercise-induced oxidative stress (EIOS; Powers & Jackson, 2008); oxidative stress defined as *“an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption in redox signalling and control and/or oxidative damage”* (Sies & Jones, 2007, p. 45).

Exercise-induced oxidative stress can cause skeletal muscle damage, inducing a localised and acute-phase inflammatory response as part of the repair and regeneration process of damaged skeletal muscle tissue (Malm, 2001). However, the accompanying negative effects can be detrimental to the athlete, translating to decreased force production, fatigue, compromised recovery, and ultimately underperformance (Ascensão et al., 2008; Powers et al., 2011; Radak et al., 2017; Reid et al., 1993). These negative effects have led to the recent interest in research investigating the underlying mechanisms of these phenomena and potential strategies to counteract these effects.

Soccer is an intermittent sport, characterised by repeated bouts of high-intensity exercise interspersed with bouts of lower intensities or rest (Bangsbo, Mohr, & Krustup, 2006). Throughout the soccer season, professional athletes train and compete daily. The main aims during the competitive in-season are performance maintenance and recovery between sessions, which is in contrast to the pre-season where training adaptations are the focus (Bangsbo et al., 2006). Evidence suggests that the metabolic and mechanical demands and strenuous training load of soccer cause oxidative stress (Fatouros et al., 2010; Finaud, Lac & Filaire, 2006; Ispirlidis et al., 2008). Therefore, strategies that counteract oxidative stress, muscle damage, and associated inflammation are vital if the negative effects of oxidant-mediated damage are to be avoided and recovery facilitated (Le Moal et al., 2016).

A potential recovery strategy currently being explored is the role of antioxidant supplementation in exercise (Braakhuis & Hopkins, 2015). It has been proposed that increasing antioxidants through supplementation may attenuate oxidative stress and inflammation, reducing muscle damage and fatigue, accelerating recovery (Bowtell & Kelly, 2019). An increasing number of athletes per year supplement with antioxidants (Antonioni, Fantini, Dimauro, & Caporossi, 2019; Gomez-Cabrera, Ristow, & Viña, 2012), yet, the use of antioxidant supplementation during exercise has been debated (Gomez-Cabrera et al., 2012; Peternelj & Coombes, 2011; Ristow et al., 2009). It is questioned whether blunting exercise-induced oxidative stress and inflammation is advantageous, given the positive role of RONS in inflammatory signalling mechanisms that promote skeletal muscle training adaptations (Gomez-Cabrera et al., 2005; Gomez-Cabrera, Domenech, & Viña, 2008; Peternelj & Coombes, 2011; Ristow et al., 2009). Radak et al. (2017) recently proposed that the action of

antioxidants to either enhance or suppress performance was dependent upon the timing of supplementation. Therefore, athletes should consider the detrimental effects of antioxidant supplementation on skeletal muscle training adaptations versus the potential benefits of promoting recovery (Owens, Twist, Cobley, Howatson, & Close, 2019). Antioxidant supplementation may potentially benefit athletes during intensified periods of exercise where recovery, as opposed to adaptation, is the focus (Antonioni et al., 2019). Thus, studies investigating antioxidant supplementation during intensified periods of exercise, such as the competitive in-season of soccer, are warranted.

A pertinent polyphenol of interest is the flavonoid quercetin, which occurs naturally in plant foods and is demonstrated to have (i) antioxidant; (ii) anti-inflammatory; and (iii) analgesic properties (Alexander, 2006; Boots et al., 2008; Nair et al., 2006). It is suggested that quercetin mediates its effects through inhibition of the inflammatory transcription factor NF- κ B, which is activated by oxidative stress during skeletal muscle activity (Chen et al., 2005; Comalada et al., 2005; Nair et al., 2006). The potential ergogenic effects of quercetin have stemmed from animal research (Davis, Murphy, Carmichael, & Davis, 2009b). Human studies have focused predominantly on prolonged endurance exercise (Dumke et al., 2009; Konrad et al., 2011; MacRae & Mefferd, 2006; McAnulty et al., 2008; Nieman et al., 2007a, 2007b, 2009; Quindry et al., 2008; Utter et al., 2009) but have shown equivocal results. Research investigating the efficacy of quercetin in intermittent exercise is scarce. Notably, Abbey & Rankin (2011) investigated the efficacy of quercetin supplementation on indices of oxidative stress and inflammation induced by repeated sprint exercise, characteristic of soccer, finding no effect of quercetin to attenuate oxidative stress or

inflammation, compared to a placebo. However, the duration of the exercise protocol used in this study was short (< 6 min), and research has yet to thoroughly investigate the efficacy of quercetin supplementation following prolonged intermittent exercise, such as 90 minutes of soccer.

This thesis used biomarker monitoring as an overarching theme throughout three studies to explore the concepts of exercise-induced oxidative stress, muscle damage, and associated inflammation in trained athletes undertaking intensified periods of soccer and simulated soccer. Specifically, this thesis examined (i) the oxidative stress response relative to high-intensity training load (study one), (ii) the response of an array of biomarkers indicative of oxidative stress, muscle damage, and inflammation following an intensified period of simulated soccer (study two), (iii) quercetin supplementation as a potential strategy to counteract these exercise-induced responses (study three), and (iv) exploration of the NF- κ B pathway to increase understanding of the mechanisms of exercise-induced inflammation (EII) and action of quercetin (studies two & three).

Practical implications of the research in this thesis may aid sports scientists/coaches in balancing the prescription of training loads and recovery periods in intensified periods of soccer and include (i) increased understanding of the biochemical response imposed on trained athletes during intensified periods of exercise, and (ii) evidence-based data regarding the efficacy of quercetin supplementation as a recovery strategy in trained athletes.

1.1 Research Aims & Hypotheses

The overall aims of this research were:

(i) to investigate exercise-induced oxidative stress, muscle damage, and inflammatory responses following intensified periods of soccer and simulated soccer in trained athletes, and

(ii) to determine whether prolonged quercetin supplementation attenuates exercise-induced oxidative stress, muscle damage, and associated inflammation, and promotes recovery, in trained athletes following a 3-day intensified period of prolonged high-intensity intermittent exercise (designed to simulate strenuous soccer activity).

1.1.1 Study one (experimental chapter 4).

Primary aim: To quantify urinary malondialdehyde (MDA), as a biomarker of exercise-induced lipid peroxidation (EILP), relative to high-intensity training load, in professional soccer players throughout a competitive in-season, in comparison to a group of recreational soccer players.

Hypotheses.

*H*₁: Urinary MDA concentration will change throughout a competitive in-season in a cohort of professional soccer players. (Bi-directional).

*H*₂: MDA concentration of professional soccer players will differ significantly to those of recreational soccer players because of differing training loads. (Bi-directional).

*H*₃: Changes in urinary MDA concentration in a cohort of professional soccer players will be relative to high-intensity training load.

1.1.2. Study two (experimental chapter 5).

Primary aim: To assess the short-term response of an array of blood biomarkers, indicative of (i) oxidative stress, (ii) muscle damage, and (iii) inflammation following a 3-day period of soccer-specific intensified exercise in athletes trained in intermittent exercise, in comparison to a control group.

Primary Hypothesis.

H_1 : A 3-day period of soccer-specific intensified exercise will significantly alter post-exercise systemic biomarkers of oxidative stress, muscle damage, and inflammation, in comparison to a resting control group, where biomarkers will remain unaltered.

H_0 : A 3-day period of soccer-specific intensified exercise will have no significant effect on systemic biomarkers of oxidative stress, muscle damage, and inflammation.

Sub-hypotheses.

H_{1a} : Post-exercise TAS and MDA will be significantly altered from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1b} : Post-exercise CK and LDH will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1c} : Post-exercise NF- κ B p65 will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1d} : Post-exercise CRP will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1e} : Post-exercise IL-6, IL-8, and MCP-1 will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1f} : Post-exercise anti-inflammatory cytokine IL-10 will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

H_{1g} : Post-exercise total leukocyte and neutrophil counts will be significantly increased from baseline following the 3-day period of prolonged high-intensity intermittent exercise, and will remain altered throughout recovery, in comparison to a resting control group.

1.1.3. Study three (experimental chapter 6).

Primary aim: To investigate the efficacy of prolonged quercetin supplementation, compared to a placebo, on blood biomarkers of (i) oxidative stress, (ii) cellular damage, and (iii) inflammation, following a controlled 3-day period of soccer-specific intensified exercise in trained athletes.

Primary Hypothesis.

H_1 : Prolonged quercetin supplementation will attenuate exercise-induced cellular damage and inflammation, and improve post-exercise recovery, following a 3-day period of soccer-specific intensified exercise in trained athletes, in comparison to a

placebo.

H_0 : Prolonged quercetin supplementation will not affect recovery measures, and indices of cellular damage and inflammation, following a 3-day period of soccer-specific intensified exercise in trained athletes.

Sub-hypotheses.

H_{1a} : Prolonged quercetin supplementation will attenuate serum LDH concentration following three days of prolonged high-intensity intermittent exercise in trained athletes, in comparison to a placebo.

H_{1b} : Prolonged quercetin supplementation will attenuate inflammatory indices (CRP, NF- κ B p65 activation, IL-6, total leukocyte & neutrophil counts) following three days of prolonged high-intensity intermittent exercise in trained athletes, in comparison to a placebo.

H_{1c} : Prolonged quercetin supplementation will alter indices of oxidative stress (TAS; MDA) following three days of prolonged high-intensity intermittent exercise in trained athletes, in comparison to a placebo.

H_{1d} : Prolonged quercetin supplementation will improve subjective measures of perceived fatigue and muscle soreness following three days of prolonged high-intensity intermittent exercise in trained athletes, in comparison to a placebo.

Literature Review

This literature review will discuss exercise-induced oxidative stress, muscle damage, and associated inflammation with specific reference to prolonged high-intensity intermittent exercise, which is performed in team sports, in this case, soccer¹. The potential beneficial role of prolonged quercetin supplementation as a recovery strategy that targets the NF- κ B pathway to counteract these phenomena will also be considered.

2.1 Physiological Demands of Soccer

A current area of research interest is the physiological demands imposed on professional soccer players throughout a competitive season. High training loads, congested match schedules, and short between-match recovery periods associated with modern-day soccer puts these athletes at an increased risk of residual fatigue and incomplete recovery (Silva et al., 2018). Training and competing in a state of incomplete recovery could increase injury risk and lead to underperformance in subsequent training sessions or competitive matches (Nédélec et al., 2012; Silva et al., 2018). Therefore, understanding mechanisms of factors that cause fatigue and hinder recovery in this type of exercise is vital, as these may serve as targets to modulate with a recovery intervention or may provide knowledge to aid coaches and sports scientists in the prescription of altered training loads.

Soccer is characterised by prolonged high-intensity intermittent exercise; that is, repeated bouts of high-intensity exercise (such as running & sprinting), interspersed

¹ Throughout this thesis English football will be referred to as *soccer*, as football refers to American football in the literature.

with recovery periods of lower intensity exercise (i.e., bouts of walking & jogging), and rest (Drust, Atkinson, & Reilly, 2007). Match analyses have recorded the total distance covered during a competitive soccer match to be ~10 km (10 - 12 km outfield players; Bangsbo et al., 2006; Bradley et al., 2010). The physiological load of this type of exercise is high, with the average intensity of a competitive soccer match performed at ~85% maximum heart rate (HR_{max}); equating to ~70% $\dot{V}O_2$ max (maximal oxygen uptake; Bangsbo, 1994; Mohr, Krusturup, & Bangsbo, 2003). Soccer uses all three energy systems (i) phosphocreatine system, (ii) anaerobic glycolysis, and (iii) aerobic metabolism (Bangsbo et al., 2006). There is a high reliance on aerobic metabolism; to support the low intensities of walking and jogging and reach higher intensities of running and sprinting (Bangsbo et al., 2006). In addition to the repeated bouts of high-intensity running and sprinting, a high number (~220 per match; Souglis et al., 2015) of high-intensity muscle actions, such as accelerations, decelerations, directional changes, and jumps, are also performed (Akenhead, Hayes, Thompson & French 2013; Bangsbo et al., 2006; Nédélec et al., 2012); approximately 20% of a 90 min match is played at high-intensities (Akenhead et al., 2013). A reduction in the (i) total distance covered; (ii) percentage of time spent high-intensity running; and (iii) total number of high-intensity actions has been observed in the second half of a match, attributed to cumulative fatigue (Mohr et al., 2003).

During microcycles of a competitive season, professional soccer players frequently train daily (once or twice per day) and play up to two (occasionally three) competitive matches per week (Bangsbo et al., 2006; Jeong et al., 2011). The training load prescribed is periodised, dependent upon the phase of the season. The main aim of pre-season training is increased fitness, whereas, during the competitive period, the

focus is on maintenance of performance (Jeong, Reilly, Morton, Bae, & Drust, 2011), facilitating between match recovery and avoiding maladaptation is fundamental during this time. The time period of recovery between successive training sessions and competitive matches is typically 24 - 72 hr matches (Bangsbo et al., 2006; Nédélec et al., 2012; Reilly & Ekblom, 2006). Given that the physiological demands of training must simulate competitive match play (i.e., intensity, frequency, volume, & duration [Iaia, Rampini, & Bangsbo, 2009]), current recovery periods may be insufficient to allow complete recovery between successive sessions. Recovery from training/competition can be defined as complete when an athlete meets an individualised performance target in a sports-specific test (Bishop, Jones, & Woods, 2008). Previous research has shown the kinetics of fatigue and recovery markers following exercise characteristic of soccer to be longer than 72 hr (Nédélec et al., 2012). The implication of training in a state of incomplete recovery is that residual fatigue may accumulate (Silva et al., 2013). As a consequence, there is an increased risk of overreaching and injury, which may impact subsequent performances (Bengtsson, Ekstrand & Hagglund, 2013; Nédélec et al., 2012). Thus, the ability to recover quickly is vital for these athletes.

The physiological demands of the high-intensity intermittent nature of soccer have been well documented (Bangsbo, 1994; Heisterberg et al., 2013). The high metabolic and mechanical stress associated with the high number of eccentric muscle actions involved in repeated accelerations and decelerations has been shown to induce muscle damage (Ascensão et al., 2008; Ispirlidis et al., 2008; Souglis et al., 2015), which is associated with a localised and systemic inflammatory response (Fatouros & Jamurtas, 2016) that can induce oxidative stress and further

secondary muscle damage (Andersson et al., 2008; Fatouros et al., 2010; Finaud, et al., 2006a; Ispirlidis et al., 2008; Souglis et al., 2015; refer to section 2.6). These exercise-induced phenomena have negative implications for the athlete, contributing to peripheral fatigue (i.e., fatigue localised within skeletal muscle; fatigue commonly defined as a reduction in muscular force or power [Gandevia, 2001]), and muscle soreness that can lead to decreased neuromuscular performance, impaired recovery, and underperformance (Fatouros et al., 2010; Finaud et al., 2006a; Ispirlidis et al., 2008).

High-level soccer players are at an increased risk of exercise-induced muscle damage, oxidative stress, and inflammation during a competitive in-season due to congested schedules (Le Moal et al., 2016; Nédélec et al., 2012). Monitoring the training load concomitant with biomarkers of exercise-induced muscle damage (EIMD), exercise-induced oxidative stress (EIOS), and EII would aid in the prescription of balanced training loads and recovery periods that ensures athletes are in a state of readiness to train (Le Moal et al., 2016).

2.1.1 Quantifying training load.

Quantifying the training load (team & individual athletes) throughout a competitive season is essential to (i) determine the effectiveness of training; (ii) understand individual responses; (iii) prescribe training loads that optimise performance; (iv) minimise fatigue and injury risk; and (v) determine complete recovery (Borresen & Lambert, 2009; Halson, 2014; Jeong et al., 2011; Scott, Lockie, Knight, Clark, & Janse de Jonge, 2013; Sparks, Coetzee, & Gabbett, 2016). There are two types of training load (TL); internal training load (ITL) and external training load (ETL [Halson,

2014]). Internal training load is based on a measure of physiological/psychological stress (e.g., heart rate [HR], often weighted by duration); in contrast, external training load is a measure of physical stress that can be defined as the work done and is often based on movement (i.e., speed & distance covered; Gabbett, 2016; Halson, 2014; Scott et al., 2013). To assess the overall response to the prescribed training load, it is important to assess both types of training load (Halson, 2014; Impellizzeri, Rampini, & Marcora, 2005; Sparks et al., 2016) as a “disassociation” (Halson, 2014, p. 139) between the two could be a cause of fatigue. Furthermore, it is important to quantify the intensity of the training load; however, no set definition of high-intensity exists within soccer literature. High-intensity is usually defined by heart rate or speed zones (as a percentage of time e.g., > 80% HRmax or > 19.8 km·h⁻¹; Bradley et al., 2009; Dwyer & Gabbett, 2012).

There is no *gold standard* method to quantify training load in team sports such as soccer, with several methods available, including heart rate monitoring, Rating of Perceived Exertion (RPE) based methods (e.g., Bannister’s TRIMPS; Edward’s TRIMP; session-based RPE; Foster et al., 2001), questionnaires, biochemical and video analysis (Halson, 2014). Each method has advantages and limitations (e.g., cost-effectiveness; practicality; subjectivity; for a review of methods monitoring training load in athletes specific to fatigue readers are referred to Halson, 2014). Global Positioning Systems are now widely used in team sports and have become the standardised method used to quantify training load in professional soccer (Aughey, 2011; Cummins, Orr, O’Connor, & West, 2013; Scott et al., 2013) and in the literature. Global Positioning Systems allow simultaneous quantification of ETL and ITL (due to built-in heart rate monitoring) in individual athletes, enabling

comparisons between athletes and training sessions, aiding coaches/sports scientists in the prescription of training load and fatigue detection (Aughey, 2011).

2.1.2 Monitoring recovery.

Recovery can be assessed through monitoring exercise-induced alterations from baseline in sports-specific tests (Bishop et al., 2008). Standard tests in team sports such as soccer include (i) jump testing, (ii) isokinetic knee extensor/flexor strength testing, (iii) repeated sprint tests (objective measures), or subjective measures, such as (iv) RPE, and (v) perceived lower body muscle soreness (Nédélec et al., 2012). A battery of sports-specific tests is recommended; the reliability, sensitivity, and the impact of the tests on recovery should also be considered (McLean, Coutts, Kelly, McGuigan, & Comack, 2010; Nédélec et al., 2012).

Another method of monitoring recovery is biomarker monitoring (Nédélec et al., 2012).

2.1.2.1 Biomarker monitoring.

Biomarkers are now routinely used in exercise research to reflect exercise-induced responses (Halson, 2014), and provide a useful objective assessment of recovery (Nédélec et al., 2012). A biomarker (short for biological/biochemical marker) has been defined by the World Health Organisation as “*any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease*” (Strimbu & Tavel, 2010, p. 2). This definition is health-related, but there has been an interest in regular biomarker monitoring of athletes (as a tool to optimise training by determining a balance between training

load and recovery [Heisterberg et al., 2013; Lee et al., 2017]); therefore, redefining this definition in the context of exercise-induced responses is potentially warranted. In team sports such as soccer, monitoring recovery is essential because of the demands of training and congested match fixtures (Heisterberg et al., 2013). Once a basal value of a biomarker is known, the impact of training/match play can be monitored to facilitate prescription of training loads that maximise performance while minimising fatigue and the risk of injury, or provide evidence to support the implementation of a recovery strategy (Heisterberg et al., 2013; Huggins et al., 2018; Lee et al., 2017). Research is yet to determine *gold standard* biomarkers for detecting exercise-induced responses. The ideal model is the simultaneous assessment of an array of biomarkers (Lee et al., 2017; Nédélec et al., 2012; Powers, Smuder, Kavazis & Hudson, 2010 [as was used in chapters 5 of this thesis, which assessed biomarkers of oxidative stress, muscle damage, & pro- & anti-inflammation]). Importantly, chosen biomarkers should be sensitive enough to detect exercise-induced changes, as even minor fluctuations from basal values could have implications for the athlete (Heisterberg et al., 2013; Lee et al., 2017).

Biomarkers can be assessed in urine, saliva, sweat, and exhaled breath (non-invasive procedures), or skeletal muscle tissue and blood components (invasive methods). The advantages of non-invasive methods include practicality for the field setting and less disruption for the athlete/coach. Furthermore, spot urine sampling is a method most high-level athletes are familiar with (providing samples for hydration & drug monitoring). Thus, it is prudent that future research considers the efficacy of such non-invasive methods (Lindsay & Costello, 2017 [as was the focus of chapter 4 of this thesis, which assessed a biomarker of lipid peroxidation in urine]). However,

blood components and skeletal muscle tissue are the gold standard sample type (Lindsay & Costello, 2017). Of the invasive methods, the ease of blood sampling in comparison to muscle biopsies makes it preferable. Each sample type and analytical method has advantages and disadvantages (namely cost & method), with choice of biomarker usually logistics dependent (Marrocco, Altieri, & Peluso, 2017). Several different biomarkers have been used within the exercise recovery literature (established biomarkers are presented, Table 2.1), making comparisons of results difficult; a more consistent approach to biomarker monitoring is required (Lee et al., 2017). A common issue with biomarker monitoring is false-positive values arising due to exercise-induced changes in sweat rate and hydration status. Therefore, it is imperative to correct for exercise-induced changes in blood volume or urine concentration (Dill & Costill, 1974; Matömaki, Kainulainen & Kyröläinen, 2018; chapters 4, 5 & 6 of this thesis focused on quantifying post-exercise changes in biomarkers following intensified periods of soccer, correcting for urine creatinine concentration & haemoconcentration). Care should also be taken when using indirect measures; for example, post-exercise increases in a systemic biomarker may not accurately reflect skeletal muscle tissue (Powers et al., 2010b). There are limited sports-specific reference ranges for biomarkers, which is surprising given that biomarker responses in trained athletes are usually outside of normal reference ranges (Mougios, 2007). It is also essential to contextualise exercise-induced alterations in biomarkers to performance measures to determine the impact on performance (Lee et al., 2017; as was an original focus of chapter 6 of this thesis [CMJ data removed]).

An overarching theme of this thesis was monitoring biomarkers of (i) exercise-induced oxidative stress, (ii) biomarkers that provide indirect evidence of exercise-induced skeletal muscle damage, and (iii) biomarkers of pro- and anti-inflammatory responses.

Table 2.1

Common Biomarkers of Oxidative Stress, Muscle Damage, & Associated Inflammation in Exercise Studies

	Oxidative Stress	Muscle Damage	Inflammation
Antioxidants	Oxidative Stress By-products		
Ferric Reduction Ability of Plasma (FRAP)	Aldehydes	Alanine Transaminase (ALT)	C-Reactive Protein (CRP)
Glutathione (GSH)	Conjugated dienes	Aldolase	Cytokines, myokines, chemokines
Glutathione Peroxidase (GPX)	EPR Spectroscopy	Aspartate Aminotransferase (AST)	
Oxygen Radical Absorbance Capacity (ORAC)	8-Hydroxy-2-deguanosine (8-2-OH-dG)	Carbonic Anhydrase (CAIII)	NF-κB (p65/p50)
Protein carbonyls (PC)	F2-isoprostanes	Creatine Kinase (CK)	Prostaglandins
Superoxide Dismutase (SOD)	4 Hydroxyl-noneal (4-HNE)	Lactate Dehydrogenase (LDH)	Total Leukocyte Count
Total Antioxidant Status (TAS)	Lipid Hydroxides (LOOH)	Myoglobin	Total Neutrophil Count
Trolox Equivalent Antioxidant Capacity (TEAC)	Lipid Hydroperoxides	Urea	
Uric Acid (UA)	Oxidised LDL		
Vitamin C	Pentane Gas		
Vitamin E	Thiobarbituric Acid Reactive Substances (TBARS)		
	Ratio of oxidised glutathione to reduced glutathione (GSH: GSSG)		

Note. EPR = Electron Paramagnetic Resonance; LDL = Low-density Lipoprotein; NF-κB = Nuclear Factor light-chain-enhancer of B cells; (Brancaccio, Lippi, & Maffulli, 2010; Powers & Jackson, 2008; Powers, Duarte, Kavazis, & Talbert, 2010a).

2.2 Defining Exercise-Induced Oxidative Stress

Exercise-induced oxidative stress first came to provenance in 1978, when Dillard, Litov, Savin, Dumelin, and Tappel discovered that endurance exercise increased oxidative stress (assessed via expired pentane) and Davies et al. (1982) demonstrated increased free radical production in rat skeletal muscle tissue following exhaustive exercise. Since then, studies have shown that contracting skeletal muscle produces oxidants in a dose-dependent manner (Powers et al., 2016), and exercise-induced oxidative stress has been shown to occur following unaccustomed, prolonged, or high-intensity exercise (Powers & Jackson, 2008). Oxidative stress occurs when the amount of Reactive Oxygen and Nitrogen Species (RONS²) overwhelm the body's antioxidant defences (Powers & Jackson, 2008) and has more recently been redefined in the literature as *“an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption in redox signalling and control and/or oxidative damage”* (Sies & Jones, 2007, p. 45). It is important to note that this definition of oxidative stress clearly denotes that oxidative stress is not simply oxidants outweighing antioxidants, as an elevation in oxidants may not necessarily lead to oxidative damage. In this thesis, EIOS (chapters 5 & 6) is defined as a post-exercise alteration in a redox-sensitive marker, as evidenced by either (i) a significant post-exercise increase in a biomarker of oxidative stress or (ii) a significant decrease in a biomarker of antioxidant status, compared to pre-exercise

² Terms in redox biology are often used interchangeably. Throughout this thesis, RONS is used collectively to describe reactive oxygen & nitrogen species derived from oxygen and nitrogen during exercise. The terms oxidative stress, oxidant-mediated damage, oxidative damage will also be representative of nitrosative stress/damage. It should be noted that not all RONS are free radicals (e.g., H₂O₂). For clarification of terminology, readers are referred to a review by Nikolaidis et al. (2012).

values (Powers & Jackson, 2008). Reductive stress can also occur, when antioxidant defences exceed RONS (Powers, DeRuisseau, Quidry, & Hamilton, 2004).

2.2.1 Sources of exercise-induced RONS.

Several sources of RONS production during exercise have been proposed; however, the primary source of RONS production remains unclear (Henríquez-Olguín et al., 2019). It is theorised that the principal source of RONS generation within contracting skeletal muscle was mitochondrial (Davies et al., 1982; Packer, 1997), although this theory has recently been debated with non-mitochondrial sources now proposed (Henríquez-Olguín, Meneses-Valdes, & Jensen, 2020; Powers et al., 2016). Superoxide radical (O_2^-) generation by cytosolic enzymes (i.e., Nicotinamide Adenine Dinucleotide Phosphate [NADPH] oxidase [NOX] isoenzymes [NOX-2 & NOX-4]) has been suggested to be the predominant source of RONS generation during exercise, with mitochondrial superoxide generation associated with post-exercise recovery (Henríquez-Olguín et al., 2020). NOX generates superoxide radicals through the oxidation of NADPH and reduction of oxygen and are located within several cell organelles, including the sarcolemma, sarcoplasmic reticulum, and transverse tubules (Powers et al., 2011). Evidence to support NOX as the primary source of RONS production during contracting skeletal muscle is provided indirectly by murine and in vivo studies using mitochondrial-specific redox probes in muscle fibres (Henríquez-Olguín et al., 2019; Michaelson, Shi, Ward, & Rodney, 2010). In 2019, Henríquez-Olguín et al. found mitochondrial generated hydrogen peroxide (H_2O_2) during moderate-intensity treadmill running to be decreased in comparison to a resting condition.

The mitochondria were originally proposed as the main source of RONS production within contracting skeletal muscle (Davies et al., 1982; Powers et al., 2016). Approximately 2 - 5% of oxygen consumed was thought to be reduced during oxidative phosphorylation to form superoxide radical (Powers et al., 2011; Powers et al., 2018). In brief, exercise increases oxygen consumption; thus, oxygen flux through the mitochondria in contracting skeletal muscle is increased. During the reduction of oxygen to water, electrons passing through the electron transport chain leak (possibly mostly from complex III ubiquinone/cytochrome level, although 11 different sites have been identified Henríquez-Olguín et al., 2020), and reduce oxygen via electron addition, forming superoxide (Gomes, Silva, & Rubino du Oliveria, 2012), in a dose-dependent manner (Powers & Jackson, 2008). Superoxide is a highly reactive and unstable free radical (Powers et al., 2011). Superoxide radicals can dismutase (catalysed by superoxide dismutase) into hydrogen peroxide (H_2O_2), which can react further to create hydroxyl radicals (HO^\cdot), another highly reactive species due to its mobility, formed through the Fenton and Haber-Weiss transition metal-based reactions (Packer, 1997; Powers et al., 2016). Collectively these oxygen-derived species are known as *reactive oxygen species (ROS)*. Exercise-induced Nitric Oxide released from endothelial cells can also react with hydroxyl radicals to form peroxynitrates (Powers et al., 2011), another highly reactive species, known as a *reactive nitrogen species (RNS)*, together these can be termed *RONS (reactive oxygen and nitrogen species; Powers et al., 2011)*.

It is theorised that RONS may exert effects locally within their subcellular compartments (mitochondria vs. cytoplasm; Henríquez-Olguín et al., 2020). For example, the location of NOX-2 in the sarcolemma and transverse tubules may lead

to RONS being generated in the cytoplasm and extracellular space, whereas superoxide radicals generated within the mitochondria may dismutate to H₂O₂ via superoxide dismutase isoenzymes located within the mitochondria matrix and inner membrane (Henríquez-Olguín et al., 2020; Powers et al., 2011). Furthermore, it is also suggested that redox signalling of exercise-induced training adaptations within skeletal muscle is likely to be compartmentalised (Henríquez-Olguín et al., 2020; for an overview of compartmentalised RONS production, readers are referred to Henríquez-Olguín et al., 2020).

Other proposed sources of exercise-induced RONS production include (i) ischemic-reperfusion hypoxanthine conversion of xanthine oxidase; (ii) phospholipase A₂-dependent processes; and (iii) catecholamine auto-oxidation (Finaud et al., 2006a; Packer, 1997; Powers & Jackson, 2008; Powers, Duarte, Kavazis, & Talbert, 2010). A secondary, non-muscle source of RONS production is superoxide production during the *neutrophil respiratory burst*, which occurs as part of the acute-phase inflammatory response to exercise-induced muscle damage (Powers et al., 2010a; refer to section 2.2.4). At present, sources of RONS contributing to the presence of RONS in blood and urine are unclear (Henríquez-Olguín et al., 2019).

Sources of RONS production during exercise is likely to be exercise mode and intensity dependent (Henríquez-Olguín et al., 2019). The exact source of RONS production in prolonged high-intensity intermittent exercise, performed in team sports such as soccer, is unknown. Given that the high-intensity intermittent nature of this type of exercise involves aerobic pathways, hypoxia, reoxygenation, and repetitive eccentric muscle actions that could lead to muscle damage and associated

inflammation, it should be considered that a combination of sources contributes to RONS generation, including (i) NOX isoenzymes; (ii) aerobic metabolism; (iii) increased catecholamines; (iv) ischemia-reperfusion; and (v) the neutrophil respiratory burst (Ascensão et al., 2008; Henríquez-Olguín et al., 2020; Magalhães et al., 2010). It should also be considered that because soccer is a field-based sport, pollution is a factor that could also contribute to RONS production (Lamprecht, Greilberger, & Oettl, 2004).

Increased production of RONS during exercise can lead to exercise-induced oxidative stress, with potential positive and negative consequences for the athlete.

2.2.2 Negative & positive roles of exercise-induced RONS.

This area of research is ever-evolving, and only recently have the positive and negative roles of exercise-induced RONS been understood. Originally, RONS production was thought to be an undesirable effect of exercise because evidence suggests that EIOS can cause cellular damage to lipids, proteins, and DNA which results in structural or functional changes and possibly cell apoptosis and tissue necrosis (Halliwell & Gutteridge, 2015; Powers & Jackson, 2008; Powers et al., 2010a). Oxidation of lipids, *lipid peroxidation*, is a source of oxidant damage during exercise that is commonly assessed (Packer, 1997). Polyunsaturated fatty acids (PUFAs) located in the sarcolemma are highly susceptible to oxidation due to (i) location (near sites of RONS generation) and (ii) double bonds containing highly reactive hydrogen atoms. Lipid peroxidation is a *chain reaction* of events (as described by Powers & Jackson, 2008; Figure 2.1). In brief, the process involves three steps (i) initiation, (ii) propagation, and (iii) termination, involving the production

of a fatty acid radical (usually initiated by superoxide or hydroxyl radical abstracting an H[•] atom from an unsaturated carbon located in a methylene bridge of a PUFA), that reacts with oxygen to form a peroxy-fatty acid radical (propagation). The chain reaction is terminated when two radicals react to produce a non-radical species, or alternatively, an antioxidant neutralises the free radical (Powers & Jackson, 2008).

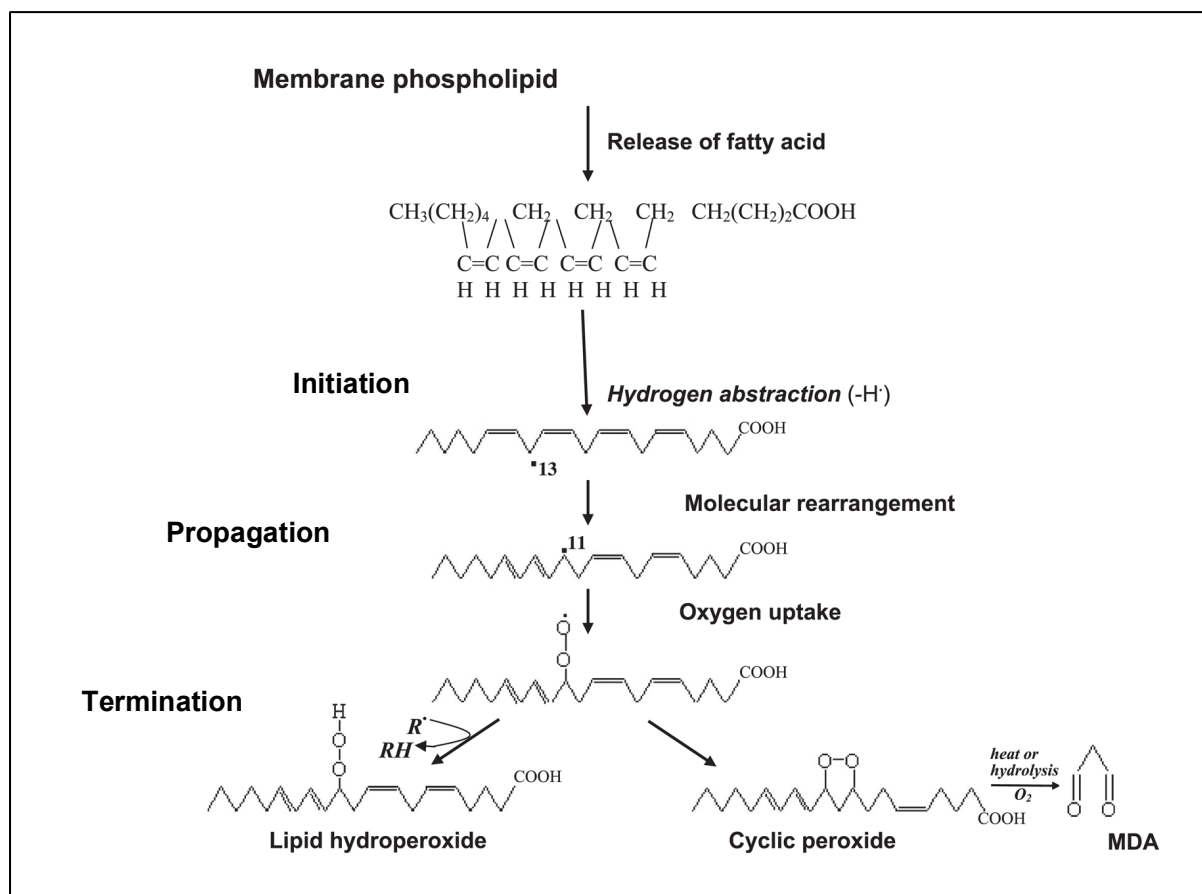


Figure 2.1. Overview of Lipid Peroxidation, adapted from Powers & Jackson (2008), p. 50. Malondialdehyde (MDA) is formed as an end-product of lipid peroxidation.

For the athlete, oxidant damage to skeletal muscle can translate to skeletal muscle and endothelial dysfunction, and is associated with muscle damage, inflammation, muscle soreness, decreased force production, fatigue, and ultimately impaired performance (Le Moal et al., 2016; Powers et al., 2010a; 2011; Radak et al., 2017;

Reid et al., 1993; Reid, Stokić, Koch, Khawli, & Leis, 1994). Stemming from work by Reid et al. (1993) on the inverted U relationship between redox state and force production (Figure 2.2; note the inverted U represents levels of oxidants), a beneficial, hormetic³ role of exercise-induced RONS has now emerged (Ji et al., 2006; Powers et al., 2016; Radak, Chung, Koltai, Taylor, & Goto, 2007; Radak et al., 2017). While excessive RONS leading to EIOS can cause skeletal muscle damage, it is now theorised that RONS play a role in cell signalling (Gomez-Cabrera et al., 2008; Gomez-Cabrera, Viña, & Ji, 2016; Powers et al., 2016; Radak, Chung, & Goto, 2008). It is proposed that RONS activate transcription pathways (e.g., NF- κ B; Nrf-2; MAPK) that upregulate expression of genes that promote training adaptations within skeletal muscle, for example, inducing antioxidant enzymes (promoting tolerance to oxidative stress) and PGC-1 α (stimulating mitochondrial biogenesis; Gomez-Cabrera et al., 2015).

³ *Hormesis*: “low dose ... results in a beneficial adaptation whereas a high dose results in a toxic effect” (Powers, Radak & Ji, 2016, p. 5087).

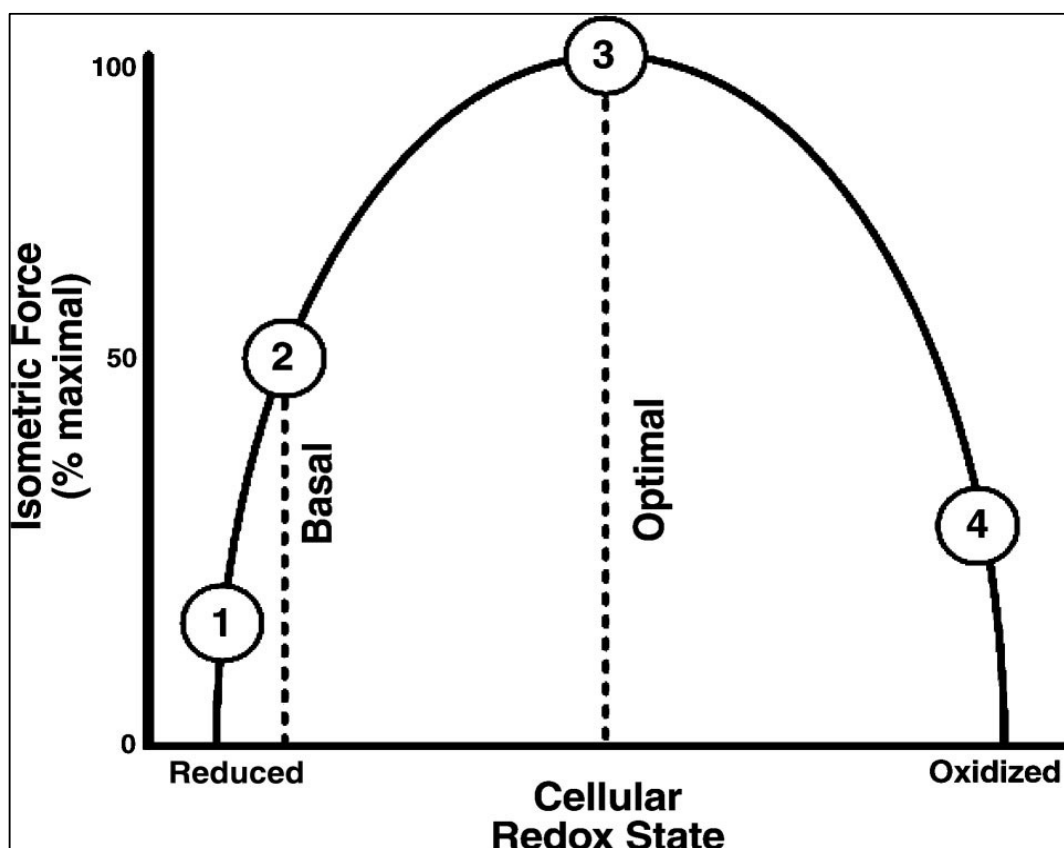


Figure 2.2. The concept of hormesis applied to the role of reactive oxygen species (ROS) in force production (Reid et al., 1993). Recopied from Powers & Jackson (2008), p. 1259. Point 4 represents the detrimental excessive effects of ROS on force production.

2.3 Defining Antioxidants

The function of the antioxidant defence system is to protect cells from oxidant damage (Powers et al., 2004). Antioxidants have been defined in the literature by the prominent leaders in the field as “*molecules that can be present in small concentrations compared to other oxidisable biologically-relevant molecules and prevent or reduce the extent of oxidative damage to other biologically-relevant molecules*” (Halliwell & Gutteridge, 1989, as cited in Burke & Deakin, 2015 p. 297).

More recently, this has been updated to “*any substance that delays, prevents or removes oxidative damage to a target molecule*” (Halliwell & Gutteridge, 2015, p. 78). There are two classes of antioxidants (i) endogenous (cellular, produced within the body) and (ii) exogenous (dietary, derived from plants; Burke & Deakin, 2015 [Table 2.2]), and it can be deduced that this updated definition of antioxidants now incorporates both classes. Antioxidants protect against oxidative stress through several mechanisms, including directly scavenging RONS into less harmful species, inhibiting the formation of harmful RONS, elimination, and by binding to metal ions (Burke & Deakin, 2015; Powers et al., 2004; Tejada et al., 2017).

Antioxidants are strategically located in lipid and aqueous, intra-cellular (cell membranes, cytoplasm, mitochondria) and extra-cellular compartments (plasma; Powers & Jackson, 2008; Figure 2.3 [note the location of exogenous antioxidants in the cell membrane]). For example, vitamin E is located in cell membranes and protects PUFA from lipid peroxidation by scavenging radicals causing termination of lipid peroxidation chain reactions (Powers & Lennon, 1999). Endogenous and exogenous antioxidants work synergistically to provide protection (Packer, 1997). For example, vitamin E radicals are formed when vitamin E scavenges RONS. Vitamin C reduces vitamin E radicals back to vitamin E (Brites et al. 1999; Packer, 1997; Powers et al., 2010a); however, a vitamin C radical is then produced but can be regenerated by glutathione (Packer, 1997).

Table 2.2

Classes of Common Antioxidants

	Endogenous Sources	Exogenous Sources	Endogenous/ Exogenous Sources
Non-enzymatic	Bilirubin Uric acid	α -Lipoic Acid Carotenoids e.g. β -carotene Polyphenols e.g. flavonoids Selenium Vitamins A, C, E, K Zinc	Co-enzyme Q ₁₀ Glutathione
Enzymatic	Primary: Catalase Glutathione Peroxidase Superoxide Dismutase Secondary: Glutathione Reductase Glutathione-6-Phosphate Dehydrogenase Accessory: Glutaredoxin Peroxiredoxin Thioredoxin		

Note. Recreated from Powers et al. (2004); Powers & Jackson (2008).

Under normal exercising conditions, the body's antioxidant defences can cope with the amount of RONS generated (Burke & Deakin, 2015). Redox homeostasis is maintained by exercise-induced RONS signalling the upregulation of antioxidant enzymes via redox-sensitive pathways (Gomez-Cabrera et al., 2016; Powers et al., 2016) and mobilisation of antioxidants to sites of oxidants (Burke & Deakin, 2015;

Powers & Jackson, 2008). However, during intensified periods of exercise (such as a soccer competitive in-season), the antioxidant defence system may be compromised, leading to EIOS. The magnitude of oxidative stress is dependent not only upon the amount of RONS produced but the level of antioxidants (Burke & Deakin, 2015; Halliwell & Gutteridge, 2015); thus, EIOS can occur as a result of excessive RONS generation, a lack of antioxidant defenses, or both. Exercise-induced oxidative stress is intrinsically linked with muscle damage and an inflammatory response (Brancaccio et al., 2010; Fatouros & Jamurtas, 2016).

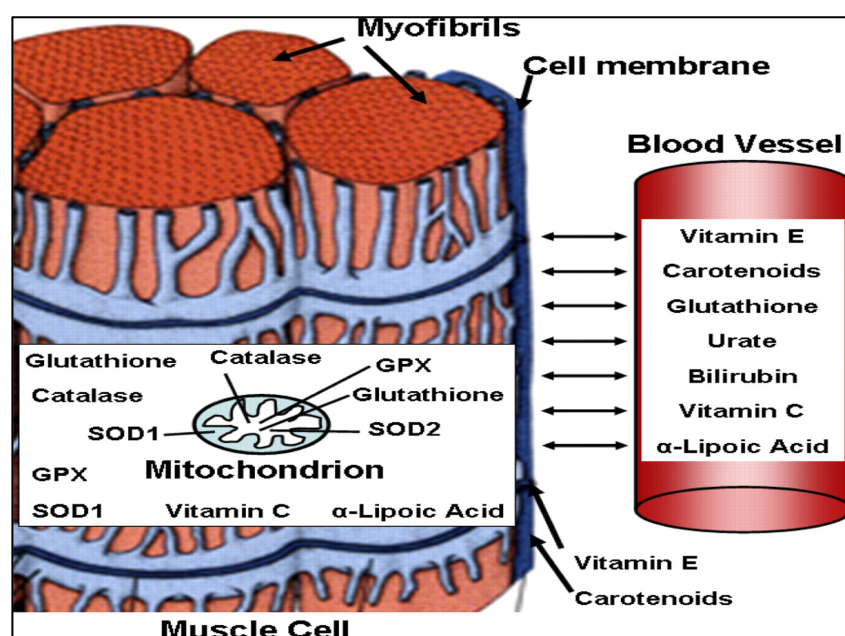


Figure 2.3. Locations of primary antioxidants. Recopied from Powers & Jackson (2008), p. 1248. Antioxidants are located in intra- & extra-cellular compartments.

2.4 Exercise-Induced Muscle Damage & Associated Inflammation

Exercise-induced muscle damage is associated with a concomitant localised inflammatory response within skeletal muscle, accompanied by a systemic inflammatory response, known as the *acute-phase response* (Pederson 2000;

Petersen & Pedersen, 2005). Exercise-induced muscle damage and associated inflammation are part of the repair and regeneration process of adaptive remodeling of skeletal muscle tissue that promotes training adaptations (Fatouros & Jamurtas, 2016; Malm, 2001; Peake et al., 2017).

2.4.1 Primary & secondary muscle damage.

Exercise-induced muscle damage following eccentric exercise is well documented and is observed in untrained and trained athletes (Proske & Allen, 2005; Proske & Morgan, 2001). Eccentric muscle contractions (i.e., lengthening under tension [Proske & Morgan, 2001]), for example, the action of the hamstrings during decelerating, recruit fewer myofibres when compared to concentric contractions (Peake et al., 2017). The resulting increased mechanical load per fibre can lead to ultrastructural damage that is the predominant cause of primary muscle damage (Peake et al., 2017; Proske & Allen, 2005). Type II myofibres are mainly affected (Jamurtas, 2018; Owens et al., 2019; Twist & Eston, 2005). Mechanisms of muscle damage are complex. Proposed underlying mechanisms of primary muscle damage include (i) overstretching of sarcomeres resulting in cell membrane damage (*sarcomere popping* [Proske & Morgan, 2001]); (ii) disturbances in sarcoplasmic Ca^{++} homeostasis resulting from membrane damage; and (iii) excitation-contraction coupling dysfunction (Owens et al., 2019; Peake et al., 2017; Proske & Allen, 2005; Proske & Morgan, 2001). Z band streaming provides evidence of myofibre disruption but is perhaps evidence of skeletal muscle remodeling, indicative of the beneficial role of exercise-induced muscle damage (Peake et al., 2017).

Exercise-induced muscle damage response is bimodal; following primary muscle damage, secondary muscle damage can occur as a result of (a) proteolysis and (b) RONS produced during inflammation (Owens et al., 2019; Peake et al., 2017; Proske & Allen, 2005; Proske & Morgan, 2001). The *acute phase* inflammatory response is characterised by upregulation of cytokines/chemokines, acute phase proteins, immune cells, and antioxidant enzymes responsible for skeletal muscle tissue repair and regeneration (Fatouros & Jamurtas, 2016; Giudice & Gangestad, 2018; Pyne, 1994b; Gomez-Cabrera et al., 2016). Moreover, interleukin-six (IL-6), a *myokine* (cytokine released from myocytes [Pedersen & Febbraio, 2008]) released from contracting skeletal muscle stimulates the hepatic release of C-Reactive Protein (CRP) as part of the acute-phase response (Giudice & Gangestad, 2018; Pedersen, 2000; Petersen & Pedersen, 2005). A variety of other cells also produce cytokines (e.g., endothelial & blood cells; Pedersen et al., 2001; Suzuki, 2018) at sites of inflammation, inducing a pro-inflammatory cytokine cascade. Cytokines act as intracellular signalling molecules regulating inflammation (Petersen & Pedersen, 2005), with several functions, including (i) chemotactic activity of leukocytes, (ii) angiogenesis, (iii) expression of adhesion cells, and (iv) glucose homeostasis (Pedersen et al. 2001; Petersen & Pedersen, 2005; Suzuki, 2018).

Following primary muscle damage, neutrophils (& leukocyte subsets) migrate and infiltrate sites of skeletal muscle tissue damage, intentionally releasing superoxide radicals, H_2O_2 , and proteases (e.g., myeloperoxidase) to remove damaged tissue as part of the neutrophil respiratory burst (Peake et al., 2017; Powers et al., 2010b). However, neutrophil production of ROS at sites of skeletal muscle damage is a double-edged sword, potentially causing secondary muscle damage to undamaged

skeletal muscle tissue at the sites of primary muscle damage (Pyne, 1994a). This localised leukocytosis causes swelling and is a proposed cause of delayed onset of muscle soreness (DOMS; Brancaccio et al., 2010; Howatson & Van Someren, 2008).

It is proposed that exercise-induced RONS regulate inflammation, acting as cell signalling molecules to activate the NF- κ B pathway, which induces expression of cytokine IL-6 in a downstream manner (Kramer & Goodyear, 2007 [the role of NF- κ B activation during exercise is further explored, refer to section 2.7]).

The pro-inflammatory response is counteracted by the release of anti-inflammatory cytokines and cytokine inhibitors (e.g., IL-10; IL-1ra), which suppress further synthesis of pro-inflammatory cytokines (Pedersen et al., 2001; Petersen & Pedersen, 2005) to restore homeostasis (Suzuki, 2018). Interleukin-six also exerts an autocrine effect; hence, IL-6 exhibits pro- and anti-inflammatory effects and is known as a *mixed* cytokine (Suzuki, 2018). In this thesis, pro-inflammatory is used to describe the induction of an inflammatory response, whereas anti-inflammatory is a term used to describe the suppression of inflammation. Scott, Khan, Roberts, Cook, and Duronio (2004) have defined exercise-induced inflammation as a response that causes “clinical, physiological, cellular and molecular” (p. 372) changes in injured skeletal muscle tissue after exercise. However, an updated definition encompassing the regulation and beneficial effects of exercise-induced inflammation is warranted (Peake et al., 2017). In the context of this thesis, inflammation is deemed as a detrimental process to athletes undertaking intensified periods of exercise where recovery is a key focus.

2.4.1.1 Symptoms of muscle damage and associated inflammation.

While EIMD and associated inflammation are regular exercise-induced responses that stimulate the repair and regeneration of damaged skeletal muscle tissue (Fatouros & Jamurtas, 2016; Malm, 2001; Peake et al., 2017) leading to long-term training adaptations, there are implications for performance. No set definition of exercise-induced muscle damage exists within the literature. Moreover, muscle damage and inflammation are characterised by a milieu of symptoms resulting from myofibre disruption, which could compromise recovery and subsequent performance. Symptoms include (i) impaired neuromuscular function, (ii) increased fatigue, (iii) localised swelling, (iv) muscle soreness/DOMS, and, (v) decreased ROM/stiffness (Fatouros & Jamurtas, 2016; Harty, Cottet, Malloy, & Kerksick, 2019; Jamurtas, 2018; Owens et al., 2019).

The magnitude and time course of recovery of exercise-induced muscle damage and the associated inflammatory response is dependent on the exercise modality, duration, and trained status of the athlete (Nédélec et al., 2012; Peake et al., 2017). The consensus is a delayed peak in the symptoms of these responses, which can persist for days following muscle-damaging exercise but is resolved with sufficient rest (Peake et al., 2017 [discussed further relative to prolonged high-intensity intermittent exercise characteristic of soccer, refer to section 2.6]).

Metabolic and mechanical stresses have been implicated in causing muscle damage in soccer (Ispirlidis et al., 2008). Athletes participating in team sports such as soccer are predisposed to primary muscle damage because of (a) the mechanical stress associated with performing repeated high-intensity eccentric muscle actions (i.e.,

accelerations; decelerations; directional changes; jumping), and secondary muscle damage because of (b) metabolic stress associated with aerobic metabolism and the inflammatory response. It should be considered that oxidative damage might also be a cause of primary muscle damage during prolonged and strenuous aerobic exercise. Given the time-course of recovery and associated negative symptoms, monitoring exercise-induced muscle damage and associated inflammation during intensified periods of exercise is essential, as current recovery periods may be insufficient, and these transient effects may manifest into chronic inflammation. Implementation of a recovery strategy that targets EIMD and associated inflammation during this time may be warranted.

2.5 Assessment of EIOS, EIMD, & Associated Inflammation

2.5.1 Oxidative stress.

Determining and quantifying oxidative stress *in vivo* is challenging because of (a) the short half-life of RONS and (b) the cost of methods, such as electron paramagnetic resonance (Halliwell & Gutteridge, 2015). Indirect methods commonly used to assess EIOS include assessment of post-exercise elevations in biomarkers of (i) by-products (primary, secondary, or end products) of oxidant-mediated damage to lipids, proteins, or DNA (e.g., F₂-isoprostanes, 4-HNE, the comet assay, respectively), (ii) decreases in antioxidants (individual or combined, e.g., glutathione or Total Antioxidant Status), or, (iii) a ratio of the pro-oxidant: antioxidant balance (e.g., GSH: GSSG; Powers & Jackson, 2008 [Table 2.1]). The most commonly used biomarkers are measurements of lipid peroxidation products, such as Malondialdehyde (MDA), a secondary by-product formed during the lipid peroxidation of polyunsaturated fatty acids (PUFAs).

Malondialdehyde is measured via the thiobarbituric acid reactive substances (TBARS) assay, a relatively inexpensive, spectrophotometric technique based on a Knoevenagel-type condensation reaction between MDA and thiobarbituric acid (Halliwell & Gutteridge, 2015). Previous criticism of the TBARS method is reduced specificity (Cobley, Close, Bailey, & Davison, 2017; Powers et al., 2010b) as thiobarbituric acid has the potential to generate further MDA during the reaction (artificial lipid peroxidation [Cobley et al., 2017]). Despite criticism, the TBARS assay remains popular in exercise literature (chapters 4, 5, & 6 of this thesis assayed MDA in urine & serum). There is no single optimal biomarker of oxidative stress, with multiple biomarkers of oxidative stress (Cobley et al., 2017) and a combination of biomarkers representative of pro-oxidant and reductive stress recommended (Powers et al., 2010b; as was used in chapters 5 & 6 of this thesis; common biomarkers are presented Table 2.1).

2.5.2 Muscle damage & associated inflammation.

Evidence and quantification of skeletal muscle damage can be provided indirectly by post-exercise elevations in markers of EIMD compared to pre-exercise values. Several markers of muscle damage have been used in the literature, including (i) tests of neuromuscular function, (ii) muscle soreness assessment, and (iii) biomarkers (Brancaccio et al., 2010; Howatson & van Someren, 2008; Nédélec et al., 2012; Owens et al., 2019). Focusing on biomarkers, post-exercise elevations in systemic intramuscular proteins (e.g., Creatine Kinase [CK]; Lactate Dehydrogenase [LDH]; myoglobin; or non-proteins such as urea [Table 2.1]) that are usually located within the cytoplasm provide indirect evidence of exercise-induced muscle damage

(Brancaccio et al., 2010). Damage to the sarcolemma, or increased permeability, from strenuous or prolonged exercise, results in leakage of these components into the interstitial fluid (Brancaccio et al., 2010). A limitation of this assessment method is specificity, as these proteins are not specific to myocytes and other sources of tissue damage (i.e., brain & cardiac tissue). An assay that specifically assesses muscle isoforms of these biomarkers should be considered (Lee et al., 2017). Nevertheless, CK assessment in blood is the most commonly assessed biomarker of muscle damage (Brancaccio et al., 2010). However, it has been criticised as individuals can be high or low responders (Brancaccio et al., 2010).

Lee et al. (2017) propose that biomarkers of muscle damage be assessed in conjunction with biomarkers of inflammation. Similar to EIOS and EIMD, exercise-induced inflammation is characterised by a post-exercise systemic increase in inflammatory biomarkers, which are mobilised into systemic circulation following muscle damage (Lee et al., 2017). Biomarkers of inflammation include (i) pro-inflammatory cytokines (including chemokines & myokines), (ii) acute-phase proteins (e.g., CRP), (iii) adhesion molecules (e.g., sVCAM-1), and (iv) immune cells (e.g., leukocyte & neutrophils), assessed in blood components or skeletal muscle tissue (Lee et al., 2017; Table 2.1).

There appear to be no optimal biomarkers of exercise-induced muscle damage or inflammation recommended in the literature. The timing of sampling biomarkers of exercise-induced responses should also be considered.

2.5.3 Sampling time points.

Few studies have been conducted to determine optimal sampling time points of biomarkers of exercise-induced responses. Most studies sample biomarkers immediately pre- and post-exercise, +24, and +48 hr throughout recovery. Inconsistent results in recovery kinetics of the biomarkers have been reported, possibly due to the differences in the release and clearance of biomarkers (Powers et al., 2010b) or the control of confounding variables prior to sampling. To combat these research shortcomings, Michailidis et al. (2007) measured time course changes of commonly used biomarkers of oxidative stress (i.e., TAC; TBARS; GSH: GSSG; catalase; PC) over a 24 hr period following strenuous aerobic exercise. The authors concluded there to be no optimal time point that would apply to all biomarkers because biomarkers peak at different time points (over a 1 - 4 hr window). In experimental chapters 4, 5, and 6, biomarkers were sampled 42 hr post-exercise. The rationale for this time point was because it is representative of the maximal post-match recovery time typically used in professional soccer (Mohr et al., 2015). This time point is when the athletes would next be expected to train, following a recovery day, and was chosen to provide interesting information on recovery and athlete readiness.

Given that strenuous and prolonged exercise has been shown to induce oxidative stress, and that eccentric exercise causes muscle damage and inflammation, these responses are expected as a normal part of participation in prolonged high-intensity intermittent exercise performed in team sports such as soccer. Several studies have explored biomarker responses of EIOS, EIMD, and EII following bouts of soccer-specific exercise.

2.6 EIOS, Muscle Damage, & Inflammation following Soccer-Specific Prolonged High-Intensity Intermittent Exercise

Research investigating exercise-induced oxidative stress, muscle damage, and associated inflammatory responses following bouts of soccer-specific exercise (i.e., match play; simulated match play; soccer-specific exercise; studies are presented [Table 2.3]) has increased over the past 20 years, possibly due to advances in cheaper, less complex, more practical methods of biomarker monitoring alongside increases in technology, such as the use of GPS in sport to quantify the physiological demands of the sport.

2.6.1 Single bouts.

Research has predominantly focused on these exercise-induced responses following a single bout of exercise characteristic of soccer in trained male soccer players. Marked increases in common biomarkers of oxidative stress, muscle damage, and inflammation have been demonstrated (Ascensão et al., 2008; Bell, Stevenson, Davison, & Howatson 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Mello et al., 2017; Romagnoli et al., 2016; Souglis et al., 2015; Sureda et al., 2009). There appears to be a specific magnitude and time course in the response of these biomarkers, as it is evident that there is a transient post-exercise increase in biomarkers of oxidative stress, muscle damage, and inflammation that is resolved with sufficient recovery.

Biomarkers of exercise-induced oxidative stress have been shown to peak 24 hr post-match and remain elevated above pre-match concentrations for up to 72 hr

(Ascensão et al., 2008; Fatouros et al., 2010; Ispirlidis et al., 2008). Suerda et al. (2009) demonstrated the magnitude of oxidative stress in prolonged high-intensity intermittent exercise, characteristic of soccer, to be exercise intensity-dependent. Eighteen male semi-professional soccer players undertook 60 min of match play at varying intensities. Lymphocyte MDA and H₂O₂, total neutrophil counts, and Haem oxygenase-1 gene expression were observed to increase in a dose-dependent manner, with the highest values observed, alongside reductions in antioxidant vitamin C, when more than 50% of the match was performed at > 90 % HRmax.

A delayed peak (24 - 48 hr) in exercise-induced muscle damage (evidenced by elevated systemic CK and/or LDH concentrations) has also been observed in response to a single bout of soccer (Ascensão et al., 2008; Ispirlidis et al., 2008; Romagnoli et al., 2016; Souglis et al., 2015) and simulated soccer (Bell et al., 2016). The magnitude of muscle damage reported is consistent, with CK reported to be > 300 IU immediately post soccer bout, increasing to ~ 800 IU throughout recovery periods; the recovery time course relative to the magnitude of the peak (Nédélec et al., 2012). Exercise-induced inflammation has also been observed in response to a bout of soccer or simulated soccer. Delayed increases in inflammatory cytokines and acute-phase proteins, alongside leukocytosis, provide evidence of an exercise-induced acute-phase response (Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Romagnoli et al., 2016; Souglis et al., 2015). Varying responses in the magnitude and time course kinetics of inflammation can be attributed to methodological differences, including (i) type of soccer-specific exercise; (v) trained status of participants, (vi) sampling time points, (vii) sample types, (vii) analytical methods; and (viii) expression of results. It is evident that biomarkers of

EIOS, EIMD, and EII are increased post-exercise and are elevated throughout recovery. Current recovery periods may be insufficient to restore homeostasis within skeletal muscle (Ispirlidis et al., 2008; Fatouros et al., 2010).

Several of the studies have also contextualised biomarker data through assessment of performance and recovery measures assessed concomitantly (Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Romagnoli et al., 2016; Table 2.3). These studies showed impairments in performance and recovery throughout the considered recovery periods that paralleled the alterations in biomarker data. Thus, providing evidence that exercise, characteristic of soccer, upregulates EIOS, EIMD, and EII that has implications for performance and recovery.

2.6.2 Intensified periods.

Few studies have explored EIOS, EIMD, and associated inflammation throughout intensified periods of soccer, such as microcycles of a competitive season or tournaments, probably due to the inherent difficulties of longitudinal monitoring of athletes. The practical significance of investigating these exercise-induced responses following intensified periods of exercise is that in the real-world setting, athletes would perform back-to-back sessions, and a competitive match or training session may be influenced by residual fatigue from a prior session or microcycle (Mohr et al., 2015). Increasing understanding of these responses is important as recovery periods may not be optimal to resolve the negative effects associated with EIOS, EIMD, and EII during this time, and performance may be affected.

In 2015, Mohr et al. investigated these exercise-induced responses throughout an 8-day simulated microcycle that consisted of three competitive matches separated by two and then three, respectively, training sessions, in 20 male professional soccer players, with a control group also consisting of professional soccer players. Results showed elevated biomarkers of oxidative stress, muscle damage, and inflammation, together with swelling, muscle soreness, decreased knee range of motion, and increased repeated sprint time to be greatest after the second match, which was performed 3 days after the first match. Results of this study suggest that a two-day recovery period between consecutive matches (with daily training) to be insufficient to allow complete recovery. Interestingly, research conducted in elite female soccer players by Andersson et al. (2010) observed an inflammatory response following a competitive soccer match, assessed via an array of plasma pro-inflammatory cytokines (IL-6; IL-12, TNF- α ; IFN- γ ; IL-17), chemokines (MCP-1; IL-8; MIG), and anti-inflammatory cytokines (IL-2R; IL-4; IL5; IL-7; IL-10; IL-13; INF- α) alongside leukocytosis and neutrophilia. However, found a dampened inflammatory response (excluding IL-12; IL-6; IL-8; MIG & MCP-1, leukocytes & neutrophils) following a second match performed 72 hr later, possibly indicative of a protective *repeated bout effect* (RBE; an adaptive effect associated with eccentric muscle-damaging exercise that has been shown to protect skeletal muscle from further muscle damage during subsequent exercise bouts [Howatson, van Someren, & Hortobágyi, 2007; McHugh, 2003; Proske & Allen, 2005]). The time course of recovery for inflammation was resolved at 21 hr post-match, which also suggests the exercise-induced inflammatory response following consecutive bouts may differ from that following a single bout. This study assessed female athletes; inflammatory responses may differ between sexes; however, Souglis et al. (2015) demonstrated no differences in EII

between sexes. Andersson et al. (2010) also investigated the effects of active recovery during the 72 hr recovery period between the two matches but found no effect of active recovery compared to passive recovery on inflammatory responses. The authors attribute the observed increases in pro-inflammation (i.e., IL-6; IL-8; IL-12; MCP-1; MIG) following both competitive matches to be part of the repair and regeneration of skeletal muscle tissue; however, muscle damage per se was not assessed.

Furthermore, studies (Becatti et al., 2017; da Silva Barbosa et al., 2017; Ferrer et al., 2009) that have explored oxidative stress and muscle damage responses throughout longitudinal periods of soccer (35 days; 3 months; competitive season, respectively), have shown regular participation promotes exercise tolerance, demonstrated by a reduction in lipid peroxidation. Additionally, trained athletes demonstrate (a) an enhanced antioxidant capacity (Brites et al., 1999; Cazzola, Russo-Volpe, Cervato, & Cestaro, 2003; Metin, Gümüştas, Uslu, Belce, & Kayserilioglu, 2003; Mukherjee & Chia, 2009), (b) less exercise-induced muscle damage (Brancaccio et al., 2010; Mougios, 2007), and (c) lower basal inflammation in comparison to untrained age- and sex-matched cohorts (Souglis et al., 2015).

To date, only one study (Le Moal et al., 2016) has monitored redox status (assessed via plasma GSH: GSSG) in professional soccer players ($n = 19$; male) throughout a soccer season relative to training load (quantified by the subjective RPE method). Data revealed that periods with increased training loads were associated with increased oxidative stress ($r^2 = .84$; $p < .001$). Support for these results is provided by a similar study conducted in rugby (Finaud et al., 2006b), demonstrating the

importance of biomarker monitoring and potential implications for recovery. Sports scientists/coaches should consider the link between oxidative stress and high training loads to prescribe a balance between training loads and recovery periods. Further longitudinal research in this area is warranted (as was the focus of chapter 4, which quantified training load using an objective measure alongside the assessment of exercise-induced lipid peroxidation).

The majority of research in this area has assessed an array of biomarkers of these exercise-induced responses. Results from studies that have only assessed pro-oxidant and pro-inflammatory responses should be treated with caution, as antioxidant or anti-inflammatory responses may have been increased to counteract these responses, restoring homeostasis. Several studies have also not included a control group in the study design, probably due to the inherent difficulties of athletes acting as resting controls. A control group would provide information regarding resting and post-exercise variance in biomarker data. However, the majority of studies have used a repeated measures design, comparing differences in biomarkers pre- to post-exercise, with athletes, therefore, acting as their own controls. Whether a one-off sample collected immediately pre-exercise accurately represents an athlete's baseline value of a biomarker is questionable.

Although limited research has explored these exercise-induced responses following intensified periods of soccer, several studies have demonstrated transient increases in these responses in other team sports (e.g., basketball [Chatzinikolaou et al., 2014]; rugby [Finaud et al., 2006b]) and intensified periods of steady-state exercise

(i.e., cycling & running [Bell, Walshe, Davison, Stevenson, & Howatson, 2014; Nieman et al., 2014; Shing et al., 2007]).

2.6.3 Summary.

There is clear evidence from the research that prolonged high-intensity intermittent exercise, as performed in soccer, induces a transient alteration in biomarkers of EIOS, EIMD, and EII. The magnitude and time course of these exercise-induced responses are dependent on the methods used. Nonetheless, it appears to be sports-specific with an acute increase in biomarkers of oxidative stress, muscle damage, and inflammation in the days following exposure, which are resolved back to pre-exercise levels with sufficient rest. The beneficial role of these exercise-induced responses is now recognised, but there are potential implications for recovery and performance during intensified periods where recovery time is limited. Further exploration of these phenomena during intensified periods, such as the competitive in-season or tournaments, is required, as residual effects from a prior session may impact the magnitude or time course of these responses following subsequent sessions. It should be considered that there might be a potential publication bias in this research area, as there is a lack of published studies with non-significant findings.

Table 2.3

Summary of Studies Investigating EIOS, EIMD, & EII in Soccer-specific Exercise

Authors	Study Design	Participants	Exercise Modality	Sample Type	Outcome	Conclusion
Andersson et al. (2010)	Repeated measures Independent groups	<i>N</i> = 20 ♀ Elite soccer players Exp: <i>n</i> = 10 Con: <i>n</i> = 10	2 x soccer matches, 72 hr active or passive recovery	Bloods: Post match 1 15 min, 21 hr, 45 hr, 69 hr; post match 2 15 min	↑ total leukocyte & neutrophil counts ↑ IL- 12, TNF-α, INF- γ, IL-17, MCP-1, IL-8, MIG, ↑ IL-2R, IL-4, IL-5, IL-7, IL-10, IL-13, & mixed cytokine IL-6, INF-α after 1 st match ↑IL-6, IL-8, IL-12, MCP-1, MIG after 2 nd	↑ inflammatory response, dampened response after a 2 nd match performed 72 hr later. No effect active recovery.
Ascensão et al. (2008)	Repeated measures	<i>N</i> = 16 ♂ Soccer players	Friendly soccer match	Bloods + performance + muscle soreness: pre match, post match, 30 min, 24 hr, 48 & 72 hr	↑ plasma TAS, Mb, neutrophils 30 min ↓-SH 48 hr ↑ CK, MDA, UA, DOMS 72 hr ↓ Lower limb isokinetic strength & 20 m sprint time 72 hr	↑ muscle damage & soreness altered redox status ↓ performance
Becatti et al. (2017)	Prospective cohort	<i>N</i> = 27 ♂ Elite soccer players	Soccer season	Bloods: Pre/beg/mid/end season	Leukocyte ROS, TAC & GSH: GSSG correlated with CK	Oxidative stress correlated with muscle damage

Bell et al. (2016)	Randomised Double-blind placebo controlled Independent groups	<i>N</i> = 16 ♂ Semi-pro soccer players Exp: <i>n</i> = 8 Con: <i>n</i> = 8	LIST _{adapted} performed on day 5 of supplementation Montmorency tart cherry concentrate (MC) vs. placebo (PLA) 8 d 60 mL·d ⁻¹ before exercise & during recovery	Bloods: baseline, post-exercise + 1, 3, 5, 24, 48 & 72 hr Performance + recovery measures: baseline, + 24, 48 & 72 hr	Exercise effect ↑ IL-6, IL-8, CK, hsCRP, TNF-α, DOMS, No exercise effect LOOH ↓ MVIC, CMJ, agility, 20 m sprint, no effect IL-1β ↑ MVIC, CMJ, agility, ↓ DOMS & IL-6 with MC	LIST induced inflammation & muscle damage but no effect on oxidative stress MC promoted recovery & performance following the LIST
da Siva Barbosa et al. (2017)	Randomised Double-blind placebo controlled Independent groups	<i>N</i> = 20 ♂ Semi-pro soccer players Exp: <i>n</i> = 10 Con: <i>n</i> = 10	28 d soccer training, 6 days per wk, 3 hr·d ⁻¹ Sesame vs. PLA 28 d	Bloods + VO ₂ max: pre, 48 hr post, 28 d	Exercise ↓ hsCRP & MDA, ↑ CK, vitamin E Sesame ↓ CK, LDH, CRP, MDA ↑ SOD, vitamins C & E	Difference in baseline CK, LDH, CRP Exercise improves redox status Sesame reduces oxidative stress
Fatouros et al. (2010)	Repeated measures	<i>N</i> = 30 ♂ Elite U21 soccer players Exp: <i>n</i> = 20 Con: <i>n</i> = 10	Soccer match (Exp 10 vs. 10)	Bloods: pre, post, + 24, 48, 72	↑ CK, MDA, UA, PC, TAC, catalase, GPX, leukocytes ↓ GSH: GSSG ↑ DOMS ↓ 20 m sprint, CMJ	Soccer match upregulated oxidative stress, muscle damage, inflammation & performance impairments for up to 72 hr

Ferrer et al. (2009)	Randomised Double-blind Placebo controlled Independent groups	<i>N</i> = 19 ♂ Pro soccer players Exp: <i>n</i> = 8 Con: <i>n</i> = 11	3 month soccer training, followed by 60 min match	Bloods: pre, post 3 month training, post 60 min match	Training ↓ lymphocyte MDA, catalase, α-tocopherol, ↑ glutathione reductase exercise no effect vitamin C, H ₂ O ₂ match ↑ H ₂ O ₂	Regular training improves redox status, but match induces oxidative stress No effect antioxidant supp.
Ispirlidis et al. (2008)	Randomised	<i>N</i> = 24 ♂ Pro soccer players Exp: <i>n</i> = 14 Con: <i>n</i> = 10	Soccer match	Bloods, performance, + recovery measures: pre match, immed post + 24, 48, 72, 96, 120 & 144 hr	Post match peak in total leukocyte counts, IL-6, IL- 1β, & cortisol, 24 hr peak in CRP, TBARS, & DOMS, 48 hr peak in CK, LDH, & PC, 72 hr peak in UA ↓ muscle strength, vertical jumping, speed, ↑ DOMS, muscle swelling	↑ transient muscle damage & inflammation Impaired performance up to 4 days post match
Le Moal et al. (2016)	Non- intervention Prospective cohort study	<i>N</i> = 19 ♂ Pro soccer players	Soccer season	Bloods: T1, T2, T3, T4, T5 weekly TL quantified via RPE	Whole blood GSH: GSSG altered across time points changes in GSH: GSSH correlated with cumulated mean TL SOD, GPX, vitamin E & beta-carotene not altered	Redox status is altered according to training period, correlated with TL

Mello et al. (2017)	Repeated measures	<i>N</i> = 22 ♂ Soccer players	Simulated match	Bloods: pre & post	↓ Reduced GSH ↑ ALT no change CK, AST, GGT, TAC, MDA in plasma	Redox homeostasis maintained, ALT recovery marker
Mohr et al. (2015)	Repeated measures Randomised	<i>N</i> = 40 ♂ Soccer players Exp: <i>n</i> = 20 Con: <i>n</i> = 20	1-week microcycle, 3 matches separated by 2 & 3 days	Bloods, performance, & recovery measures: pre & post match, during recovery post match + 24, 48 hr	CK, CRP, sVCAM-1, sP-Selectin, cortisol peaked 48 hr, Total leukocyte count, testosterone, IL-1β & IL-6 altered 24 hr, ↑ TBARS & PC post match, GSH: GSSG ↓24 hr, TAC & GPX up 48 hr, RSA 3 day post match, ↑ muscle soreness, KJRM ↓ match 2 elicited the greatest performance impairment & slowest recovery	2 d recovery insufficient to resolve inflammation, muscle damage, & performance decrements between consecutive matches
Romagnoli et al. (2016)	Repeated measures	<i>N</i> = 20 ♂ Pro soccer players	Soccer match	Bloods + CMJ: pre & 30 min, 24, & 48 hr post match	↓ jump height post match time points ↑ CK, hs-IL-6 & total neutrophil counts 24, 48 hr, TDC correlated with post match IL-6 & cortisol at 24 & 48 hr	IL-6 & cortisol could be used as biomarkers of fatigue to monitor recovery

Souglis et al. (2015)	Repeated measures	<i>N</i> = 83 Elite soccer players: 22 ♂; 21 ♀ Control: 20 ♂; 20 ♀	Soccer match	Bloods: pre match, immed post + 24, 48 hr	↑ IL-6 & TNF-α returned to pre match within 24 hr; TNF-α peak higher in ♂, CRP & CK peaked 24 hr post match, CK elevated 48 hr; Basal IL-6, TNF-α, & CRP Soccer players < controls	↑ inflammation & muscle damage Elite soccer players had reduced inflammation compared to a control group No sex differences in EII
Sureda et al. (2009)	Matched groups	<i>N</i> = 18 ♂ Semi-pro players low <i>n</i> = 6 med <i>n</i> = 6 high <i>n</i> = 6	Training match 60 min 3 different intensities	Bloods: pre & post	High-intensity ↑ lymphocyte MDA, H ₂ O ₂ vitamin C ↓ vitamin C & E, HO-1 ↑ med & high-intensity neutrophils & ROS ↑with exercise intensity	Oxidative stress is intensity-dependent

Note. ALT = Alanine Transaminase; AST = Aspartate Transaminase; CK = creatine kinase; CMJ = countermovement jump; CON = control group; CRP = C-Reactive Protein; d = days; DOM = delayed onset of muscle soreness; diff = difference; EII = exercise-induced inflammation; EXE = exercise group; GGT = Gamma-Glutamyl Transferase; GPX = Glutathione Peroxidase; GSH = Glutathione; GSH:GSSG = ratio of reduced glutathione to oxidised glutathione; HO-1 = haemoxygenase-1; hr = hour; hs = high sensitivity; H₂O₂ = Hydrogen peroxide; IL = interleukin; immed = immediately; INF-γ = Interferon-gamma; KJRM = knee joint range of motion; LIST = Loughborough Intermittent Shuttle Test; LOOH = lipid hydroperoxides; Mb = myoglobin; MCP = Monocyte Chemoattractant Protein; MDA = Malondialdehyde; med = medium; min = minutes; mL·d⁻¹ = milliliters per day; MVIC = Maximal Voluntary Isometric Contraction; *N* = number of participants; *n* = number of participants per group; PC = Protein Carbonyls; pro = professional; ROS = Reactive Oxygen Species; RPE = Rating of perceived exertion; RSA = repeated sprint ability; semi-pro = semi-professional; sVCAM-1 = circulating vascular cell Adhesion Molecule-1; sP = soluble platelet; -SH = sulfhydryl; supp. = Supplement; T = time point; TAC = Total Antioxidant Capacity; TAS = Total Antioxidant Status; TBARS = Thiobarbituric Acid Reactive Substances; TDC = Total Distance Covered; TL = training load; TNF-α = Tumor Necrosis Factor alpha; U = under; UA = Uric Acid; ♂ = male; ♀ = female.

It is proposed that the redox-sensitive transcription factor Nuclear Factor Kappa-Light-chain-enhancer of activated B cells (NF- κ B) is the master regulator of inflammation and can be activated by RONS during exercise (Kramer & Goodyear, 2007). The role of NF- κ B in exercise is further explored.

2.7 Nuclear Factor Kappa-Light-chain-enhancer of activated B cells (NF- κ B)

Nuclear Factor Kappa-Light-chain-enhancer of activated B cells is a redox-sensitive transcription factor that plays a fundamental role in regulating cellular inflammatory and redox responses by altering gene production (Kramer & Goodyear, 2007; Niemen et al., 2007a). The term NF- κ B refers to a family of five proteins (i.e., NF- κ B 1 [p50]; NF- κ B 2 [p52]; RelA [p65]; RELb; cREL), two of which must form a heterodimer to enable NF- κ B DNA binding activity (Kramer & Goodyear, 2007). There are three pathways by which NF- κ B can be activated, (i) classical (canonical); (ii) alternative (non-canonical), and a third pathway, (iii) pathway 3, has more recently been identified (Gilmore, 2006). Focusing on the classical pathway (p65/p50 heterodimer; as this is theorised to be activated by exercise [Kramer & Goodyear, 2007]), NF- κ B is sequestered in the cytoplasm of cells, bound to inhibitory proteins, I κ Bs (specifically I κ B α & I κ B β [inhibitor alpha & beta]), and is activated by I κ B kinases (IKK; i.e., IKK α , IKK β , IKK γ); enzymes that phosphorylate I κ B α , initiating ubiquitination and degradation, enabling the NF- κ B p65/p50 heterodimer to translocate into the nucleus (Kramer & Goodyear, 2007). Once in the nucleus, NF- κ B binds to the corresponding DNA promoter region of target genes to induce mRNA expression of the corresponding protein (Kramer & Goodyear, 2007; Figure 2.4).

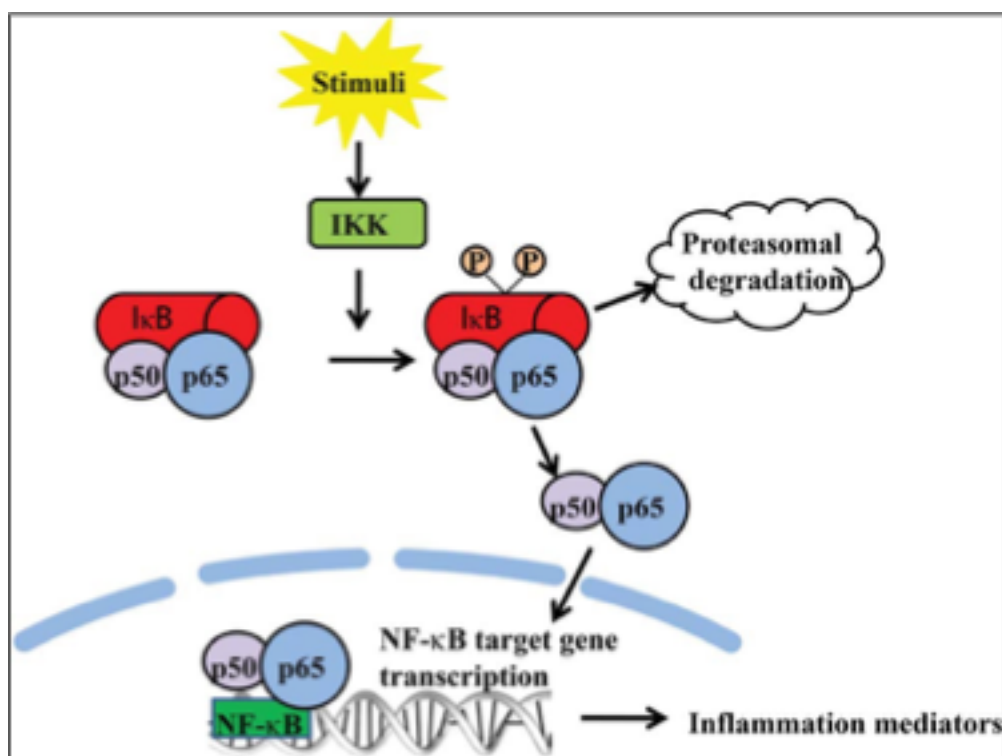


Figure 2.4. Schematic of NF- κ B classical pathway. Recopied from Zhang, Sun, Sun, Chen, & Chen (2014) p. 415. Exercise-induced RONS are a proposed stimulus of NF- κ B activation.

2.7.1 Exercise-induced NF- κ B activation.

Exercise-induced activation of the NF- κ B classical pathway has been documented (Kramer & Goodyear, 2007), although the alternative pathway has now also been implicated as being activated during exercise (Vella et al., 2014). It is postulated that a number of exercise-induced responses activate NF- κ B via IKK activation, including (i) oxidative stress (hence, NF- κ B is redox-sensitive), (ii) pro-inflammatory cytokines, (iii) endotoxins, (iv) muscle damage, (v) energy depletion, and (vi) increased intracellular CA^{2+} (Cuevas et al., 2005; Ji et al., 2004, Ji, Gomez-Cabrera, & Viña, 2007; Kramer & Goodyear, 2007; Nieman et al., 2007a; Vider et al., 2001). The role of NF- κ B activation during exercise is unclear. It is speculated that NF- κ B induces post-exercise inflammatory responses (e.g., increased transcription of acute-phase

proteins, pro-inflammatory cytokines, cyclooxygenases, adhesion molecules) to promote the repair and regeneration of damaged skeletal muscle tissue (Gomez-Cabrera et al., 2006; Ji et al., 2004; Kramer & Goodyear, 2007; Niemen et al., 2007a). Another role of NF- κ B activity during exercise may be maintenance of skeletal muscle redox homeostasis, as NF- κ B increases gene expression of antioxidant enzymes (i.e., manganese-dependent Superoxide Dismutase [MnSOD], inducible Nitric Oxide Synthase [iNOS], glutathione peroxidase [GPX] & catalase [CAT]; Gomez-Cabrera et al., 2006; Ji et al., 2004; Zhou, Fragala, McElhaney, & Kuchela, 2010). A third potential role of exercise-induced NF- κ B activity may be substrate regulation, as NF- κ B increases expression of the myokine IL-6, therefore, enhancing glucose transport and lipid oxidation within skeletal muscle (Kramer & Goodyear, 2007).

Exercise-induced NF- κ B activation has been documented in murine models (Aoi et al., 2004; Ji et al., 2004), yet; human exercise studies have been limited and have demonstrated equivocal results (human studies are summarised, Table 2.4). Vider et al. (2001) conducted the first human study providing evidence of exercise-induced NF- κ B activation. In a well-controlled study, NF- κ B p65 activity in peripheral blood mononuclear cells (PBMCs) was increased > 50% in 8 out of 12 trained male participants following a strenuous treadmill running protocol (60 min; 80% $\dot{V}O_2$ max). Cuevas et al. (2005) and Parker, Trewin, Levinger, Shaw, & Steptoe (2017) also observed increased NF- κ B binding activity following high-intensity anaerobic exercise (4 x 30 s Wingate sprints) in professional cyclists and recreationally active participants, respectively. More recently, Vella et al. (2012) demonstrated a transient increase in NF- κ B p65 activation in skeletal muscle tissue in response to leg-based

resistance exercise (80% 1RM) in recreational participants, with peak NF- κ B activity observed 2 hr post-exercise, concomitant with transient increases in MCP-1, IL-6, and IL-8 mRNA expression.

Several studies have also demonstrated post-exercise NF- κ B activation concomitant with exercise-induced oxidative stress (Cuevas et al., 2005; Gomez-Cabrera et al., 2006; Vider et al., 2001; evidenced by increased TBARS & conjugated dienes; increased 8-OH-2-dG & decreased GSH; & increased xanthine oxidase, respectively). Thus, providing evidence that NF- κ B activation may be redox-sensitive. Furthermore, Ji et al. (2004) also demonstrated increased NF- κ B binding activity alongside increased mRNA and MnSOD protein expression in rat skeletal muscle following exercise. Although, García-López et al. (2007) found an eccentric leg exercise protocol activated the NF- κ B classical pathway in moderately trained males, which was concomitant with increased muscle damage (CK), muscle soreness, and decreased jump height, but the eccentric exercise protocol did not alter biomarkers of oxidative stress (assessed via protein carbonyls & TBARS).

In contrast, several studies have observed no increase in NF- κ B activation following exercise (Buford et al., 2009; Koenig et al., 2015; Nieman et al., 2007a). In 2009, Buford et al. investigated NF- κ B p50 activity following an eccentric 45 min downhill treadmill protocol (60% $\dot{V}O_2\text{max}$) in 29 recreationally trained male participants, finding no significant post-exercise increases in NF- κ B p50 activation in skeletal muscle tissue, three or 24 hr post-exercise, despite exercise-induced inflammation, muscle damage, and increased muscle soreness. Using a similar exercise protocol (60 min downhill run; 75% $\dot{V}O_2\text{max}$) Koenig et al. (2015) also reported no activation

of NF- κ B p65 in PBMCs in moderately trained participants, and Nieman et al. (2007a) observed no effect of a 3-day prolonged cycling protocol (3 hr·d⁻¹; ~57% max work rate) on NF- κ B activity in skeletal muscle tissue using trained participants. Taken together, results of these studies suggest that exercise-induced NF- κ B activity may be exercise intensity related. However, there is a lack of performance and recovery measures to contextualise the findings. Conflicting findings in exercise studies can generally be attributed to methodological differences. The studies summarised in Table 2.4 include differences in (i) the trained status of participants, (ii) exercise modalities, (iii) sample type (skeletal muscle tissue vs. PBMCs), (iv) NF- κ B analyses (e.g., Western blotting; electrophoresis; ELISA), and (v) sampling time points.

To the lead investigator's knowledge, no study has investigated NF- κ B activity in response to prolonged high-intensity intermittent exercise, as performed in team sports such as soccer. It is postulated that NF- κ B activity may be increased following intensified periods of soccer due to the strenuous nature of the exercise, inducing oxidative stress, muscle damage, and associated inflammation. Exercise-induced activation of NF- κ B is transient, and like muscle damage, has been shown to be resolved with sufficient recovery (Kramer & Goodyear, 2007). NF- κ B is also auto-regulatory, as NF- κ B activates gene expression of I κ B (Gilmore, 2006). However, during intensified periods of exercise, where recovery time is limited, NF- κ B activity may become chronic and could lead to fatigue (Figure 2.5), impacting recovery and subsequent performances, therefore, modulation of NF- κ B activity with a recovery strategy that reduces oxidative stress and inflammation may be beneficial to athletes during this time. (Chapters 5 & 6 of this thesis attempted to address this gap in the

literature by investigating NF- κ B activation in response to a 3-day intensified period of soccer-specific prolonged high-intensity intermittent exercise and by modulating NF- κ B activity with a nutritional intervention, respectively).

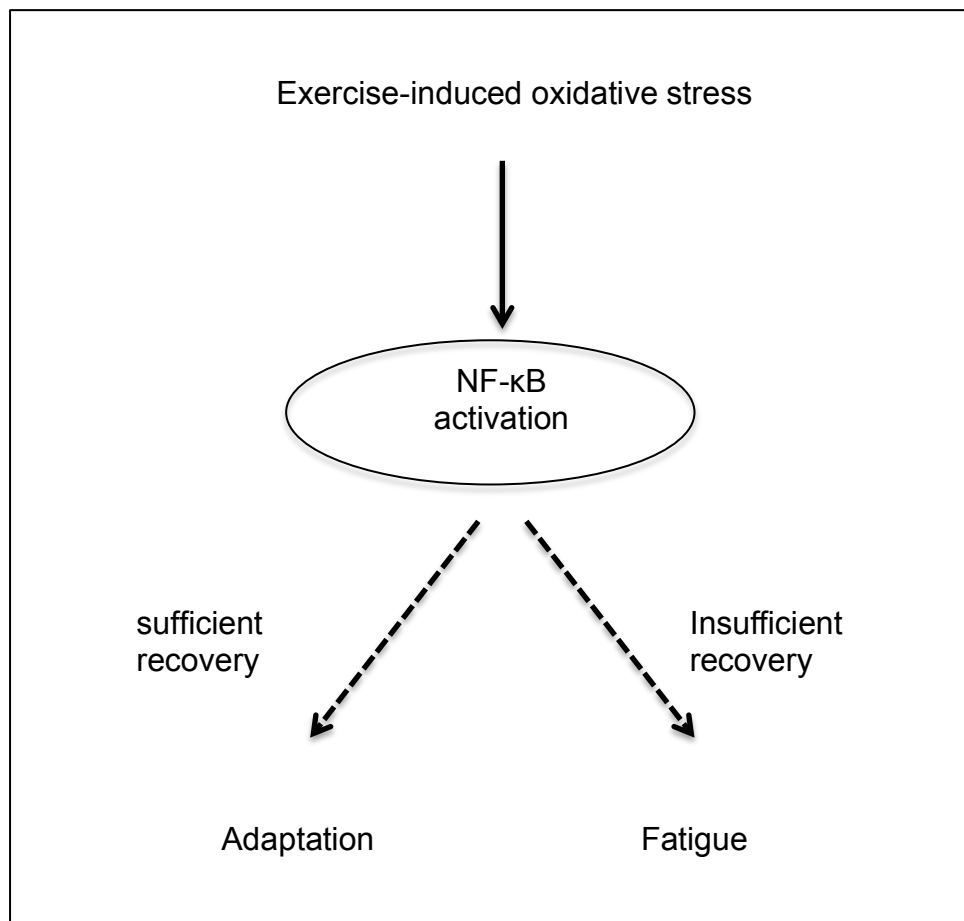


Figure 2.5. Simplified schematic of exercise-induced NF- κ B activation. Sufficient recovery leads to beneficial adaptations within skeletal muscle, but insufficient recovery between successive bouts may lead to chronic NF- κ B adaptation and fatigue. Adapted from Kramer & Goodyear (2007), p. 392.

Table 2.4

Summary of Studies Investigating NF- κ B Activation in Response to Human Exercise

Authors	Study Design	Participants	Exercise Modality	Sample Type	NF- κ B Analysis	Outcome	Significant Exercise-induced NF- κ B activity?
Buford et al. (2009)	Single group Repeated measures	$N = 29$ ♂ recreationally active	45 min downhill run 60% $\dot{V}O_2$ max	Bloods & muscle biopsies: pre, 3 hr & 24 hr post-exercise	RT-PCR	↑ IL-6, IL-8, COX-2 mRNA, serum CK, muscle soreness compared to baseline No change NF- κ B p105/50, IL-12, IL-1 β , TNF α	N
Cuevas et al. (2005)	Single group Repeated measures	$N = 8$ ♂ professional cyclists	1 x Wingate 4 x Wingate	Serial blood samples pre & post-exercise	Electrophoresis	↑ PBMC NF- κ B 8-OH-2-dg, TBARS, ↓ I κ B, GSH 24 hr post	Y

García-López et al. (2007)	Single group Repeated measures	<i>N</i> = 11 ♂ moderately active	Eccentric exercise protocol: negative squats 12 x 10 reps set 60% MVIC	Bloods: pre & 2, 6, & 24 hr post eccentric protocol (separated by 6 wks training)	Electrophoresis	↑ PBMC NF-κB p50/65 plasma CK, MnSOD, MS, ↓ JH post 1 st bout ↓ CK after the second bout (evidence of RBE) but ↑ PBMC NF-κB p65 no exercise effect on TBARS or PC	Y
Gomez-Cabrera et al. (2006)	Randomised Double-blind Matched groups: A vs. P	<i>N</i> = 25 ♂ runners A: <i>n</i> = 11 P: <i>n</i> = 14	Marathon	Bloods: 1 d pre & immed. Post	ELISA	Exercise ↑ PBMC NF-κB p50, plasma XO & MDA	Y
Koenig et al. (2015)	Randomised Double-blind Matched groups	<i>N</i> = 16 ♀ (8 per group) moderate exercisers AVA vs. P	60 min downhill run 75% $\dot{V}O_2$ max	Bloods: Pre & Post-exercise +24 hr	ELISA	No exercise effects IL-6, NF-κB p65 ↓ 24 hr post with AVA	N

Nieman et al. (2007a)	Randomised Double-blind Independent groups Q + cho 1,000 mg·d ⁻¹ 21 d	<i>N</i> = 40 ♂ trained cyclists (20 per group)	3 hr·d ⁻¹ cycling 57% Work rate max 3 d	Bloods: pre & post exercise Muscle biopsies: pre & post day 1 & 3	ELISA	No exercise effect on muscle NF-κB, cytokine mRNA, COX-2 mRNA ↓ leukocyte IL-10 & IL-8 mRNA post-exercise with Q compared to CON	N
Parker et al. (2017)	Randomised Cross over	<i>N</i> = 8 (6 ♂; 2 ♀) recreationally active	SIE: 4 x 30 s Wingate HIIE: 5 x 4 min 75% Wmax CMIE: > 30 min 50% Wmax	Muscle biopsies: pre & post-exercise + 3 hr	Western blotting	SIE ↑ NF-κB p65 post-exercise compared to HIIE & CMIE	Y
Vella et al. (2012)	Repeated measures Parallel groups	<i>N</i> = 10: EXE: <i>n</i> = 5 ♂ CON: <i>n</i> = 4 ♂; 1 ♀ recreationally active, but not strength trained	Resistance exercise: squat/leg press/leg extension 3 sets 10 reps 80% 1 RM	Muscle biopsies: pre & post-exercise + 2 hr + 4hr	Western blotting	↑ NF-κB p65 ↓ IκBα 2 hr post resolved 4 hr post ↑ MCP-1, IL-6, IL-8 mRNA & NF-κB binding No change CON	Y

Vider et al. (2001)	Single group Repeated measures	$N = 12$ ♂ endurance/aerobically trained athletes	60 min treadmill run 80% $\dot{V}O_2\text{max}$	Bloods: pre & post- exercise	Electrophoresis	↑ TBARS, CD, NF- κ B p65, Neutrophils, TNF α	Y
------------------------	--------------------------------------	---	---	---------------------------------------	-----------------	--	---

Note. A = Allopurinol; AVA = Avenanthramides; CD = conjugated dienes; cho = carbohydrate; CK = creatine kinase; CMIE = continuous moderate interval exercise; CON = control group; COX-2 = cyclooxygenase synthase 2; d = days 8-OH-2-dg = 8-oxo-2-deoxyguanosine; EXE = exercise group; GSH = Glutathione; HIIE = high-intensity intermittent exercise; hr = hour; hr·d⁻¹ = hours per day; I κ B = inhibitors of kappa B; IL = Interleukin; immed. = immediately; JH = jump height; min = minutes; MCP = monocyte Chemoattractant protein; MDA = Malondialdehyde; mg·d⁻¹ = milligrams per day; mRNA = messenger ribonucleic acid; ms = muscle soreness; N = number of participants; n = number of participants per group; NF- κ B = nuclear factor kappa-light-chain-enhancer of activated B cells; Q = quercetin; RM = rep max; RT-PCR = real-time polymerase chain reaction; SIE = sprint interval exercise; TBARS = Thiobarbituric acid reactive substances; TNF α = Tumor Necrosis Factor alpha; P = placebo; PC = Protein Carbonyls; $\dot{V}O_2\text{max}$ = maximal aerobic power; Wmax = Wingate anaerobic Watt max; XO = Xanthine Oxidase; ♂ = male; ♀ = female.

2.8 Recovery Strategies

The main aims of a recovery strategy are to (i) reduce post-exercise fatigue, (ii) promote rapid recovery, (iii) optimise performance, and (iv) reduce injury risk (Nédélec et al., 2013; Silva et al., 2018). Several recovery strategies have been implemented to promote recovery following soccer, including (i) nutritional interventions, (ii) active recovery, (iii) compression, (iv) ice baths, (v) stretching, (vi) sleep hygiene, and (vii) massage (Nédélec et al., 2013).

Focusing on nutritional recovery strategies in soccer, the emphasis has been on specific match day recommendations (i.e., fluid & macronutrient intake; Heaton et al., 2017; Nédélec et al., 2013; Oliveria et al., 2017; Ranchordas, Dawson & Russell, 2017a). However, it is now recommended that nutritional strategies be periodised in line with the demands of the training (Oliveira et al., 2017), as dietary intake may not be optimal (Ranchordas et al., 2017a). While hydration, electrolyte replacement, glycogen refueling, and protein intake still take precedence, there is now an interest in nutritional recovery strategies that support intensified periods of training and competition (Braakhuis & Hopkins, 2015; Owens et al., 2019; Ranchordas et al., 2017a). Implementation of a nutritional recovery strategy that promotes sufficient recovery could be fundamental in optimising performance when recovery time is limited. One such nutritional recovery strategy proposed is antioxidant supplementation (Braakhuis & Hopkins, 2015).

2.8.1 Dietary antioxidant supplementation in athletes.

A potential recovery strategy currently being explored is the role of antioxidant supplementation in exercise (Braakhuis & Hopkins, 2015). It is proposed that increasing dietary antioxidant intake (via supplementation or whole foods) would enhance endogenous antioxidants, which may be beneficial to athletes during intensified periods of training and competition when athletes may be at an increased risk of exercise-induced oxidative stress (Bowtell & Kelly, 2019; Powers et al., 2004; Ranchordas et al., 2017a). Increasing antioxidants could help to restore redox homeostasis, reducing muscle damage and inflammation to promote recovery. Evidence to support increased dietary antioxidant intakes in athletes is provided by studies inducing a restricted dietary antioxidant intake. Two such studies (Plunkett, Callister, Watson, & Garg, 2010; Watson, MacDonald-Wicks, & Garg, 2005) have demonstrated that athletes following antioxidant-restricted diets (i.e., avoiding high antioxidant foods for 2 wks) had (i) reduced levels of plasma antioxidants (Total Antioxidant Capacity; carotenoids), (ii) elevated post-exercise oxidative stress (F₂-isoprostanes), (iii) elevated inflammation (TNF- α ; IL-6), and (iii) an increased perception of effort during exercise, in comparison to age- and sex-matched control groups who followed a habitual high-antioxidant diet. These studies have been limited because of ethical considerations; however, further evidence for the benefits of increased dietary antioxidants in athletes is provided by antioxidant supplementation studies. Since the first study by Dillard et al. (1978) demonstrated that vitamin E (1,200 IU·d⁻¹ for 2 wks) reduced resting and exercise-induced pentane, and the study by Reid et al. (1994) showed that intravenous N-acetylcysteine (an antioxidant) inhibited muscular fatigue (attributed to oxidative stress), several studies have demonstrated the positive effects of antioxidant supplementation use in

athletes, predominantly focusing on vitamins A, C, and E (MacRae & Mefferd, 2006). An in-depth review of antioxidant exercise studies is beyond the scope of this literature review, which focuses on quercetin, a polyphenol (subclass: flavonoid) that has antioxidant and anti-inflammatory properties and thus, could be beneficial to athletes undertaking periods of prolonged high-intensity intermittent exercise.

2.8.1.1 Polyphenols.

Polyphenols are phytochemicals occurring naturally in plants (Somerville, Bringans, & Braakhuis, 2017). There are four subclasses of polyphenols, classified by their structure: (i) flavonoids, (ii) stilbenes, (iii) lignans, and (iv) phenolic acids (Figure 2.6); with flavonoids being the largest subclass (Woodward, Draijer, Thijssen & Low, 2018). Focusing on flavonoids, interest in the use of flavonoid supplements in athletes has stemmed from health research, where flavonoids have demonstrated antioxidative and anti-inflammatory effects to counteract oxidative stress and inflammation in disease (e.g., polyphenols have been shown to improve cardiovascular health [Woodward et al., 2018]). Six subclasses of flavonoids exist: (i) flavonols; (ii) flavanols; (iii) anthocyanins; (iv) flavanones; (v) flavones; and (vi) isoflavones, with flavonols being the most common subclass (Woodward et al., 2018). Accumulative evidence from several research studies has demonstrated the potential ergogenic effect of an increased flavonoid intake (via whole food or supplementation) in athletes/recreational cohorts. For example, anthocyanins (found in berries), and epicatechins (flavanols found in green tea), have demonstrated positive performance effects that have been attributed to the antioxidant or anti-inflammatory properties of flavonoids (Howatson et al., 2010; Levers et al., 2016;

Morgan, Barton, & Bowtell, 2019; Roberts, Roberts, Tarpey, Weekes, & Thomas, 2015).

Regarding exercise that is characteristic of soccer, research has focused on the efficacy of flavonoid supplementation to modulate oxidative stress and inflammation, thereby reducing muscle damage and muscle soreness to promote recovery. Catechins, present in cocoa, have been shown to modulate biomarkers of oxidative stress and muscle damage (i.e., plasma MDA & LDH) in 28 male youth soccer players following 14 days consumption of chocolate (M & M's® containing 168 mg·d⁻¹ flavanols; Fraga et al. 2005). Furthermore, Bell et al. (2016) showed an increased anthocyanin intake (30 mL tart cherry juice, twice per day [total anthocyanin content 540 mg·L⁻¹], consumed 5 days before & 3 days after exercise) promoted recovery (i.e., reduced DOMS & IL-6) and increased functional performance (assessed via a battery of sports-specific tests) in 8 male semi-professional soccer players following an adapted version of the Loughborough Intermittent Shuttle Test (LIST), in comparison to a placebo control group.

Another flavonoid that may be beneficial to athletes participating in exercise that is characteristic of soccer is quercetin.

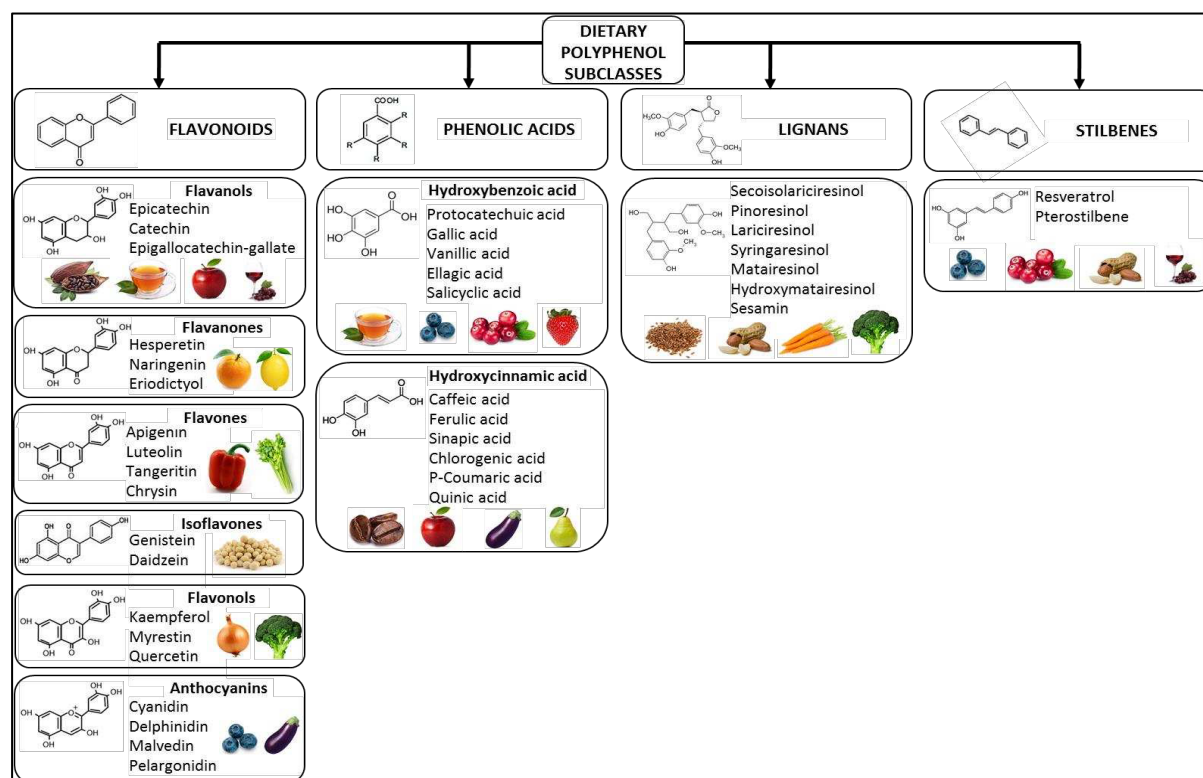


Figure 2.6. Polyphenol subclasses with basic chemical structure & picture examples of dietary sources, recopied from Woodward et al. (2018) "Polyphenols and Microvascular Function in Humans: A Systematic Review". *Current Pharmaceutical Design*, 24 (2), p. 206. Flavonoids are plant pigments that have been shown to exert an ergogenic effect in athletes.

2.8.1.1.1 Quercetin.

Quercetin (3,3',4',5,7-pentahydroxyflavone; subclass flavonols) is the main flavonoid in the human diet (Nieman et al., 2007a). Quercetin occurs in the skin of plant foods, for example, onions and apples (Nieman et al., 2007a; significant food sources of quercetin are presented, Table 2.5) as quercetin glycosides (i.e., bound to a sugar [Manach, Williamson, Morand, Scalbert, & Rémésy, 2005]). There is no Reference Nutrient Intake (RNI) for quercetin. Quercetin intake varies, with western diets reported to be low in quercetin (ranging between 20 - 35 mg·d⁻¹ [Manach et al., 2005]; ~50 mg·d⁻¹ [Boots et al., 2008]). Quercetin supplementation is popular in comparison to the consumption of whole foods containing quercetin; given the

amount of food required to consume recommended amounts (i.e., 1,000 mg·d⁻¹ quercetin) would be impractical (Kressler, Millard-Stafford & Warren, 2011).

Table 2.5

Food Sources of Quercetin

Food Source	Quercetin content (mg·100g ⁻¹ edible portion)
Capers	233.84
Wild rocket, raw	66.19
Red onion, raw	31.77
Watercress, raw	29.99
Red grapes	22.85
Kale, raw	22.58
Cocoa powder extract	10.00
Apples, raw, with skin	4.01
Black tea	2.19
Red wine	2.11

Note. Adapted from Halvorsen et al. (2006) & Database for the Flavonoid Content of Selected Foods (Release 3.2). U.S. Department of Agriculture (2015). Retrieved from <https://data.nal.usda.gov/system/files/Flav3.2pdf>.

Research (in vitro; in vivo; murine) has demonstrated quercetin to have multiple biological effects, including (i) antioxidant (Boots et al., 2008), (ii) anti-inflammatory (Nair et al., 2006), and (iii) analgesic properties (Alexander, 2006), with the antioxidant capacity of quercetin demonstrated to be five-fold that of vitamin C (Chen et al., 2005).

2.8.1.1.2 Mechanisms of action.

Understanding the mechanisms of quercetin is necessary to determine the potential effectiveness. Quercetin has gained prominence in exercise literature as it is hypothesised that quercetin could potentially promote recovery and performance through several mechanisms, including: (i) increased endurance via mitochondrial biogenesis (Davis, Murphy, & Carmichael, 2009a), (ii) accelerated recovery through a reduction in oxidative stress and inflammation (Nieman et al., 2007a), and (iii) a reduction in perceived pain via reduced central fatigue (MacRae & Mefferd, 2006).

Interest in the beneficial role of quercetin supplementation in exercise stemmed from works by Davis' research group, who demonstrated the positive effects of quercetin on endurance performance. Davis et al. (2009b) showed that quercetin feedings ($12.5 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) induced mRNA expression of biomarkers of mitochondrial biogenesis (i.e., SIRT-1 & PGC-1 α) in sedentary mice, and went on to demonstrate that 7 days of quercetin supplementation in humans (dose based on that given to the mice scaled up to an 80 kg human [$1,000 \text{ mg}\cdot\text{kg}\cdot\text{d}^{-1}$]), increased $\dot{V}\text{O}_2\text{max}$ in recreationally trained humans (Davis, Carlstedt, Chen, Carmichael, & Murphy, 2010).

Research suggests that the primary function of quercetin is to act as an antioxidant due to its phenol structure (presence of electron donating hydroxyl groups & catechol type B ring; Davis et al., 2009a; Harwood et al., 2007). It is proposed that quercetin exerts antioxidant effects by (i) scavenging RONS through donation of hydrogen atoms or electrons, (ii) chelating metal ions, (iii) inducing expression of antioxidant enzymes via signalling pathways (i.e., Nrf-2, NF- κ B), (iv) inhibition of xanthine oxidase and nitric oxide synthase, and (v) working synergistically with other

antioxidants (Davis et al., 2009a; Tejada et al., 2017). Underlying mechanisms for the anti-inflammatory properties of quercetin include inhibition of NF- κ B and COX-2 pathways (García-Mediavilla et al., 2007; Tejada et al., 2017). Regarding the NF- κ B pathway, it has been proposed that quercetin may mediate its effect by blocking I κ B phosphorylation, inhibiting NF- κ B nuclear translocation, and NF- κ B DNA binding activity (Nair et al., 2006; Nieman et al., 2007a [Figure 2.7]). Inhibition of NF- κ B activity would reduce inflammatory responses that are regulated by NF- κ B, such as the expression of cytokines and acute-phase proteins (Chen et al. 2005; Dias et al., 2005). Evidence to support this mechanism has been demonstrated in murine studies (Aoi et al., 2004; Dias et al., 2005; Ji et al., 2004). Another potential mechanism of quercetin, demonstrated in vitro (Alexander, 2006), is that quercetin may act as an adenosine A₁-receptor antagonist, and therefore, act similarly to caffeine, reducing the perception of pain.

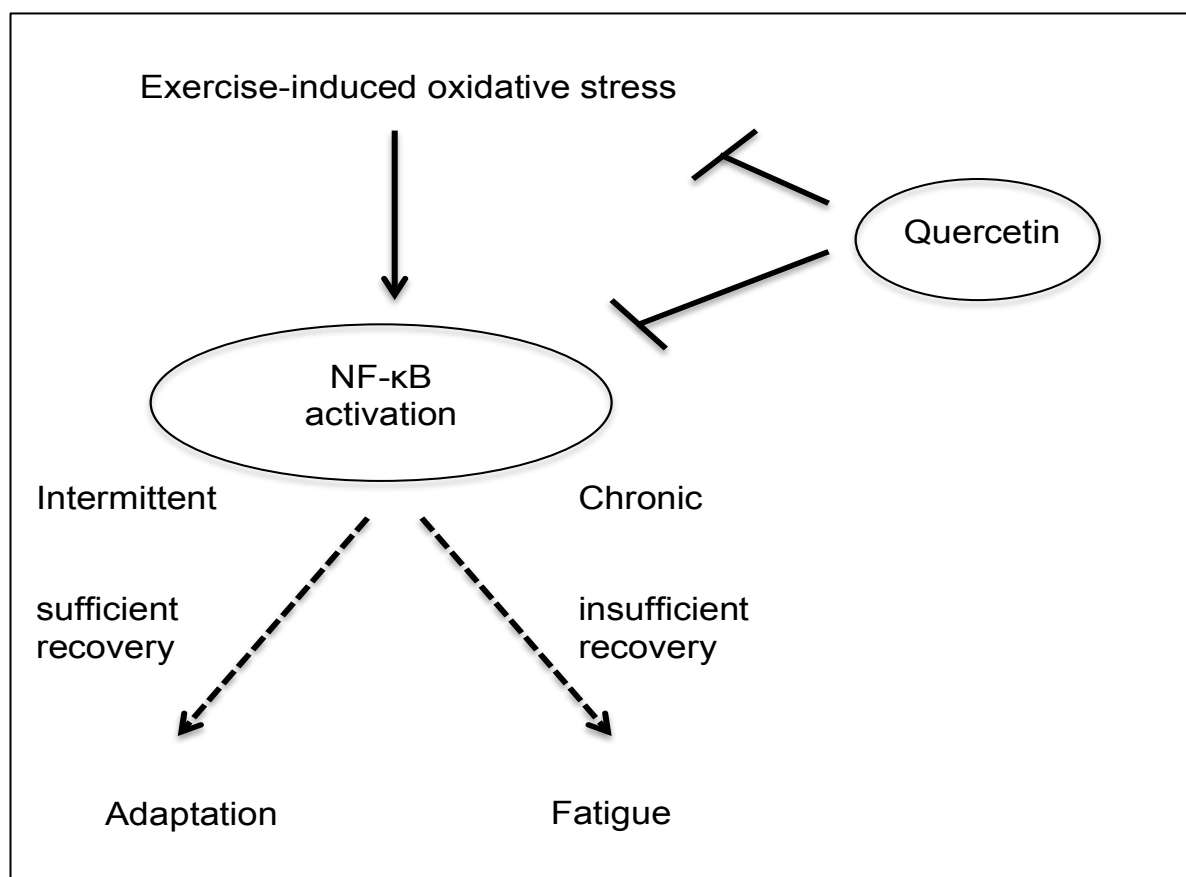


Figure 2.7. Quercetin supplementation may counteract exercise-induced oxidative stress & blunt NF- κ B activation. During intensified periods of exercise blunting oxidative stress & NF- κ B activity may be beneficial for athletes. Adapted from Kramer & Goodyear (2007), p. 392.

2.8.1.1.3 Quercetin Pharmacokinetics.

The pharmacokinetics (i.e., absorption, metabolism, & excretion) of quercetin is complicated. Quercetin originates in plants as glycosides (associated with a sugar moiety [Manach et al., 2005]). Absorption occurs in the small intestine where glycosides are hydrolysed to form quercetin aglycone, which is then conjugated into sulfate, glucuronide, and methyl forms of quercetin (known as quercetin metabolites), which appear in plasma (Justino et al., 2004; Figure 2.8 shows the molecular structure). Quercetin bioavailability has been demonstrated in humans (Jin et al., 2010; Quindry et al., 2008) but is highly individual, with dietary intake (i.e., fat) and gut microflora influencing quercetin absorption rates (Egert et al., 2008; Jin et

al., 2010; Justino et al., 2004; Manach et al., 2005). In vivo quercetin metabolites appear to exert less antioxidant effect than that demonstrated in vitro, attributed to either the location of formation of quercetin metabolites or an insufficient quantity of quercetin metabolites (Manach et al., 2005; McAnulty et al., 2008; Nieman et al., 2007a). At present, the exact form and dosage of quercetin supplementation that are beneficial to athletes is unknown. Based upon Davis' work, human exercise studies commonly use an intervention dosage of 1,000 mg·d⁻¹, loaded for 7 days (Somerville et al., 2017). Single doses of quercetin ingested before exercise are not purported to show an effect (Cheuvront et al., 2009; Konrad et al., 2011). Therefore, it is likely that quercetin accumulates in blood with repeated daily ingestion, which is further supported by increased plasma quercetin following prolonged, repeated oral ingestion (i.e., 28 d; Conquer, Malani, Azzini, Raguzzini, & Holub, 1998). Quercetin has been reported to peak in plasma ~2 hr following ingestion (Davis et al., 2009a; Egert et al., 2008), with a slow elimination rate (~16 hr [Egert et al., 2008]; ~11 - 28 hr [Manach et al., 2005]). At present, there is limited evidence to demonstrate that quercetin accumulates in skeletal muscle tissue (Nieman et al., 2007a).

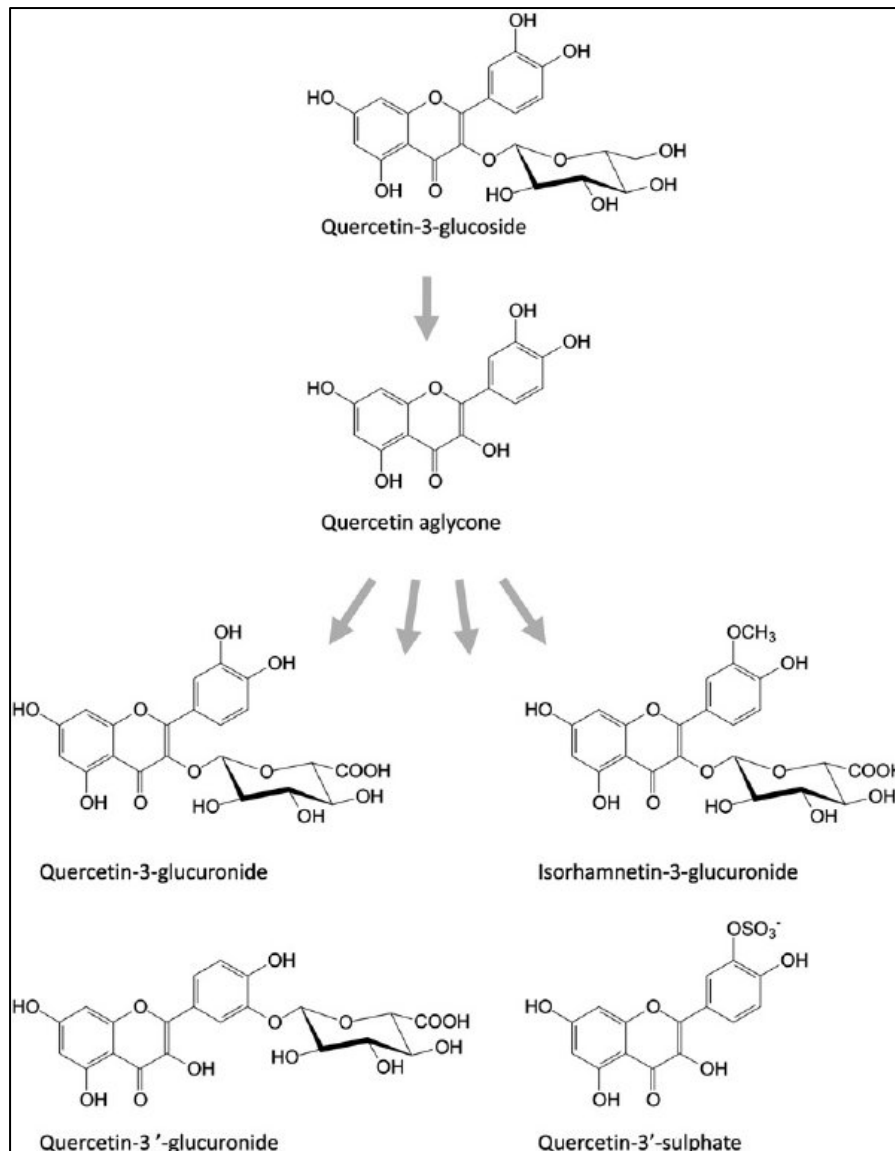


Figure 2.8. Molecular structure of quercetin & quercetin metabolites. Recopied from “Plant Science and Human Nutrition: Challenges in Assessing Health-Promoting Properties of Phytochemicals” Traka & Mithen (2011). *The Plant Cell*. 23, p. 2487.

2.8.1.1.4 Quercetin supplementation in human exercise studies.

Several studies have investigated the efficacy of quercetin supplementation to attenuate oxidative stress, muscle damage, or inflammation or promote recovery or performance following exercise (studies are summarised; Table 2.6). The potential of quercetin to enhance oxidative capacity has led to studies focusing mostly on endurance exercise, such as prolonged steady-state cycling or running, using predominantly male cohorts (conceivably to avoid the antioxidant properties of oestrogen confounding results). Studies exploring the efficacy of quercetin supplementation in prolonged high-intensity intermittent exercise, characteristic of soccer, are lacking, providing a rationale for chapter 6 of this thesis. The results of quercetin exercise studies have been inconclusive, with studies demonstrating a beneficial effect (Askari, Ghiasvand, Feizi, Ghanadian, & Karimian, 2012; Bazzuchi et al., 2019; Davis et al., 2010; MacRae & Mefferd, 2006; McAnulty et al., 2013; Nieman et al., 2007a, 2009, 2010; Ramezani & Moonikh, 2017) versus no beneficial effect on performance or recovery (Abbey & Rankin, 2011; Bigelman et al., 2010; Cheuvront et al., 2009; Cureton et al., 2009; Ganio et al., 2010; Konrad et al., 2011; McAnulty et al., 2008; Nieman et al., 2007b; O'Fallon et al., 2012; Quindry et al., 2008; Utter et al., 2009). There appear to be no significant benefits of quercetin supplementation on perceived exertion, performance, oxidative stress, inflammation, muscle damage, or muscle soreness in ultra-endurance exercise (160 km race [Nieman et al., 2007b; Quindry et al., 2008; Utter et al., 2009]), with the authors attributing a lack of effect to (i) individual differences in dietary intake, (ii) reduced antioxidant effect of quercetin metabolites, or, (iii) decreased plasma quercetin with ultra-endurance exercise.

Prolonged high-intensity intermittent exercise, performed in team sports such as soccer, is interesting as it incorporates an aerobic component and a high number of eccentric (& concentric) muscle actions (Bangsbo et al., 2006). Focusing on studies that have used (a) aerobic exercise performed on repeated days or (b) exercise with a predominant eccentric component (as this is similar to the exercise modalities used in chapters 4, 5, & 6 of this thesis [i.e., prolonged high-intensity intermittent exercise, characteristic of soccer performed on repeated days]), a well-controlled study by McAnulty et al. (2008) showed that plasma quercetin increased following supplementation in trained male athletes ($N = 20$; $1,000 \text{ mg}\cdot\text{d}^{-1}$ for 6 wks) compared to a placebo. However, found no significant effects of quercetin, compared to a placebo, on elevated biomarkers of oxidative stress (assessed via serum F_2 -isoprostanes, FRAP, TEAC, nitrite) and inflammation (serum CRP) following a 3-day period of prolonged intensified cycling ($3 \text{ hr}\cdot\text{d}^{-1}$ at $\sim 57\%$ maximum power), where quercetin was administered before and during the 3-day exercise protocol. Using a similar study design (male trained cyclists; $1,000 \text{ mg}\cdot\text{d}^{-1}$ for 3 weeks; $3 \text{ hr}\cdot\text{d}^{-1}$ cycling at $\sim 57\%$ maximum power), Nieman et al. (2007a) also reported no effect of quercetin, finding quercetin did not attenuate post-exercise increases in plasma cytokines (IL-6; IL-8; IL-10; IL-1ra; TNF- α ; MCP1). Interestingly, Nieman et al. (2007a) did find that quercetin supplementation diminished post-exercise increases in leukocyte IL-8 mRNA and IL-10 mRNA expression, suggesting perhaps that quercetin exerts an effect in blood cells. Furthermore, Nieman et al. (2009) found quercetin ($1,000 \text{ mg}\cdot\text{d}^{-1}$ combined with additional antioxidants [i.e., isoquercetin, 400 mg; EGCG, 120 mg; $N3$ -PUFA, 400 mg]) supplemented for 21 days before and during a 3-day intensified period of cycling ($3 \text{ hr}\cdot\text{d}^{-1}$ at $\sim 57\%$ work rate max) counteracted exercise-induced inflammation (evidenced by decreased plasma IL-6;

IL-10; serum CRP; whole blood total leukocyte counts) compared to a placebo and isolated quercetin supplement in 14 male trained athletes. However, the reduction in inflammation did not translate to a performance improvement (time trial performance) or improved recovery (DOMS assessment). Furthermore, no effect of quercetin on PGC-1 α mRNA expression in skeletal muscle was demonstrated.

Eccentric exercise has been shown to induce muscle damage and inflammation, attributed to mechanical and metabolic stress (Fatouros & Jamurtas, 2016); therefore, it is theorised that quercetin could be beneficial in athletes undertaking this type of exercise. Using eccentric elbow flexor exercise protocols, O'Fallon et al. (2012) found no beneficial performance effect of quercetin (1,000 mg·d⁻¹ administered in bars for 7 days before & 5 days after exercise), in comparison to a placebo, on eccentric elbow flexor strength performance, muscle damage, muscle soreness, or inflammation in sedentary/recreationally trained athletes, but more recently, Bazzuchi et al. (2019) demonstrated quercetin supplementation (1,000 mg·d⁻¹ for 14 days) increased isometric strength using a similar eccentric exercise protocol in trained athletes. However, the study designs differed, with Bazzuchi et al. (2019) using a crossover design with trained athletes where the learning effect of the exercise may have potentially confounded the results.

Studies investigating the efficacy of quercetin in intermittent exercise have been limited. To the lead investigator's knowledge, only two studies have investigated the efficacy of quercetin supplementation in athletes undertaking high-intensity intermittent exercise characteristic of soccer. Abbey and Rankin (2011) investigated the efficacy of quercetin supplementation (quercetin glycoside administered in a

carbohydrate beverage form, 1,000 mg·d⁻¹ for 7 d) in a double-blind, crossover design study using male team sport athletes (*N* = 15). Following a repeated sprint protocol characteristic of soccer (12 x 30 m; < 6 min), the authors found quercetin supplementation to be ineffective in attenuating exercise-induced increases in xanthine oxidase, serum IL-6, RPE, or increasing repeated sprint performance, compared to a placebo. In contrast, a recent clinical trial by Ramezani and Moonikh (2017) demonstrated that 6 weeks of quercetin supplementation (1,000 mg·d⁻¹ quercetin capsule vs. 1,000 mg·d⁻¹ dextrose placebo) significantly improved performance (Bruce protocol time to exhaustion) and reduced oxidative stress (evidenced by increased serum SOD, GPX, & catalase; decreased serum MDA) in 11 amateur soccer players undertaking 6 weeks of regular soccer training (3 x per wk), compared to a matched placebo group.

A proposed anti-inflammatory mechanism of quercetin is inhibition of NF-κB activation (Chen et al., 2005; Comalada et al., 2005; Nair et al., 2006). In their 2007a study, Nieman et al. investigated the efficacy of prolonged quercetin supplementation (1,000 mg·d⁻¹ quercetin in a carbohydrate beverage, 21 d before & 3 d during exercise) on exercise-induced NF-κB activation in 20 male trained cyclists, following a 3-day period of intensified cycling (3 hr·d⁻¹; ~57% work rate max). The authors reported no effect of the 3-day exercise protocol or prolonged quercetin supplementation on NF-κB p65 activity in skeletal muscle compared to a placebo control group. Results of this study are inconclusive as there was no activation of NF-κB in response to the 3-day exercise protocol. Therefore, the impact of quercetin supplementation on exercise-induced NF-κB activation and inflammatory responses in athletes is currently unknown and warrants further research. Chapter 6 (study

three) attempted to address this gap in the literature by investigating the effect of prolonged quercetin supplementation on NF- κ B p65 activation following a 3-day intensified period of soccer-specific exercise.

Table 2.6

Summary of Studies Investigating Quercetin Supplementation in Human Exercise

Authors	Study Design	Participants	Supplement Protocol	Exercise Modality	Plasma Q assessed?	Outcome Effect of Q Compared to a Placebo	Significant Effect?
Abbey & Rankin (2011)	Randomised Double-blind Crossover	$N = 15$ ♂ trained team sports athletes	Q glycoside + cho beverage $1,000 \text{ mg}\cdot\text{d}^{-1}$ 7 d	12 x 30 m repeated sprint test	N	No effect on serum XO, UA, IL-6, RPE or performance	N
Askari et al. (2012)	Randomised Double-blind Independent groups Clinical trial	$N = 60$ ♂ non-professional athletes (15 per group)	Q $500 \text{ mg}\cdot\text{d}^{-1}$ Q + AO vitamin C Placebo 56 d	Normal exercise routine 8 weeks	N	Q + AO ↓ IL-6, CRP, E-selectin & F ₂ isoprostanes	Y
Bazzucchi et al. (2019)	Randomised Double-blind Crossover	$N = 12$ ♂ undertaking regular resistance training	Q $1,000 \text{ mg}\cdot\text{d}^{-1}$ 14 d	Eccentric elbow flexor protocol 10 x 10 reps sets	N	↑ isometric strength	Y
Bigelman et al. (2010)	Randomised Double-blind Independent groups	$N = 58$ (29 per group) (♀ = 7; ♂ = 22) moderately trained	Q + AO chew $1,000 \text{ mg}\cdot\text{d}^{-1}$ 42 - 54 d	Military physical training	Y	No effect on mood state, energy, or fatigue	N

Cheuvront et al. (2009)	Randomised Double-blind Crossover	$N = 10$ ♂ moderately trained	Q energy bars $2,000 \text{ mg}\cdot\text{d}^{-1}$ 1 d	40°C 15 min cycling TT after 30 min cycling 50% $\dot{V}\text{O}_2\text{peak}$	Y	No effect on performance or perceptual responses	N
Cureton et al. (2009)	Randomised Double-blind Independent groups	$N = 30$ ♂ recreationally active (15 per group)	Q sports beverage $1,000 \text{ mg}\cdot\text{d}^{-1}$ 7 - 16 d	10 min cycling TT performance following 60 min cycling 50% $\dot{V}\text{O}_2\text{peak}$	Y	No effect on RPE, TT performance, or knee extensor strength	N
Davis et al. (2010)	Randomised Double-blind Crossover	$N = 12$ (♀ = 5; ♂ = 7) active not highly trained	Q + cho beverage $1,000 \text{ mg}\cdot\text{d}^{-1}$ 7 d	Cycling to fatigue $75\% \dot{V}\text{O}_2\text{max}$	N	$\uparrow \dot{V}\text{O}_2\text{max}$ \uparrow ride time to fatigue	Y
Ganio et al. (2010)	Randomised Double-blind Crossover	$N = 11$ (♀ = 6; ♂ = 5) untrained	Q bars $1,000 \text{ mg}\cdot\text{d}^{-1}$ 5 d + 1 d during exercise	$\dot{V}\text{O}_2\text{max}$	N	No effect on $\dot{V}\text{O}_2\text{max}$	N
Konrad et al. (2011)	Randomised Double-blind Crossover	$N = 20$ (♀ = 9; ♂ = 11) runners	Q + AO chews $1,000 \text{ mg}\cdot\text{d}^{-1}$ single dose 15 min prior to exercise	2 hr treadmill run $70\% \dot{V}\text{O}_2\text{max}$	Y	No effect on plasma CRP, inflammatory cytokines, whole blood leukocyte count	N

MacRae & Mefferd (2006)	Randomised Double-blind Crossover	$N = 11$ ♂ elite cyclists	Q + AO 600 mg·d ⁻¹ 42 d + 3 d during exercise	30 km cycling TT	N	↑ TT ↑ power output	Y
McAnulty et al. (2008)	Randomised Double-blind Independent groups	$N = 40$ ♂ trained cyclists (20 per group)	Q + cho 1,000 mg·d ⁻¹ 6 wks + 3 d during exercise	3hr·d ⁻¹ cycling 57% work rate max 3 d	Y	No effect on plasma F ₂ isoprostanes, FRAP, TEAC, CRP, or nitrite	N
McAnulty et al. (2013)	Randomised Double-blind Crossover	$N = 14$ ♂ trained athletes (defined by $\dot{V}O_{2max}$)	Q + AO 225 mg·d ⁻¹ d 1 - 6 higher dose on d 7	1 hr run 3% gradient ~80% $\dot{V}O_{2max}$	N	↓ F ₂ isoprostanes ↓ PC ↑ FRAP, TEAC No effect on IL-8	Y
Nieman et al. (2007a)	Randomised Double-blind Independent groups	$N = 40$ ♂ trained cyclists (20 per group)	Q + cho 1,000 mg·d ⁻¹ 21 d before + 3 d during exercise	3 hr·d ⁻¹ cycling ~57% work rate max 3 d	Y	Q no effect on ↑ COX-2 mRNA No effect exercise or Q on muscle NF-κB or plasma cytokines Q ↓ leukocyte IL-10 & IL-8 mRNA	Y

Nieman et al. (2007b)	Randomised Double-blind Independent groups	<i>N</i> = 39 (race finishers) ultra-endurance runners Q: <i>n</i> = ♀ = 4, ♂ = 14 P: <i>n</i> = ♀ = 3, ♂ = 18	Q + AO chews 1,000 mg·d ⁻¹ 21 d before	160 km ultra-endurance race	Y	No effect on plasma pro- & anti-inflammatory cytokines, serum CK, CRP, urea, DOMS	N
Nieman et al. (2009)	Randomised Double-blind Independent groups	<i>N</i> = 39 (♀ = 7; ♂ = 32) trained cyclists P: <i>n</i> = 12 Q: <i>n</i> = 13 Q + AO: <i>n</i> = 14	Q Q + AO 1,000 mg·d ⁻¹ 21 d + 3 d during exercise	3 hr·d ⁻¹ cycling ~57% work rate max 3 d	Y	↓ serum CRP, IL-6, IL-10, total leukocytes No effect on cycling TT or DOMS, or PGC-1α mRNA expression	Y
Nieman et al. (2010)	Randomised Double-blind Crossover	<i>N</i> = 30 ♂ untrained (26 completed the study)	Q beverage 1,000 mg·d ⁻¹ 14 d	12 min performance run 15% incline following 60 min run 60% $\dot{V}O_2$ max	Y	↑ running performance	Y
O'Fallon et al. (2012)	Randomised Double-blind Independent groups	<i>N</i> = 30 Sedentary/recreational naïve to resistance Q: ♀ = 7, ♂ = 8 P: ♀ = 8, ♂ = 7	Q bars 1,000 mg·d ⁻¹ 12 d; 7 d pre- 5 d post-exercise	24 x eccentric elbow flexor contractions	Y	No effect Q on Isometric/kinetic strength, ms, swelling, ROM, Serum CK/IL-6	N

Quindry et al. (2008)	Randomised Double-blind Independent groups	$N = 63$ (♀ = 15; ♂ = 48) ultra-runners	Q + AO $1,000 \text{ mg} \cdot \text{d}^{-1}$ 21 d before & 3 d during	160 km ultra-endurance race (39 finishers)	Y	No effect of exercise or Q+AO on plasma PC, F ₂ isoprostanes, TEAC, FRAP, or performance time	N
Ramezani & Moonikh (2017)	Randomised Double-blind Independent groups Clinical trial	$N = 22$ ♂ amateur soccer players (11 per group)	Q $1,000 \text{ mg} \cdot \text{d}^{-1}$ (500 mg twice daily) 42 d	Regular soccer training 3 x wk	N	↑ time to exhaustion (Bruce protocol) ↑ serum SOD, GPX, CAT ↓ serum MDA	Y
Scholten & Sergeev (2013)	Randomised Double-blind Independent groups	$N = 8$ ♂ trained endurance Q: $n = 5$ P: $n = 3$	Q dihydrate $1,000 \text{ mg} \cdot \text{d}^{-1}$ 6 wks	Regular training	N	↓ serum MDA No effect TAC, PC, SOD or performance	Y
Utter et al. (2009)	Randomised Double-blind Independent groups	$N = 63$ ♀♂ ultra-runners	Q $1,000 \text{ mg} \cdot \text{d}^{-1}$ 21 d	160 km ultra- endurance race (39 finishers)	Y	No effect on RPE or performance time	N

Note. AO = antioxidants; CAT = catalase; cho = carbohydrate; CK = creatine kinase; CRP = C-Reactive Protein; d = day; DOMS = delayed onset of muscle soreness; FRAP = ferric reducing ability of plasma; GPX = glutathione peroxidase; hr = hour; $\text{hr} \cdot \text{d}^{-1}$ = hours per day; IL = interleukin; $\text{mg} \cdot \text{d}^{-1}$ = milligrams per day; mRNA = messenger ribonucleic acid; ms = muscle soreness; N = total number of participants; n = number of participants per group; N = no; NF- κ B = nuclear factor kappa-light-chain-enhancer of B cells;

P = placebo; PC = protein carbonyls; Q = quercetin; RPE = rating of perceived exertion; ROM = range of motion; SOD = superoxide dismutase; TEAC = Trolox equivalent antioxidant capacity; UA = uric acid; wks = weeks; XO = xanthine oxidase; $\dot{V}O_2\text{max}$ = maximal aerobic power; TT = time trial; Y = yes; ♂ = male; ♀ = female.

Literature Review

Equivocal results in the quercetin exercise studies may be attributed to methodological differences, including (i) the trained status of participants; (ii) exercise modalities, (iii) quercetin dosage, (iv) sampling times, (v) enhanced effects because of a reductionist dietary approach, (vi) the synergistic effect of quercetin combined with additional antioxidants, or (vii) outcome measures assessed (Davis et al., 2010; Jin et al., 2010; MacRae & Mefferd, 2006; Nieman et al., 2010). Several of the studies demonstrating beneficial effects have used lesser-trained participants (Askari et al., 2012; Cureton et al., 2009; Davis et al., 2010; Nieman et al., 2010); however, beneficial effects have also been demonstrated in studies using trained athletes (MacRae & Mefferd, 2006; McNulty et al., 2013; Nieman et al., 2007a, 2009; Ramezani & Moonikh, 2017). The literature is also inconclusive regarding dose, with studies supplementing for ≥ 7 days demonstrating an effect (Askari et al., 2012; Bazzucchi et al., 2019; Davis et al., 2010; MacRae & Mefferd, 2006; Nieman et al., 2007a, 2009, 2010; Ramezani & Moonikh, 2017), versus no effect (Abbey & Rankin, 2011; Bigelman et al., 2010; Cureton et al., 2009; Ganio et al., 2010; McNulty et al., 2008; Nieman et al., 2007b; O'Fallon et al., 2012; Quindry et al., 2008, Utter et al., 2009). Two studies supplementing quercetin in a single dose before exercise observed no beneficial effects of quercetin in comparison to a placebo. Cheuvront et al. (2009) found that 2,000 mg of quercetin dihydrate did not affect cycling time trial performance or perception of effort in ten male trained athletes exercising in the heat. Moreover, Konrad et al. (2011) showed no attenuation of exercise-induced inflammatory biomarkers following a 2 hr run in 20 trained runners after ingestion of a mixed quercetin supplement (1,000 mg of quercetin) 15 min before the run. Results of these two studies suggest repeat

ingestion of quercetin necessary to exert an effect (as also demonstrated by other studies; Table 2.6; & was therefore the strategy implemented in chapter 6).

There may be a beneficial synergistic effect of enhanced quercetin bioavailability when quercetin is combined with additional antioxidants compared to isolated quercetin supplements, as demonstrated in several studies (MacRae & Mefferd, 2006; McAnulty et al., 2013; Nieman et al., 2009). However, these aforementioned studies did not control for the independent effects of the additional antioxidants, therefore, drawing conclusions about the efficacy of quercetin is not easy. Results from a highly controlled clinical trial by Askari et al. (2012) showed quercetin (500 mg·d⁻¹) co-ingested with vitamin C (250 mg·d⁻¹) to be more effective in reducing oxidative stress (serum F₂isoprostanes), inflammatory biomarkers (serum CRP & IL-6) and endothelial adhesion molecules (E-selectin) in comparison to an isolated quercetin supplement, with the authors suggesting that vitamin C regenerates quercetin following oxidation. In chapter 6 of this thesis, quercetin supplementation was used as a nutritional intervention. The quercetin supplement used is commercially available and incorporates a small dose of vitamin C to enhance quercetin bioavailability (as specified by the manufacturer).

In summary, there is currently no clear consensus on the use of quercetin supplementation in athletes. Research has shown conflicting results, with repeated ingestion of quercetin demonstrating the most beneficial results, although information regarding the timing of the dose has been limited. Whether the positive effects of quercetin previously observed in vitro and in murine studies translate to positive effects in vivo requires further research. To date, research investigating the efficacy

of quercetin supplementation in prolonged, high-intensity intermittent exercise, characteristic of soccer, has been limited, with the two studies using soccer based exercise yielding inconclusive results. It is theorised that the antioxidant, anti-inflammatory, and analgesic properties of quercetin may potentially aid recovery between bouts of soccer exercise through the attenuation of oxidative stress and associated muscle damage and inflammation caused by metabolic and mechanical stress, further research investigating these effects is warranted (rationale for chapter 6 of this thesis).

Despite research demonstrating beneficial effects of antioxidants, such as the flavonoid quercetin, the efficacy of antioxidant supplementation in athletes has become an area of controversy.

2.8.1.2 Controversial role of antioxidant supplementation in athletes.

A high number of athletes supplement with antioxidants (Antonioni et al., 2019; Gomez-Cabrera et al., 2012). The rationale being that increasing exogenous antioxidants will increase antioxidant capacity to protect against the negative effects associated with exercise-induced oxidative stress that may lead to performance decrements (Powers et al., 2004). However, the efficacy of antioxidant supplementation in trained athletes has been debated (Antonioni et al., 2019; Gomez-Cabrera et al., 2012; Peternelj & Coombes, 2011; Radak et al., 2017; Ristow et al., 2009). Evidence from antioxidant supplementation intervention studies has been inconclusive. Blunting exercise-induced RONS is questionable, and may even be detrimental to the athlete, given that RONS theoretically play a role in cell signalling pathways that promote training adaptations within skeletal muscle

(Gomez-Cabrera et al., 2005, 2008 2012; Peternelj & Coombes, 2011; Ristow et al., 2009). For example, despite reducing inflammation, blunting exercise-induced activation of the redox-sensitive NF- κ B classical pathway may, in theory, interfere with the expression of antioxidant enzymes, a beneficial training adaptation (Kawamura & Muraoka, 2018). Evidence also suggests that high dosages or chronic antioxidant supplementation can produce a harmful pro-oxidant effect (Braakhuis & Hopkins, 2015). Reductive stress could lead to impaired muscular function; the concept of hormesis (Powers et al., 2004). In a review of 23 antioxidant exercise studies, Peternelj and Coombes (2011) found high dosages of antioxidant supplements interfered with training adaptations. However, limited information regarding the cohorts and methodological differences (i.e., supplementation strategy; outcome measures) make it difficult to draw definitive conclusions. In a 2012 letter to the editor, Gomez-Cabrera et al. questioned the efficacy of antioxidant supplementation in athletes, suggesting that antioxidant supplementation may have a detrimental effect on skeletal muscle, exercise performance, and recovery.

Radak et al. (2017) recently proposed that the action of antioxidant supplementation to enhance or suppress performance was dependent upon the timing of supplementation. Current research supports the use of antioxidant supplementation to modulate RONS when athletes are at an increased risk of EIOS (Merry & Ristow, 2016, Figure 2.9, note, the beneficial effects of ROS on the Y axis lead to training adaptations), such as during intensified periods of exercise. Owens et al. (2019) have proposed a conceptual region on the hormetic curve (Figure 2.10) where antioxidant supplementation could be applied to target the negative effects of RONS to reduce muscle damage when recovery time is limited. However, antioxidant

supplementation is not recommended during periods where training adaptations are the key focus (Antonioni et al., 2019; Gomez-Cabrera et al., 2006; Harty et al., 2019; Radak et al., 2017; Ranchordas et al., 2017a; Owens et al., 2019).

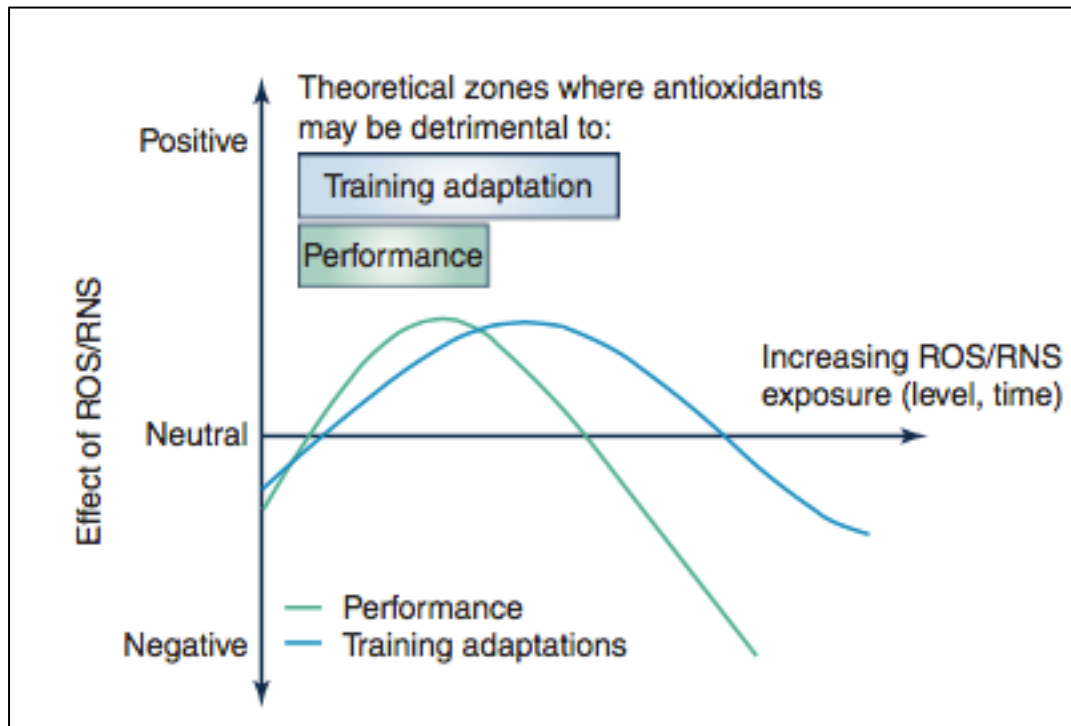


Figure 2.9. Reproduced from Merry & Ristow (2016) "Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training?" *Journal of Physiology*, 594 (18), p. 5143. Antioxidant supplementation is not recommended when training adaptation is the key focus.

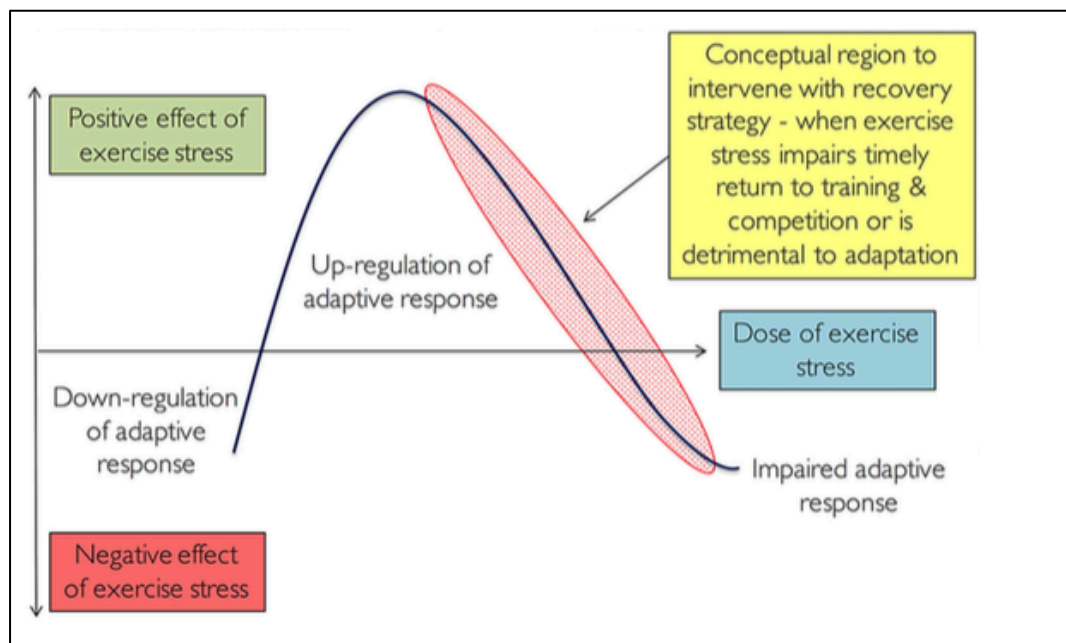


Figure 2.10. Reproduced from Owens et al. (2019) p. 80 "Exercise-induced muscle damage: What is it, what causes it, what are the nutritional solutions?" *European Journal of Sport Science*, 19 (1), p. 80. During intensified periods of exercise antioxidants could be administered in the conceptual region to reduce muscle damage.

2.9 Summary & Rationale

To summarise, the majority of literature has shown that the prolonged duration, high-intensity, intermittent nature of soccer type exercise induces a transient post-exercise increase in biomarkers of oxidative stress, muscle damage, and associated inflammation in trained athletes that is resolved with recovery. However, research investigating these responses following intensified periods of soccer has been limited, which is surprising, given athletes are unlikely to perform a single bout of soccer but rather train and compete daily across microcycles of a competitive season or throughout tournaments, providing a rationale for this thesis. Studying the magnitude and time course of these exercise-induced responses is vital, as current recovery periods may be insufficient to restore skeletal muscle homeostasis, and there may be potential implications for recovery that could impact subsequent

performances. Reducing oxidative stress, muscle damage, and associated inflammation to promote recovery is vital during this time and may provide performance improvements and success over opponents. Monitoring biomarkers of these exercise-induced responses are of interest, as these biomarkers could serve as specific targets to modulate with a recovery strategy, such as a nutritional intervention. The dietary flavonoid quercetin has demonstrated antioxidant and anti-inflammatory effects in humans and has been shown to be beneficial in prolonged aerobic exercise performed on repeated days and in eccentric exercise; therefore, it is postulated that quercetin supplementation may potentially support periods of intensified soccer by attenuating exercise-induced oxidative stress, muscle damage, and associated inflammation to facilitate recovery. Research investigating the efficacy of quercetin supplementation strategies in exercise has predominantly focused on endurance exercise. Only two studies to date have investigated quercetin supplementation in soccer-based exercise with equivocal results. Therefore, further research exploring the efficacy of quercetin supplementation in prolonged, high-intensity intermittent exercise, characteristic of soccer, is warranted.

Thus, the overall aims of the thesis were (i) to increase understanding of exercise-induced oxidative stress, muscle damage, and associated inflammation in trained athletes undertaking intensified periods of soccer-specific exercise, and (ii) to determine the benefit of prolonged quercetin supplementation to counteract the negative effects associated with these phenomena; specifically, through down-regulation of the NF- κ B classical pathway.

General Methods

General methods used to collect and analyse data are detailed in this chapter and are referred to in the *Methods* section of each of the three studies ((i) study one, chapter 4; (ii) study two, chapter 5; & (iii) study three, chapter 6).

3.1. Ethics, Informed Consent, & Health Screening

Ethics approval for each of the three studies was granted from the University of Hertfordshire's Ethics Committee (protocol numbers: (i) LS4/4/12P; (ii) aLMS/PG/UH/00164(3); (iii) aLMS/PGR/UH/02431(1)). Studies were conducted in accordance with the Declaration of Helsinki and ethical standards for sports science research (Harriss & Atkinson, 2011, 2015; Harriss, MacSween & Atkinson, 2017). Prior to participation in the studies, participants were issued a participant information sheet explaining the procedures in full. The lead investigator also briefed the participants verbally, and written informed consent was obtained. A health screen was completed prior to any exercise testing or invasive procedures (i.e., urine & blood sampling) set by the lead investigator. Additional verbal health checks were made prior to subsequent procedures following the initial health screen.

All laboratory testing was conducted in the temperature controlled Human Physiology Laboratory, University of Hertfordshire (84 m above sea level; ambient environmental conditions are displayed in Table 3.1); the lead investigator carried out data collection and analyses (unless stated).

Table 3.1

Laboratory Ambient Environmental Conditions

	Month	Temperature (°C)	Barometric Pressure (kPa)	Relative Humidity (%)
Study One	June	19.0 (\pm 2.0)	996 (\pm 8)	34 (\pm 9)
Study Two	July – Sept.	21.8 (\pm 1.5)	1013 (\pm 4)	29 (\pm 6)
Study Three	July – Sept.	21.6 (\pm 1.9)	1014 (\pm 9)	38 (\pm 8)

Note. Data presented as $M (\pm SD)$. kPa = kilopascal; Sept. = September.

3.2 Preliminary Physiological Testing

Preliminary physiological testing (including familiarisation procedures) was conducted a minimum of one-week prior to main data collection. Participants were asked to undertake testing in a hydrated state (i.e., (i) no alcohol consumption; (ii) limited caffeine intake; (iii) refraining from strenuous exercise [in the previous 24 hr]), following a period of 24 hr rest.

3.2.1 Anthropometric measures.

Anthropometric measures were conducted in accordance with standardised BASES testing guidelines (Winter, Jones, Davison, Bromley & Mercer, 2007) and the guidelines of the International Society for the Advancement of Kinanthropometry (2001).

3.2.1.1 Stature.

Stature was measured to the nearest 0.1 cm using a portable stadiometer (Model 225B, Seca, Hamburg: Germany). Participants were asked to stand on the footplate

in bare feet with heels together, heels, buttocks and upper back pushed against the measuring rod. Stature was recorded after participants undertook a maximal inhalation with the head in the Frankfurt plane hair compressed (Winter et al., 2007).

3.2.1.2 Body mass.

Body mass was measured to the nearest 0.1 kg using electrical column digital scales (Model 780, Seca, Hamburg: Germany). Participants wore minimal clothing (i.e., shorts & top/topless, bare feet). Following zeroing of the scales, participants were asked to stand in the middle of the scale with their body weight evenly distributed, facing forwards (Winter et al., 2007). Body mass was recorded.

3.2.1.3 Estimation of percentage body fat.

Two different methods were used to determine percentage body fat (%bf); chapter 4 used the method of bioelectrical impedance (choice of method dictated by the soccer club); chapters 5 and 6 used 7-site skinfold measurements (Jackson & Pollock, 1978; Jackson, Pollock, & Ward, 1980).

3.2.1.3.1 Bioelectrical impedance.

Percentage body fat was estimated via 8-electrode whole body bioelectrical impedance (Model BC-418, Tanita UK Ltd., Middlesex: UK [50 kHz; 500 mA; 150 – 1200 Ω ; range 4 – 55%bf]). Measurements were conducted according to the guidelines, with participants in a 3 hr fasted state, wearing minimal clothing, having voided their bladder. Participants were asked to stand barefoot on the metal footpads of the analyser and to hold the metal handgrips. The lead investigator ensured the inner thighs were not touching, nor their arms touching their sides. The

mode selected was group-dependent; (i) athletic mode selected for EXP group (> 10 hr intense exercise per week), (ii) standard mode for COM group (< 10 hr intense exercise per week). Percentage body fat was calculated to the nearest 0.1% using algorithms processed within the analyser. Percentage body fat measurements were accepted if total body water of participants was > 50% of total body weight (Van Loan & Boileau, 1996). Criterion validity of the Tanita Model BC-418 has previously been demonstrated ($r = .92$, body fat predicted from Dual-energy X-ray Absorptiometry; Kao et al., 2010). Test-retest reliability coefficient of variation (CV) determined from ten measurements on one male athlete was CV 0.4%. (Variance reported as CV %, using the equation: $CV = \sigma/\mu * 100$ [Hopkins, 2000]).

3.2.1.3.2 Skinfold measurements.

Percentage body fat was estimated via the skinfold caliper method (Harpenden skinfold calipers, Baty International, Sussex: UK [range 0 – 80 mm; pressure 10 g/mm²]). Skinfold thickness was measured at the following seven sites: (i) chest; (ii) triceps; (iii) subscapular; (iv) abdominal; (v) supriliac; (vi) mid-auxiliary, and (vii) thigh (Jackson & Pollock, 1978; Jackson et al., 1980). Site locations and measurements were as described (Winter et al., 2007). Participants were asked to avoid activities affecting blood flow or hydration (i.e., exercise, sauna usage, shower/bath) prior to measurement. Participants wore minimal clothing and stood in the anatomical position until instructed to move by the lead investigator (triceps, subscapular, mid-auxiliary, & thigh sites). Each skinfold site was located and marked using bony anatomical landmarks and a circumference tape measure (Seca 201 Ergonomic Circumference Measuring Tape, Seca, Hamburg: Germany). Measurements were performed on the right-hand side of the body after the calipers

were set to 0 mm and tips cleaned (70% isopropyl alcohol wipe [PDI® Healthcare, Orangeburg: NY]). Skinfold thickness of each site was recorded (nearest 0.2 mm) after full caliper pressure had been applied for 2 s. Participants were encouraged to relax throughout the procedure. Each site was measured non-consecutively (to avoid lead investigator bias & subcutaneous fat compression), two times (or 3 times if results of the first 2 measurements differed ± 2 mm; mean value recorded from duplicate measures, or median from triplicate measures). The lead investigator conducted all skinfold measurements (lead investigator intra-tester reliability was determined from repeated measurements on an energy-stable athlete on two separate occasions CV < 5%; ICC .987, 95% CI [.925, .998]). The sum of the skinfolds (mm) was used to predict body density using age- and sex-specific equations (Jackson & Pollock, 1978; Jackson et al., 1980; Equation 3.1). Body density was used to estimate percentage of body fat using the Siri equation (Siri, 1961; Equation 3.2). Results presented to 1dp.

(Equation 3.1)

Males:

$$BD = 1.112 - (0.00043499 \times \sum \text{skinfolds}) + (0.00000055 \times \sum \text{skinfolds}^2) - (0.00028826 \times \text{age})$$

Females:

$$BD = 1.097 - (0.00046971 \times \sum \text{skinfolds}) + (0.00000056 \times \sum \text{skinfolds}^2) - (0.00012828 \times \text{age})$$

Note. Body Density equations. BD = Body density ($\text{g}\cdot\text{mL}^{-1}$). Age- & sex-specific equations used to calculate body density from the sum of the skinfolds (Jackson & Pollock, 1978; Jackson et al., 1980).

(Equation 3.2)

$$\text{bf}\% =$$
$$([4.95 / \text{BD}] - 4.5) * 100$$

Note. The Siri equation (Siri, 1961). bf% = body fat percentage; BD = Body density ($\text{g}\cdot\text{mL}^{-1}$). The Siri equation was used to calculate percentage body fat from body density.

3.2.2 Determination of maximal oxygen uptake & maximum heart rate.

Following a standardised warm-up (10 min self-paced treadmill run; dynamic stretches), participants performed a continuous, graded exercise step test (Bruce-type protocol) to volitional exhaustion on a computer-controlled motorised treadmill (Quasar 5.0, with treadmill controller software, version 2.6.14, H/P/COSMOS Sports & Medical, Nussdorf-Traunstein: Germany [treadmill speed & incline were verified throughout testing, refer to section 3.3.2.2]). The initial test speed was set between $8.0 - 14.0 \text{ km}\cdot\text{h}^{-1}$ (participant-dependent), gradient was set at 1%; subsequently, gradient and speed were increased alternatively every minute (1% & $1.0 \text{ km}\cdot\text{h}^{-1}$); this protocol has previously been used with a cohort of elite soccer players (Malm et al., 2004). Variables calculated from pulmonary gaseous exchange ((i) volume of Oxygen consumption per minute [$\dot{V}\text{O}_2$]; (ii) volume of Carbon Dioxide output per minute [$\dot{V}\text{CO}_2$]; (iii) Respiratory Exchange Ratio [RER]) were recorded continuously throughout the test using an online breath-by-breath system (MetaLyzer 3BR2, in conjunction with Metasoft software, version 3.9.78R6 [Cortex Biophysik, Leipzig: Germany], facemask and headgear [Hans Rudolph, Kansas: USA]). Prior to testing, the MetaLyzer was warmed up (~45 min) and calibrated in accordance with the manufacturer's instructions (using a 3 L calibration syringe [Hans Rudolph, Kansas: USA] & a 2-point gas calibration using ambient air and a certified calibration gas [$\sim 17\% \text{ O}_2$; $\sim 5\% \text{ CO}_2$, Specialised Gases, Birmingham: UK]. Measurements of Rating

of Perceived Exertion (RPE; 6 - 20 scale; Borg, 1970) and heart rate (HR, heart rate monitor FT1 with T31 chest strap [0.2 Hz; range 15 - 240 beats·min⁻¹], Polar Electro Oy, Kempele: Finland) were recorded throughout the test (1 min intervals). Standardised verbal encouragement was provided every 15 s during the latter stages of the test (Andreacci et al., 2002). Maximum heart rate (HR_{max}) was recorded as the peak heart rate at the end-point of the test. Where required to meet maximal test criteria, a fingertip capillary whole blood sample was collected at the end of the test (within 1 min of cessation of exercise) and analysed for lactate concentration (refer to section 3.3.3.1). Tests were considered maximal if at least two of the following criteria were met: (i) $\dot{V}O_2$ plateau despite an increase in exercise intensity (< 2.1 mL·min·kg⁻¹ between stages); (ii) RER \geq 1.10; (iii) age- and sex-adjusted blood lactate accumulation \geq 7.0 mmol·L⁻¹ (females); \geq 9.0 mmol·L⁻¹ (males); and (iv) RPE 19/20 (Edwardsen, Hem & Anderssen, 2014; Howley, Bassett & Welch, 1995; Taylor, Buskirk, & Henschel, 1955). Maximal oxygen uptake was determined as peak $\dot{V}O_2$ (standardised temperature, pressure, dry [STPD]) at the end of the test, 30 s sampling period, and 10 s averaging applied (Poole & Jones, 2017).

Tests failing to meet maximal criteria were repeated until a maximal test was achieved. Participants inexperienced with maximal exercise testing and use of the RPE scale were required to undertake a familiarisation sub-maximal test first. Absolute $\dot{V}O_{2max}$ was expressed relative to body mass (STPD). MetaLyzer test-retest reliability was completed throughout testing (CV: $\dot{V}O_2 = 0.9\%$; $\dot{V}CO_2 = 1.8\%$; RER = 2.0%).

3.3 Main Experimental Testing

3.3.1 Instrumentation.

3.3.1.1 Overview of the SPI Pro System.

The SPI Pro System (GPSports, Canberra: Australia; referred to hereafter as *SPI Pro* [Figure 3.1]) is a GPS tracking device that consists of individual GPS receiver units (sampling rate: 5 Hz), integrated with tri-axial accelerometers (6 g; 100 Hz), heart rate sensors (0.2 Hz), and wireless transmitters, manufactured specifically to quantify movements of individual athletes in team sports. The SPI Pro utilises time-stamped GPS data to determine an athlete's position. Physical (external) loads of speed and distances covered can be quantified in conjunction with physiological (internal) loads based on heart rate. The SPI Pro sampling rate (5 Hz) can detect multiple changes in speed and direction; predominant movements in team sports. (A review on how GPS technology works is beyond the scope of this thesis; readers are referred to Larsson, 2003). The SPI Pro units are worn between the scapulae (thoracic vertebrae 2 - 4 of the spinal column) in purpose-built vests incorporating the GPS receiver unit and a heart rate monitor chest strap. At the time of data collection (2012 - 2013 season [study one, chapter 4]), the use of GPS units in professional sport was level-dependent; GPS systems could be worn in soccer training sessions; however, restrictions existed on the use of GPS units in professional soccer competition, hence, match data was not included in experimental chapter 4. The SPI Pro units log data that can be downloaded and analysed with Team AMS software (version 1.2, GPSports, Canberra: Australia) for a number of training load variables. The SPI Pro is one of the leading GPS team monitoring systems used in professional team sports (*note*. GPSports is now owned by Catapult).



Figure 3.1. SPI Pro unit & vest. The professional soccer players in chapter 4 wore the units & vests in all training sessions monitored.

Previous research has demonstrated test-retest reliability of the SPI Pro for sprint movements (Waldon, Worsfold, Twist, & Lamb, 2011). A pilot study, conducted by the lead investigator (similar to Waldron et al., 2011), further assessed (i) concurrent validity, (ii) intra-unit reliability, and (iii) inter-unit reliability of the SPI Pro. Following a familiarisation session, participants (male; $N = 8$; age: 20 ± 1 years; stature: 176 ± 0.1 cm; body mass: 73.8 ± 5.3 kg) were fitted with two SPI Pro units (worn simultaneously). The SPI Pro units were set up in accordance with the manufacturer's instructions (Aughey, 2011; Cummins et al., 2013; Jennings, Cormack, Coutts, Boyd, & Aughey, 2010; Waldron et al., 2011). Course outlines were marked by the lead investigator using a trundle wheel and cones (Figure 3.2; refer to section 3.3.1.1.1 for sprint protocol).

Following a standardised warm up, participants completed the WJR protocol. The WJR protocol is based on the Multi-Stage Fitness Test (Ramsbottom, Brewer, & Williams, 1988) and consists of repeated 20.0 m linear shuttles, designed to reflect intermittent sports activity patterns. The protocol is incremental in speed, starting at walking pace ($4.0 \text{ km}\cdot\text{h}^{-1}$), increasing to jogging ($9.0 \text{ km}\cdot\text{h}^{-1}$), and running ($11.0 \text{ km}\cdot\text{h}^{-1}$), every 200 m. To control inter-subject variability, pacemakers were present throughout the WJR protocol and used verbal encouragement alongside audible pacing beeps to ensure participants maintained the set speeds of the protocol and adhered to the course outline. (For a more detailed description of the WJR protocol readers are referred to Johnstone et al., 2012). Following the WJR protocol, participants performed three maximal 30.0 m sprints, with a minimum of three minutes passive recovery between sprints.

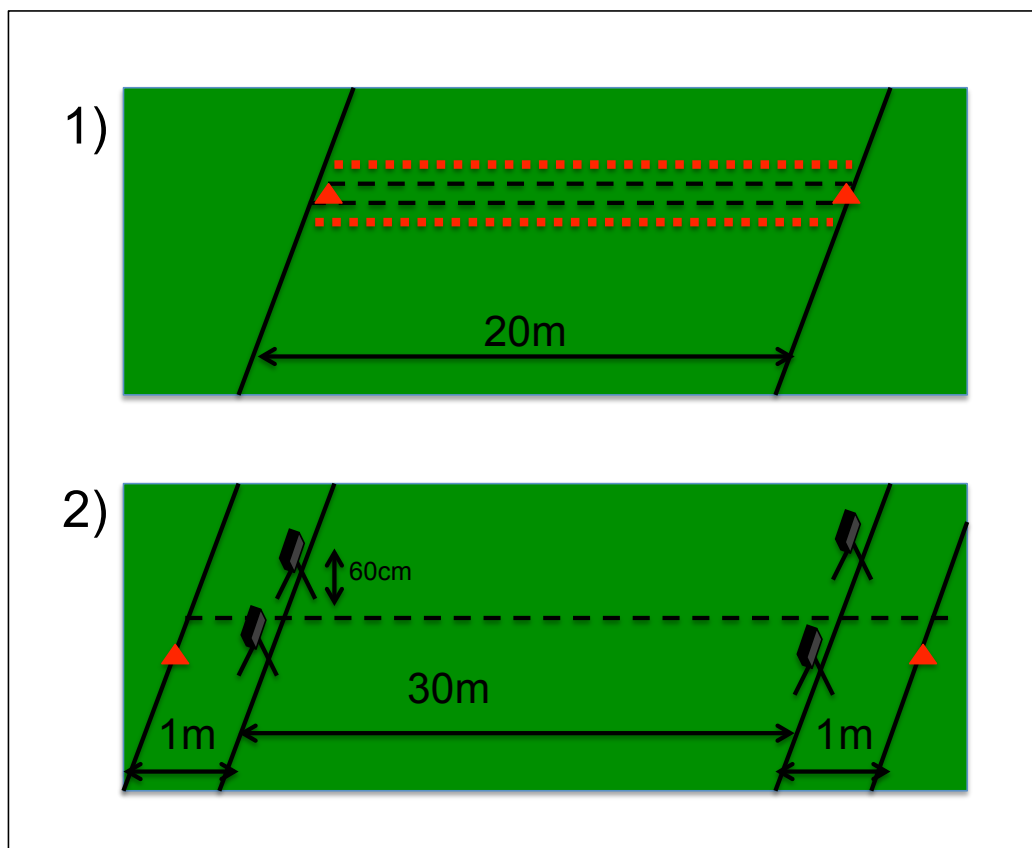


Figure 3.2. Outline of (1) the Walk-Jog-Run protocol & (2) 30 m linear sprint course used in the pilot study.

Mean sprint speed ($\text{m}\cdot\text{s}^{-1}$) was calculated (distance/time), and converted to $\text{km}\cdot\text{h}^{-1}$ for comparison with SPI Pro calculated speed ($\text{km}\cdot\text{h}^{-1}$) using the following equation: $\text{speed (km}\cdot\text{h}^{-1}) = \text{speed (m}\cdot\text{s}^{-1}) * 3.6$, (peak data used for analysis). To determine intra-unit reliability participants repeated the protocols 3 days later (ambient conditions: temperature 19.8°C ; pressure 99.6 kPa ; relative humidity 34% ; mean number of satellites detected: 10 ± 1). Global Positioning System data were downloaded and analysed with TeamAMS software for total distance covered during (i) each speed of the WJR protocol ($4.0 \text{ km}\cdot\text{h}^{-1}$; $9.0 \text{ km}\cdot\text{h}^{-1}$; $11.0 \text{ km}\cdot\text{h}^{-1}$) and (ii) 30.0 m sprint, and (iii) mean sprint speed. Validity and reliability were assessed using common analysis methods (i) 95% Limits of Agreement method (LOA [Bland &

Altman, 1986]); (ii) paired samples t-tests; (iii) typical error (expressed as CV, classification according to Coutts & Duffield, 2010: good < 5%; moderate 5 - 10%; poor < 10%); and (iv) bias (as per Waldron et al., 2011).

Results confirmed that the distances calculated by the SPI Pro agreed with measured distance, bar running (Table 3.2). SPI Pro measurements were slightly biased (1.54 - 6.8%), underestimating total distance covered during jogging, running, and sprinting, CV < 10%. The SPI Pro demonstrated agreement with the criterion measure (timing gates) for mean speed during 30.0 m linear sprinting (Figure 3.3), however, a significant difference was detected ($p \leq .05$) between measures. The SPI Pro underestimated 30.0 m mean sprint speed by $1.51 \pm 0.89 \text{ km}\cdot\text{h}^{-1}$; CV 2%.

The SPI Pro was found to be moderately reliable for all distance and speed measurements of the WJR protocol and 30.0 m sprint (CV < 10%) with all measurements within 95% LOA (Table 3.3 & 3.4). Significant differences were detected between SPI Pro units for jogging and running ($p \leq .05$).

Table 3.2

Concurrent validity of measured distance vs. SPI Pro distance (N = 16)

	Measured distance (m) ($M \pm SD$)	SPI Pro distance (m) ($M \pm SD$)	Bias (%)	CV (%)
Walking	200 \pm 0	202.9 \pm 6.1	1.44	2.9
Jogging	200 \pm 0	197.0 \pm 8.2	-1.54	3.8
Running	200 \pm 0	186.4 \pm 7.4*	-6.8	3.8
Sprint	30 \pm 0	28.5 \pm 3.4	-5.6	10.0

Note. CV = coefficient of variation. Pooled data from 2 units.

*Denotes significant difference between measured distance & SPI Pro distance, $p \leq .05$. * Adapted from Waldron et al. (2011).

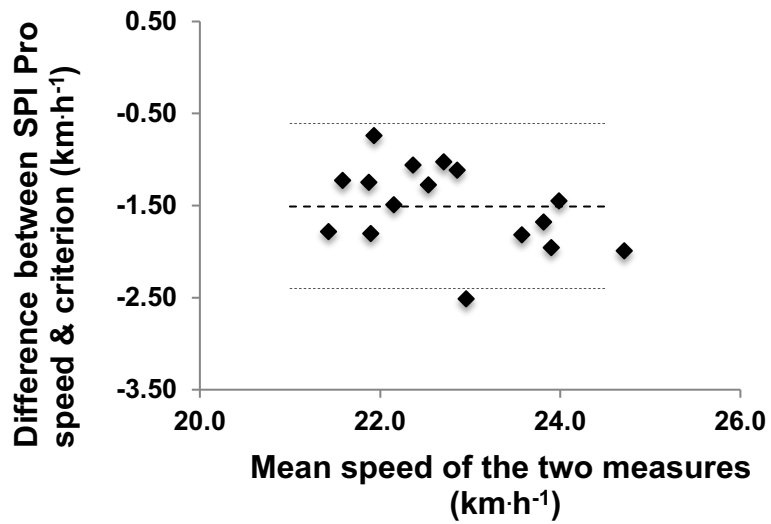


Figure 3.3. Bland-Altman plot of the difference in speed between the SPI Pro and criterion (timing gates). Dotted lines represent $M \pm 1.96 SD$ (95% LOA), $N = 16$ (pooled data from 2 SPI Pro units).

Table 3.3

Intra-unit reliability of distance and speed measured by the SPI Pro (N = 8)

	Trial 1 ($M \pm SD$)	Trial 2 ($M \pm SD$)	Mean difference \pm 95% LOA	CV (%)
Distance (m)				
Walking pace	202.9 \pm 6.1	204.4 \pm 0.4	1.48 \pm 8.23	2.1
Jogging	197.0 \pm 8.2	198.3 \pm 5.9	1.35 \pm 11.48	3.0
Running	186.4 \pm 7.4	185.4 \pm 9.7	-1.10 \pm 27.73	7.6
Sprinting	28.0 \pm 2.2	28.1 \pm 1.6	0.13 \pm 5.25	9.6
Speed (km·h⁻¹)				
Sprint	22.3 \pm 5.6	22.6 \pm 6.0	0.32 \pm 2.23	5.1

Note. CV = coefficient of variation; 95% LOA = Limits of Agreement. Adapted from Waldron et al. (2011).

Table 3.4

Inter-unit reliability of distance and speed measured by the SPI Pro (N = 8)

	SPI Pro unit 1 ($M \pm SD$)	SPI Pro unit 2 ($M \pm SD$)	Mean difference \pm 95% LOA	CV (%)
Distance (m)				
Walking pace	202.9 \pm 6.1	202.4 \pm 18.7	-0.5 \pm 29.69	8.0
Jogging	197.0 \pm 8.2	179.4 \pm 7.4**	-17.54 \pm 15.02	4.4
Running	186.4 \pm 7.4	176.0 \pm 9.1*	-10.33 \pm 21.24	6.4
Sprinting	28.0 \pm 2.2	28.4 \pm 2.4	0.57 \pm 5.39	9.8
Speed (km·h⁻¹)				
Sprint	21.6 \pm 0.7	22.1 \pm 0.8	0.47 \pm 0.60	1.4

Note. CV = coefficient of variation; 95% LOA = Limits of Agreement.

^a*Denotes significant difference between SPI Pro units, $p \leq .05$; ** $p \leq .01$. Adapted from Waldron et al. (2011).

High LOA (5 - 30 m) were reported for measured distance during shuttle running with directional changes (across all speeds). Systemic underestimation of distance during movements with a directional change is consistent with previous 5 Hz GPS research (Jennings et al., 2010) and could be attributed to the low sampling rate of the units (Waldron et al., 2011). The location of the SPI Pro units on the upper body or deviation from the course could also have been factors contributing to the underestimation of distance (Coutts & Duffield, 2010).

Taken together the results demonstrated < 10% typical error (classified as moderate validity & reliability; Coutts & Duffield, 2010) for measurements of speed and distance measured by the SPI Pro during shuttle running and linear sprinting, acceptable for quantifying team sport movements (Scott, Scott, & Kelly, 2016). Therefore, the 5 Hz SPI Pro system was used to monitor high-intensity running across repeated time points in experimental chapter 4 (study one). Due to observed inter-unit differences between SPI Pro units, athletes consistently wore the same unit (chapter 4). The 5 Hz SPI Pro was the GPS model available at the time of data collection of experimental chapter 4 (2012-13). Advancements in technology have led to GPS models with increased sampling rates (i.e., 10 & 15 Hz) demonstrating increased validity and reliability in comparison to 5 Hz GPS (readers are referred to the review by Scott et al., 2016).

3.3.1.1.1 Determination of 30.0 m sprint speed.⁴

To determine individual peak speed to calculate speed zones in chapter 4 (study one), experimental group participants completed a 30.0 m linear sprint test using timing gates while wearing the SPI Pro (as previously described by Waldron et al., 2011). The 30.0 m sprint course was measured and outlined by the lead investigator using a trundle wheel and cones. Timing gates were set at the start (0.0 m) and end of the course (30.0 m; height 0.6 m [Cronin & Templeton, 2008]), with additional cones placed 1.0 m before and after the start and finish lines (to ensure triggering of the gates and maximal sprinting (Cronin & Templeton, 2008; Waldron et al., 2011). Participants started in a stationary position so that the GPS did not detect any movement ($0.0 \text{ km}\cdot\text{h}^{-1}$), with the preferred foot positioned in line with the start cone (Waldron et al., 2011). Participants were instructed to avoid any backward movements and to start running at maximum speed when ready (Cronin & Templeton, 2008; Waldron et al., 2011). Sprint time over 30.0 m was measured concurrently with the timing gates and SPI Pro. Participants performed six maximal sprints, with a minimum of 3 minutes of active recovery between sprints. Time intervals recorded by the timing gates were used to calculate 30.0 m mean/peak speed ($\text{m}\cdot\text{s}^{-1}$) using the following equation:

speed ($\text{m}\cdot\text{s}^{-1}$) = distance (m) / time (s) (this data was requested by the fitness coach).

Raw GPS time-stamped speed data was downloaded from the SPI Pro units, and 30.0 m peak sprint detected ($\text{km}\cdot\text{h}^{-1}$).

⁴The terms *speed* & *velocity* are often used interchangeably, although velocity is a vector quantity. Both terms are used throughout this thesis in keeping with the literature.

3.3.1.2 Overview of the Drust protocol.

Assessing the physiological demands imposed on professional soccer players in an applied field setting (as in experimental chapter 4) increases ecological validity; however, is limited by constraints imposed on the research (management team, league rules; Drust et al., 2007). Thus, to further study the demands of soccer-specific intermittent exercise, researchers have developed soccer simulation protocols that replicate the movement patterns and physiological loading of soccer in (a) a controlled laboratory environment and (b) the field (Drust et al., 2007). Such protocols are categorised by Drust et al. (2007) as: (i) treadmill-based (motorised or non-motorised, e.g., the Drust protocol [Drust et al., 2000]); or (ii) field-based shuttle running (e.g., Loughborough Intermittent Shuttle Test [LIST, part A; Nicholas, Nuttall, & Williams, 2000]; Soccer Match Simulation [Russell, Rees, Benton, Kingsley, 2011]; Soccer Aerobic Fitness Test [SAFT⁹⁰; Lovell, Knapper, & Small, 2008]).

Devised by Drust et al. (2000), the Drust protocol is a laboratory-based, soccer-specific, high-intensity intermittent exercise protocol (HIIE), designed to reflect the work rate, thus physiological demands, of competitive match play in the controlled laboratory setting using a treadmill. The protocol replicates the external loading (i.e., total distance covered; frequency, intensity, & duration of intermittent bouts) and is based upon (a) motion analysis data from professional competitive matches observed by Reilly & Thomas (as cited in Drust et al., 2000), and (b) velocities observed during match play by Van Gool (as cited in Drust et al., 2000).

The protocol is performed on a motorised treadmill (in this thesis, an H/P/Cosmos Quasar 5.0 treadmill, H/P/Cosmos, Nussdorf-Traunstein: Germany) interfaced to a

computer with virtual controller software (H/P/Cosmos Paragraphics®, version 2.6.14), which runs the protocol automatically. The high-intensity intermittent nature of soccer is replicated through repeated rapid accelerations and decelerations involved in jogging, high-intensity running, and sprinting, interspersed with periods of walking and static recovery (Drust et al., 2007). A total of 47 bouts are performed at four different velocities. The duration, frequency, and velocity of bouts is as follows; (i) walking: 12 bouts @ 6.0 km·h⁻¹; (ii) jogging: 14 bouts @ 12.0 km·h⁻¹; (iii) cruising: 5 bouts @ 15.0 km·h⁻¹; and (iv) sprinting: 16 bouts @ 21.0 km·h⁻¹; periods of static recovery are also incorporated (3 bouts @ 0.0 km·h⁻¹). The duration of each bout ranges from 15 s to 2 min (increments of 30; 40; 45; 50 s; 1:05; 1:10; 1:25; 1:30; 1:45 min; Drust et al., 2000). High- and low-intensity bouts have been set in a randomised order to replicate the stochastic intermittent nature of the sport (Drust et al., 2007; Figure 3.4). The total distance covered during the protocol is 6.88 km, completed over ~46 minutes (representative of one half of a soccer match, including stoppage time [Drust et al., 2000, 2007]). Drust et al. (2000) have previously demonstrated the metabolic response of the protocol ($N = 7$; male; university soccer players) to be comparable to studies investigating the physiological demands of competitive match play in the field (Ekblom, 1986; Reilly, 1990; Van Gool et al., 1988, as cited in Drust et al., 2000). The results showed the protocol was performed at an average exercise intensity of 68% $\dot{V}O_{2\max}$ ($HR_{\text{mean}} 168 \pm 10 \text{ beats}\cdot\text{min}^{-1}$), which increased to 110% $\dot{V}O_{2\max}$ during high-intensity running and sprinting, with RPE recorded at the end of the protocol as 15 ± 2 . Blood lactate concentration sampled during recovery periods was $7.7 \pm 0.6 \text{ mmol}\cdot\text{L}^{-1}$, demonstrating the anaerobic component of the protocol. Based on previous work and pilot work from the lead investigator's laboratory, the top-end sprinting speed of 21.0 km·h⁻¹ is

difficult for trained athletes to achieve on the treadmill (even with a harness); therefore, a modified version of the protocol, with top-end sprinting speed reduced to $18.0 \text{ km}\cdot\text{h}^{-1}$, was used (Figure 3.2). Modified versions of the protocol with a reduced top-end speed have previously been used in research (Clarke, Drust, McLaren, & Reilly, 2008; Clarke et al., 2012; Taylor et al., 2014). Pilot work ($N = 3$) confirmed that the intensity of the modified version was comparable to the original, equating to $70.1 \pm 14.5\% \dot{V}O_{2\text{max}}$.

Advantages of using a motorised treadmill protocol to simulate soccer include (i) the fast acceleration and deceleration times permissible by the treadmill software and (ii) independence from the motivation of participants to achieve the set speeds (Drust et al., 2007); increasing ecological validity of the protocol as the duration of each bout is attained at the correct velocity. The main limitation of the protocol, which has been widely acknowledged (Drust et al., 2000; Drust et al., 2007; Silva et al., 2018), is the use of a treadmill that allows forward movements only. Additional soccer movements, such as, (i) backpedaling; (ii) sidestepping; (iii) multiple directional changes; (iv) jumping; (v) tackling; and (vi) ball play, are omitted (Drust et al., 2000; Drust et al., 2007; Silva et al., 2018). The protocol incorporates 92 velocity changes; however, over 1000 changes have been recorded in competitive match play (Mohr et al., 2015). Therefore, the overall loading of the protocol is lower than field-based soccer; thus, the metabolic and mechanical stress of the protocol may be underestimated in comparison. Drust et al. (2000) have previously demonstrated the metabolic loading of the protocol; however, there is a lack of studies validating the mechanical loading of the protocol or assessing reliability. Nevertheless, the Drust protocol is comparable to soccer in terms of external loading and metabolic response

and has been used in several studies as a model to assess (i) the efficacy of nutritional interventions on soccer performance (Clarke, Drust, McLaren, & Reilly, 2008; Clarke et al., 2012); (ii) thermoregulation (Taylor et al., 2014); (iii) the effects of soccer on salivary IgA (Sari-Sarraf et al., 2007); and (iv) fatigue (Rahnama, Lees, Reilly, & Graham-Smith, 2003). In their 2003 study, Rahnama et al. used the Drust protocol as a model to investigate the effects of soccer on neuromuscular fatigue in amateur male soccer players. The results demonstrated a significant progressive decrease in knee extension & flexion peak torque assessed via eccentric and concentric isokinetic dynamometry, indicating increased neuromuscular fatigue throughout the protocol, supporting the use of the protocol as a model to impair muscle function comparable to field-based soccer.

The rationale for using a laboratory-based protocol in experimental chapters 5 and 6 was to increase control of the environment and standardise potentially confounding variables (e.g., variance in external loading; standardised dietary intake). A laboratory-based protocol was also more practical given the invasive nature of the studies, which involved venous blood sampling. The primary rationale for using a motorised treadmill soccer-simulation protocol was based on standardising the external loading applied to participants. Alternatives include the use of a non-motorised treadmill protocol, which was not considered because (a) achievement of the high-intensity running velocities is dependent on the participants (Drust et al., 2007) and (b) this equipment was unavailable at the time of data collection. Similarly, field-based protocols were not considered because of (a) difficulties in ensuring participants achieve and maintain the velocities of the protocol, and (b) lack of GPS and HR equipment to monitor the intensity of the exercise in the field-based setting.

Therefore, the Drust was used as a model to simulate the demands of soccer to explore further exercise-induced muscle damage, oxidative stress, and inflammation.

3.3.2 Three-day period of intensified exercise.

In chapters 5 and 6 of this thesis, the modified Drust protocol (~45 min) was performed twice (90 min), with a 15 min recovery interval (total time 105 min), repeated on three consecutive days, and is referred to forthwith as *a 3-day period of soccer-specific intensified exercise*. The rationale for performing the protocol on three repeated days was to simulate the physiological demands imposed on professional soccer players within microcycles of a competitive in-season, where athletes are required to train and compete across consecutive days (Malone et al., 2015b) with minimal recovery periods. Previous research has primarily explored biochemical responses following single bouts of soccer-specific exercise (Mohr et al., 2015), there is paucity in the literature investigating responses following repeated bouts of soccer-specific exercise performed on consecutive days. At present, the cumulative effects of daily training and competitive match play are unclear and may have implications for recovery and subsequent performances. The three day design of the experiment was based on data from chapter 4, where the lead investigator observed the following within a two match weekly microcycle; (i) the duration of training sessions to be ~90 min; (ii) a mid-week recovery day; (iii) two training days preceded the competitive match played on a Saturday; and (iv) a 42 hr recovery period followed the Saturday competitive match (refer to Figure 4.1). Therefore, use of the Drust protocol was as a model to simulate two consecutive training sessions followed by a competitive match as part of a weekly in-season microcycle (i.e., (i) match; (ii) match day -1; (iii) match day -2). Whilst the Drust protocol was originally

designed to simulate the physiological load imposed on players during match play (Drust et al., 2000). Data on between match training loads has been limited (Malone et al., 2015a; Stevens, Twisk, Savelsbergh, & Beek, 2017). The load of training should replicate the demands of competition (Iaia et al., 2009), hence, the use of the protocol to also simulate training sessions. The lead investigator fully acknowledges that training load periodisation is club and playing level dependent. The 3-day schedule and 42 hr recovery period observed in chapter 4, and replicated in chapters 5 and 6, may be unique to the League One club observed in chapter 4 and may not be representative of other clubs or playing levels, where tapering, and strength and conditioning sessions may be included.

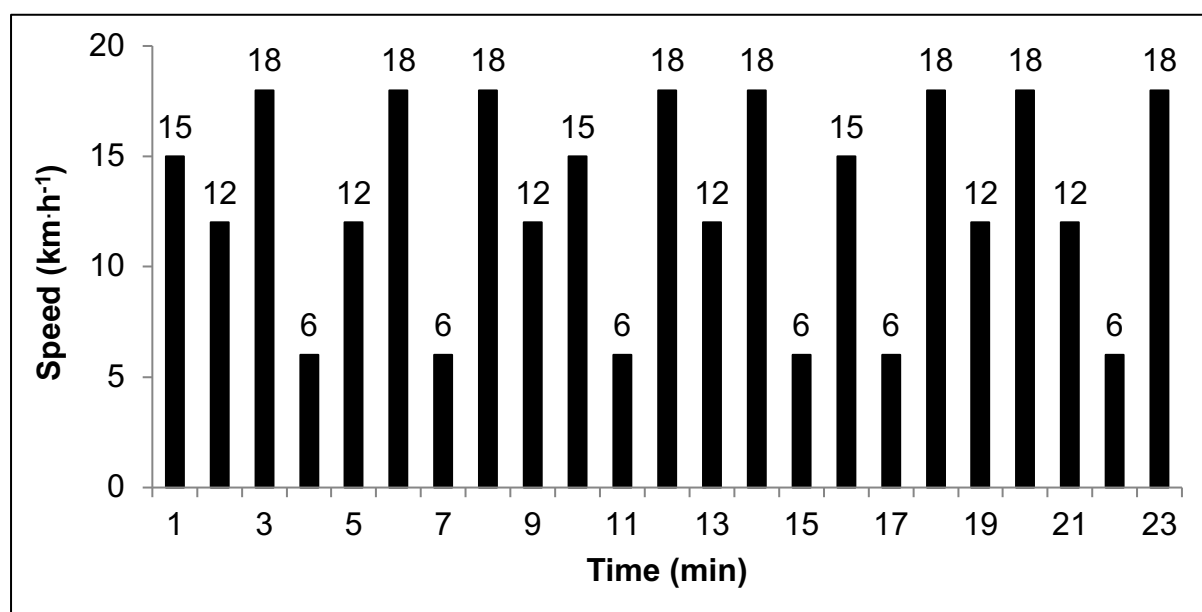


Figure 3.4. Exemplar of part of the modified Drust Protocol. Reproduced from “Physiological responses to laboratory-based soccer-specific intermittent and continuous exercise”, by Drust et al. (2000), *Journal of Sports Sciences*, (11), p. 887. The protocol was used during chapters 5 & 6.

3.3.2.1 Familiarisation session.

Participants attended the laboratory for a familiarisation session to confirm that the treadmill speeds of the protocol were achievable. Following a standardised warm-up (10 min, self-paced, sub-maximal running & lower limb, dynamic stretch routine), participants completed a 15 min block of the Drust protocol, experiencing the different speed changes (6; 12; 15; 18 km·h⁻¹).

3.3.2.2 Three-Day soccer-specific prolonged high-intensity intermittent exercise protocol.

Participants reported to the laboratory following a 24 hr rest period, were fitted with a heart rate monitor (FT1, with T31 chest strap) and followed a standardised warm-up (10 min self-paced, sub-maximal running & lower limb, dynamic stretch routine), before completing the exercise protocol with the lead investigator present. Heart rate and RPE were used to verify the intensity of the workload. Heart rate was recorded continuously throughout the protocol (1 Hz) using H/P/Cosmos Paragraphics® software. Overall RPE (chapters 5 & 6), and differential RPE-Legs (RPE-L, Borg's 15 point scale [Borg, 1998; Field et al., 2020; Pandolf et al., 1982] chapter 6) were recorded throughout the protocol at 15 min intervals during bouts of walking. Water was permitted ad libitum throughout the 90 min protocol, with the amount recorded by the lead investigator (no other food or drink was permitted). Following completion of the protocol, participants recovered by walking self-paced on the treadmill until a heart rate ≤ 120 beats·min⁻¹ was achieved. Participants repeated the exercise protocol on the next two days (3 consecutive days), at approximately the same time of day (~24 hr recovery intervals).

Treadmill belt speed was verified throughout each study across a range of speeds (8.0, 10.0, & 12.0 km·h⁻¹) as per the manufacturer recommendations. In brief, the length of the treadmill belt was first measured (mm) and a line painted across the treadmill belt. Each speed was then selected, and the lead investigator recorded the duration of time (s) for ten belt revolutions using a stopwatch (Fastime 14, AST Ltd., Leicester: U.K.). This procedure was repeated with a participant running on the belt (69.5 kg). Verified speeds were calculated using the following equation:

belt length (mm) / number of revolutions of belt / time (s) x 0.36 (mean of 3). Verified speeds were within the manufacturer's range of tolerance, $\pm 5\%$ (Table 3.5). Treadmill gradients (0, 10, & 20%) were also verified as per the manufacturer's instructions (for use in $\dot{V}O_2$ max testing). In brief, the distance between the base of the front of the treadmill and the floor was measured (mm) with a precision metre rule (CMS Weighing Equipment LTD, Camden: U.K.). Verified distances for each gradient were within the manufacturer's range of tolerance (data not shown).

Table 3.5

Exemplar of Treadmill Verification

Treadmill Speed (km·h ⁻¹)	Verified speed (km·h ⁻¹)	Verified speed (km·h ⁻¹) with 69.5 kg participant
8.00	8.09	8.04
10.00	10.09	10.20
12.00	12.10	12.31

Note. Laboratory environmental conditions during verification ($M \pm SD$): 20.9 \pm 1°C; 42 \pm 6%; 99.9 \pm 6 kPa.

3.3.3 Blood sampling & handling.

During each study of the thesis, capillary blood was collected for lactate analysis using a micro-method (end criteria for maximal testing). Venous blood was collected throughout chapters 5 and 6. Blood-sampling procedures were standardised and conducted by the lead investigator. Prior to blood sampling, equipment was checked for sterility.

3.3.3.1 Capillary blood sampling, including lactate analysis.

Capillary whole blood was sampled from the lateral side of a selected fingertip (index, middle or ring finger of the non-dominant hand [without rings on]), using a single-use, sterile, disposable lancet (set to a depth of 2.3 mm, [Accu-chek Safe T Pro Plus Disposable Lancet, Accutrend®, Roche Diagnostics, West Sussex: UK]) with the participant in a standing position. In brief, the puncture site was cleansed with 70% isopropyl alcohol (PDI® Healthcare) and left to air-dry, the site punctured (in case of haemodilution/haemoconcentration/contamination the first 2 drops of blood were discarded), and a continuous blood sample (10 μL) collected into a sodium heparin plastic capillary tube (EKF Diagnostics, Cardiff: UK) without air bubbles. Immediately post blood collection, the lancet was disposed into a sharps container and pressure applied to the puncture site to promote clotting. The sample was mixed with 500 μL haemolysing solution (prefilled sample cup, EKF Diagnostics) to rupture erythrocytes. Samples were then analysed for lactate using an automated analyser (Biosen C_Line Clinic, EKF Diagnostics; range 0.50 - 40.0 $\text{mmol}\cdot\text{L}^{-1}$; calibrated in accordance with the manufacturer's instructions). Results expressed in $\text{mmol}\cdot\text{L}^{-1}$. Prior to testing, quality control (QC), sensor tests, and linearity checks were performed (analytical variance determined from ten replicates of QC material (Readycon Norm, EKF Diagnostics): $\text{CV} < 1\%$).

3.3.3.2 Venous blood sampling.

Blood samples were collected by the lead investigator (qualified phlebotomist) using a 21G Vacuette® Safety Blood Collection Set, with Vacuette® short tube holder, in conjunction with 4 mL Serum Separator Clot Activator Vacuette® tubes, 4 mL Lithium Heparin Vacuette® tubes and 4 mL di-potassium Ethylenediaminetetraacetic acid (K2EDTA) Vacuette® tubes (Greiner Bio-One GmbH; Kremsmunster: Austria). In brief, venous blood was collected from an antecubital vein (preferred vein; or basilic/cephalic veins), with participants in a standardised position (seated in the upright high Fowler position, the selected arm supported by a pillow), following a period of 15 min rest. To occlude venous flow, a tourniquet was applied 3 - 4 inches above the vein of choice. The site of insertion was cleansed with 70% isopropyl alcohol (PDI® Healthcare) and left to air dry. The needle was inserted into the chosen vein in one smooth motion (bevel up; ~30° angle). Samples were drawn in the following order: (i) serum; (ii) Lithium Heparin; (iii) K2EDTA, with the tourniquet released between the second and third blood collection tubes (2 min maximum tourniquet application). After blood collection, the needle was removed, safety lock activated, and the needle immediately disposed into a sharps container. To promote clotting of the puncture site cotton wool was held over the puncture site. To thoroughly mix the blood with the anticoagulant/serum clotting activator, blood collection tubes were mixed manually eight times using back and forth wrist inversion.

3.3.3.3 Blood processing & storage.

Serum tubes were left to clot (30 min; 21°C); following clotting, samples were centrifuged (4,000 rpm, radius 7 cm; 10 min [Hettich® EBA 21 centrifuge, Hettich UK, Manchester: UK]) and 0.5 mL serum aliquots stored at -80°C for batch analysis of analytes. Samples were deemed void if haemolysed. Serum was preferable to plasma as anti-coagulants can interfere with certain assays. Freezer storage time for each analyte was dependent on the assay manufacturer's recommendations for stability of the analyte.

Aliquots of K2EDTA whole blood (80 µL; 10 µL; 10 µL), were analysed immediately for: (i) haematocrit (Hct); (ii) haemoglobin (Hb); and (iii) total leukocyte counts, including differentials (specifically, total neutrophil counts), respectively.

Heparinised whole blood was used to isolate peripheral blood mononuclear cells (predominantly monocytes & lymphocytes) by the method of density gradient centrifugation. In brief, heparinised blood was transferred into 12 mL Leucosep™ tubes with porous barrier (density: 1.077 g·mL⁻¹; Greiner Bio-One GmbH) and centrifuged (10 min; 20°C; 1,000 g, swing out rotor centrifuge with soft brake applied [Sorvall™ Legend™ X1, Thermo-Fisher Scientific, Hertfordshire: UK]). The buffy layer (PBMC; Figure 3.5) was harvested and washed three times (10 min; 20°C; 250 g) with Phosphate Buffered Saline (PBS; [pH 7.4]). Peripheral Blood Mononuclear Cell pellets were isolated, re-suspended (5 mL PBS) and stored at -20°C for the determination of NF-κB p65 activation and nuclear protein quantification. Samples were deemed void if haemolysed.

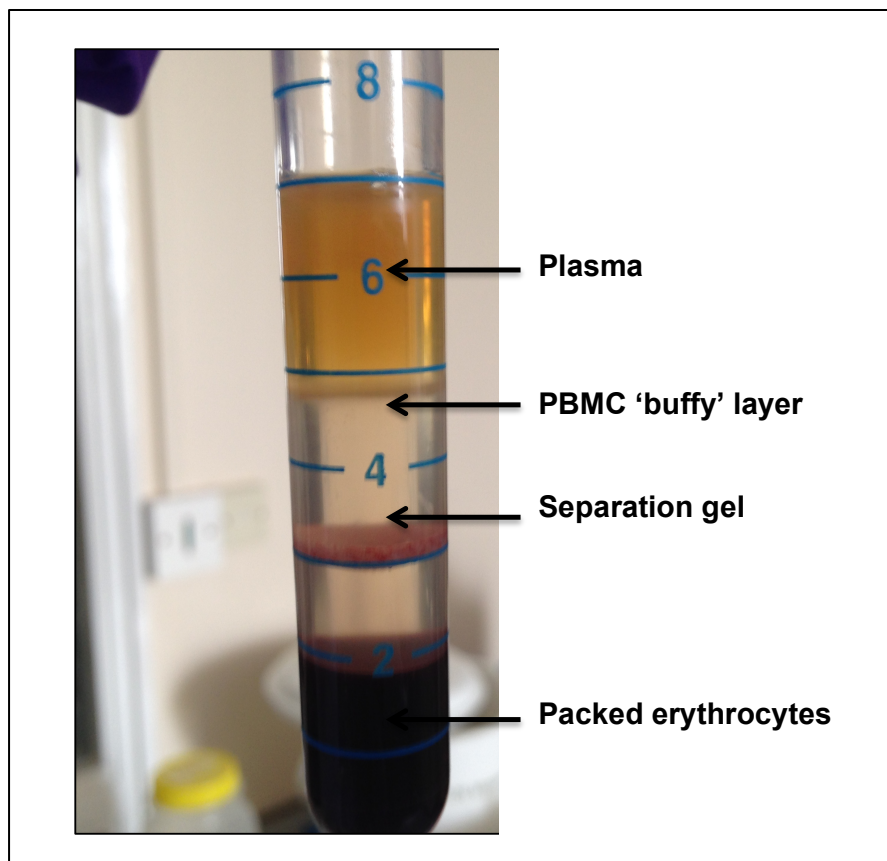


Figure 3.5. Blood separation by density gradient centrifugation. The buffy layer was isolated from peripheral venous blood & used for NF- κ B p65 activation & nuclear protein quantification.

3.3.4 Analytical methods.

The method of analysis for each biomarker is detailed below. Standardised procedures were used to reduce variance. For example, all assays were performed by the lead investigator, excluding (i) Inflammatory Cytokine Array (chapter 5), and (ii) IL-6 and full range CRP (chapter 6), which were analysed at Randox Health in London with the lead investigator present. Prior to analysis, the lead investigator's pipetting accuracy was determined from ten duplicate replicates of distilled water measured at 20°C (range: 10 - 500 μ L; CV < 5%). Analytes in serum were analysed in batches (with the exclusion of TAS). Prior to batch analysis, samples were thawed to room temperature (one freeze thaw cycle only) and vortexed briefly (Fisons

Whirlimixer™, Fisher Scientific Ltd., Loughborough: UK). Chemical reagents were equilibrated to room temperature prior to use. Assays were performed on the same day, using the same batch of reagents and the same analyser. For microplate analysis, each athlete's samples (i.e., pre, post, recovery samples [chapters 5 & 6]) were analysed on the same plate. Samples were analysed in duplicate (cost & sample volume permitting). Standard concentration curves were accepted if the correlation coefficient was $r \geq .8$. Accuracy and precision were checked through the use of QC and standard material. Analytical variance calculated from ten duplicate samples of QC. Spiked samples were also used to check recovery of analytes. Freezer stability checked using recovery of QC. Intra-individual (intra-assay) variance was calculated for each assay to assess repeatability by measuring ten duplicate replicates of a common blood sample in one run. Inter-individual variance (inter-assay) was calculated from duplicates of ten different blood samples.

3.3.4.1. Determination of MDA in serum.

A by-product of lipid peroxidation, Malondialdehyde (MDA), was used to measure oxidative stress levels in serum (chapters 5 & 6). Total MDA were assessed using a standardised 2-Thiobarbituric Acid Reactive Substances (TBARS) spectrophotometric method (Ohkawa, Ohishi, & Yagi, 1979; Yagi, 1976), via a commercially available assay kit (Quantichrom™ TBARS Assay Kit, DTBA-100, Bioassay Systems, Hayward: California [detection limit 1.0 μM ; linearity 30.0 μM]). The TBARS method is based on the principle that one molecule of MDA reacts with two molecules of 2-Thiobarbituric Acid under acidic conditions, (Knoevenagel-type condensation), forming a pink chromogen (Figure 3.6). To summarise, 100 μL serum samples were reacted with 200 μL ice cold 10% trichloroacetic acid, incubated on ice

(5 min) and centrifuged (5 min, 14,000 rpm, radius 6 cm [Anachem, Hyspin 16K, Anachem, Luton: U.K.]). The clear supernatant was harvested (200 μ L), reacted with 200 μ L 2-Thiobarbituric Acid (TBA) reagent in screw capped tubes, vortexed (Fisons Whirlimixer, Fisher Scientific Ltd.), and heated (100°C; 60 min) using a dry heat mantle (DB-3, Techne, Cambridge: UK). Tubes were allowed to cool down, briefly vortexed and centrifuged. Samples (100 μ L) were pipetted into wells of an uncoated 96 well plate and the absorbance of the resultant pink adduct was measured at 540 nm using a microplate reader (Multiskan™, FC Microplate Photometer, ThermoFisher Scientific, MA: USA). To control for background interference, samples were blanked against a reference well containing dH₂O, and net absorbance calculated (sample absorbance – blank absorbance = net absorbance). Total MDA concentration of the samples was calculated using the regression equation generated from an 8-point standard MDA concentration curve (MDA concentrations: 0.0; 0.5; 1.0; 2.5; 5.0; 10.0; 15.0; 20.0 μ M; prepared from a 6 M MDA standard diluted with dH₂O; Figure 3.7). Samples were assayed in duplicate replicate (analytical variance 18.7%; intra-assay variance 35.2%; inter-assay variance 38.1%). Post-exercise sera concentrations were controlled through normalisation of MDA to plasma volume concentrations (refer to section 3.3.4.9). The concentration of total MDA was expressed as MDA equivalents in μ M.

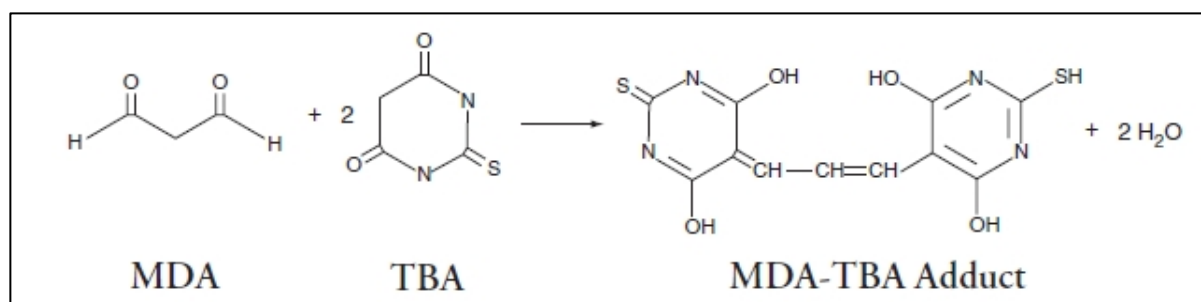


Figure 3.6. Reaction between Malondialdehyde (MDA) and 2-thobarbituric acid (TBA). The reaction forms a pink MDA-TBA adduct which can be analysed spectrophotometrically at 532 nm. FR35 TBARS Cuvette Assay Kit insert. Copyright Oxford Biomedical Research Inc., with permission.

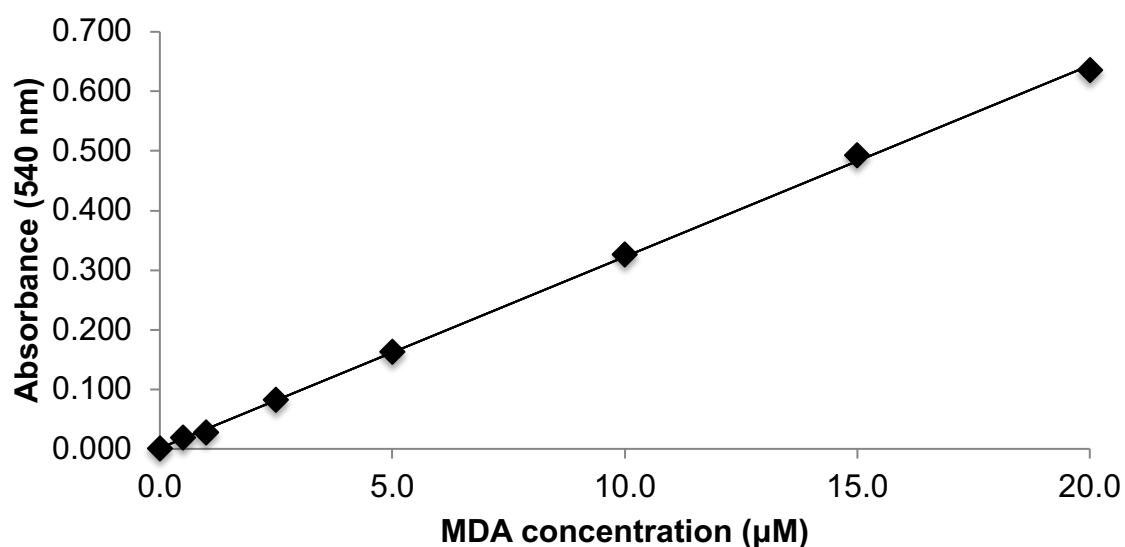


Figure 3.7. Exemplar scatterplot of standard Malondialdehyde (MDA) concentration curve. The regression equation was used to calculate total MDA in serum samples.

3.3.4.2 Determination of Total Antioxidant Status.

Serum samples were analysed for Total Antioxidant Status (TAS) using the 2, 2'-Azino-di-(3-ethylbenzthiazoline sulphonate) method (ABTS[®], alternatively known as Trolox equivalent antioxidant capacity) which is an end-point assay that is based on the principle that antioxidants inhibit the oxidation of ABTS[®] to radical cation ABTS^{®*} (Miller, Rice-Evans, Davies, Gopinathan, & Milner, 1993). This process

involves reacting metmyoglobin with hydrogen peroxide to form ferrylmyoglobin; ferrylmyoglobin incubated with ABTS[®] produces the radical cation ABTS^{®*+}, which is a stable blue-green colour that can be measured spectrophotometrically (600 nm; Figure 3.8). Antioxidants, if present within the sample, suppress the blue-green colour, proportional to concentration (Figure 3.9).

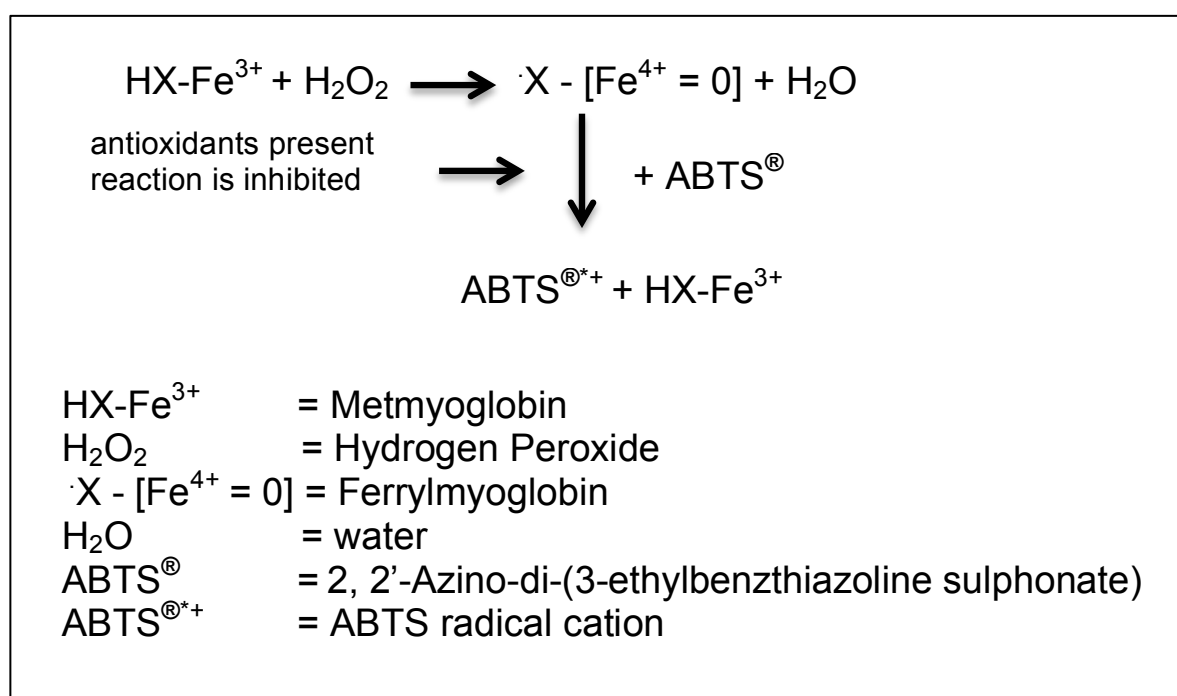


Figure 3.8. Principle of the ABTS[®] method. The method was used to analyse Total Antioxidant Status in serum; reproduced from Randox TAS kit insert.

Samples were analysed using a commercially available assay kit (Total Antioxidant Status NX2332, Randox Laboratories Ltd., Co. Antrim: Northern Ireland [linearity 2.5 mmol·L⁻¹]), in conjunction with a semi-automated spectrophotometer in cuvette mode (RX Monza™, Randox Laboratories Ltd.). Samples were analysed seven days from date of collection (in accordance with the manufacturer's instructions serum for TAS analysis can only be stored frozen for up to 14 days). First, reagents chromagen R2

and substrate R3 were prepared (Table 3.6). A gain calibration was then performed in cuvette mode to check the photometer. For sample analysis, 20 μL of serum was reacted with 1,000 μL of chromagen R2 reagent in a semi-microcuvette, placed immediately into the RX Monza™ cuvette holder to incubate (10 s; 37°C) and initial absorbance (A_1) read (600 nm). Substrate R3 reagent (200 μL) was then added to the semi-micro-cuvette, the sample mixed and absorbance (A_2) read after a 3 min incubation period (37°C; 600 nm). Change in absorbance was calculated ($\Delta A = A_2 - A_1$).

Reagent	Reagent Composition	Concentration	Amount
Chromagen R2 Reagent	PBS Metmyoglobin ABTS®	80.0 mmol·L ⁻¹ 6.1 $\mu\text{mol}\cdot\text{L}^{-1}$ 610.0 $\mu\text{mol}\cdot\text{L}^{-1}$ pH 7.4	10 mL Dry enzyme Dry enzyme
Substrate R3 Reagent	PBS Hydrogen Peroxide	80.0 mmol·L ⁻¹ 255.0 $\mu\text{mol}\cdot\text{L}^{-1}$ pH 6	1.5 mL 1 mL

Note. PBS = Phosphate Buffered Saline.

Total Antioxidant Status was calculated automatically (Equation 3.3) from a 2-point standard calibration curve (Figure 3.10) generated using (i) double distilled water (ddH₂O) as a reagent blank, and (ii) a standard calibrator (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid [concentration 2.33 mmol·L⁻¹]). Blanks and standards were analysed using the same protocol as samples. Samples, standards

and blanks assayed in duplicate (analytical variance CV 6.8%; intra-assay variance CV 8.5%; inter-assay variance CV 22.2%), results expressed in $\text{mmol}\cdot\text{L}^{-1}$ of Trolox equivalents (reported as $\text{mmol}\cdot\text{L}^{-1}$ hereafter within this thesis). Post-exercise samples were corrected for plasma volume change (refer to section 3.3.4.9).

(Equation 3.3)

$$\text{Factor} = \frac{\text{concentration of standard}}{(\Delta A \text{ blank} - \Delta A \text{ standard})}$$

$$\text{TAS (mmol}\cdot\text{L}^{-1}) = \text{Factor} \times (\Delta A \text{ blank} - \Delta A \text{ sample})$$

Note. A = absorbance.

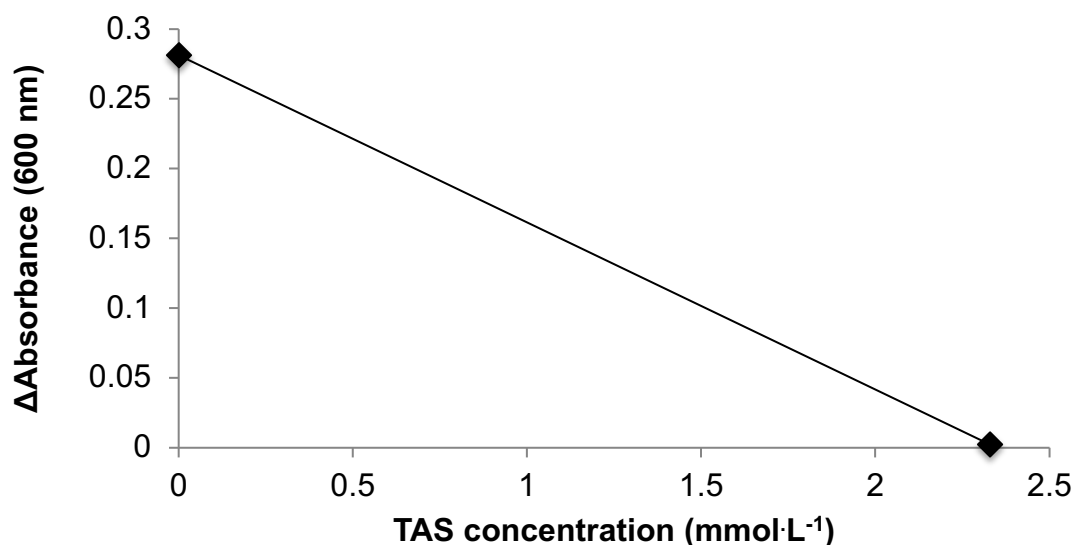


Figure. 3.10. Exemplar scatterplot of Total Antioxidant Status (TAS) standard concentration curve. Serum TAS concentration was automatically calculated using the equation generated from the above curve.

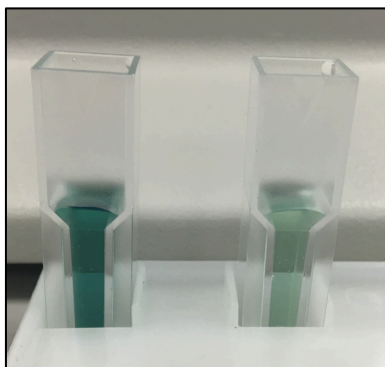


Figure 3.9. Antioxidant decolourisation of ABTS[®] in the TAS assay. The TAS assay was used in chapters 5 & 6.

3.3.4.3 Determination of Creatine Kinase.

Serum samples were analysed for Creatine Kinase (CK) as a biomarker of muscle damage, using a commercially available assay kit (CK110, Randox Laboratories Ltd [sensitivity 21.7 U·L⁻¹; linearity 2804.0 U·L⁻¹]) in conjunction with the RX Monza™ in flow cell mode. This method utilises the standardised kinetic method for CK activity recommended by the German Society for Clinical Chemistry (1972), and is based on a modified version of the original *N*-acetyl-*L*-cysteine (NAC) method developed by Oliver (1955). Creatine Kinase activity is determined by a coupled enzyme reaction (Figure 3.11): (i) Creatine Kinase catalyses Creatine Phosphate and ADP to produce ATP; (ii) ATP phosphorylates glucose to glucose-6-phosphate (G-6-P) via Hexokinase; (iii) G-6-P is oxidised by glucose-6-phosphate dehydrogenase (G-6-PDH), and (iv) simultaneously, Nicotinamide Adenine Dinucleotide Phosphate (NADP) is reduced to Nicotinamide Adenine Dinucleotide Phosphate Reduced (NADPH). The rate of NADPH production is directly proportional to CK present in the sample, measured at 340 nm.

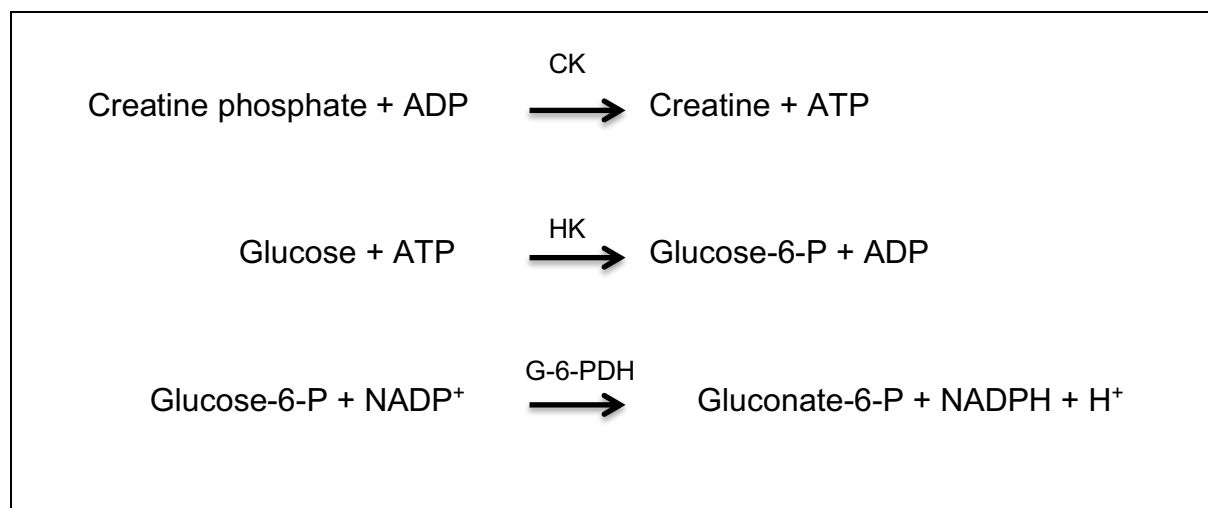


Figure 3.11. Principle of the Creatine Kinase (CK) assay. ADP = adenosine diphosphate; ATP = adenosine triphosphate; Glucose-6-P = glucose-6-phosphate; G-6-PDH = glucose-6-phosphate dehydrogenase; NADP = Nicotinamide Adenine Dinucleotide Phosphate; H = Hydrogen; NADPH = Nicotinamide Adenine Dinucleotide Phosphate Reduced. This method was used to analyse CK present in sera. Reproduced from Randox CK110 kit insert.

Creatine Kinase reagents were first prepared in accordance with the manufacturer's instructions; R1B reagent was reconstituted with 2.5 mL R1A reagent (Table 3.7), and the RX Monza™ calibrated in accordance with the manufacturer's instructions. First, a gain calibration was performed in flow cell mode to check the photometer. A 2-point calibration was then performed using: (i) 10 µL saline (as a standard blank); and (ii) 10 µL universal standard (CK concentration ~535 U·L⁻¹ [Randox Calibration Sera Level 3, CAL2351, Randox Laboratories Ltd.]). Standards were reacted in test tubes with 500 µL of reconstituted R1B reagent, aspirated into the RX Monza™ to incubate (37°C; 60 s), and absorbance read (after 1, 2, & 3 min; 340 nm), and a standard concentration curve generated (Figure 3.12); standards assayed in duplicate.

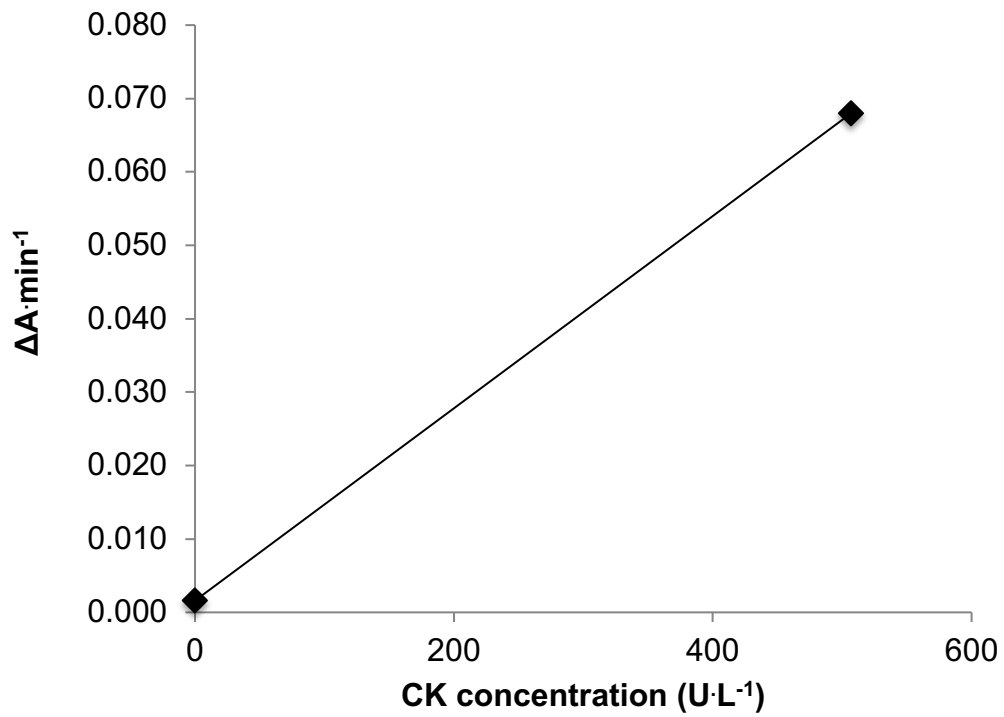


Figure 3.12. Exemplar scatterplot of Creatine Kinase (CK) standard concentration curve. Serum CK concentration was automatically calculated using the regression equation generated from the curve in chapter 5. A = absorbance.

Reagent	Reagent Composition	Concentration	Amount
R1A reagent Buffer/glucose	Imidazole buffer Glucose Mg-acetate EDTA	0.1 mmol·L ⁻¹ ; pH 6.7 20.0 mmol·L ⁻¹ 10.0 mmol·L ⁻¹ 2.0 mmol·L ⁻¹	2.5 mL
R1B reagent Enzymes/coenzymes/substrates	ADP AMP Diodenosine pentaphosphate NADP HK G-6-PDH N-acetylcysteine Creatine Phosphate	2.0 mmol·L ⁻¹ 5.0 mmol·L ⁻¹ 10.0 mmol·L ⁻¹ 2.0 mmol·L ⁻¹ ≥ 2.5 U·mL ⁻¹ ≥ 1.5 U·mL ⁻¹ 20.0 mmol·L ⁻¹ 30.0 mmol·L ⁻¹	Dry enzyme

Note. ADP = Adenosine phosphate; AMP = Adenosine monophosphate; EDTA = Ethylenediaminetetracetic acid; Mg-acetate = Magnesium-acetate; G-6-PDH = Glucose-6-phosphate dehydrogenase; HK = Hexokinase; NADP = Nicotinamide Adenine Dinucleotide Phosphate Reduced. N-acetyl-L-cysteine (NAC) is present in the reaction as an enzyme reactivator to increase stability of the assay. Adenosine-5'-monophosphate and diadenosine pentaphosphate are present to suppress interference from myokinase activity.

In brief, the assay procedure for samples (10 μL serum) was as per standards. Creatine Kinase concentration was calculated from the regression equation generated by the standard curve (Figure 3.12) and calculation: $8095 * \Delta\text{absorbance}$. Samples assayed in duplicate (analytical variance CV 11.8%; intra-assay variance CV 15.8%; inter-assay variance CV 51.0%). For consistency with the literature, results were expressed in conventional units, $\text{U}\cdot\text{L}^{-1}$ (International Units per litre; the amount of enzyme activity required to catalyse 1 μM of phosphate from Creatine Phosphate to ADP per minute, under specified temperature and pH). Post-exercise samples corrected for plasma volume change (refer to section 3.3.4.9).

3.3.4.4 Determination of Lactate Dehydrogenase.

Serum samples were analysed for Lactate Dehydrogenase (LDH) using a semi-automated spectrophotometer (RX Monza™) in flow cell mode, in conjunction with a commercially available assay kit (Lactate Dehydrogenase P-L, Randox Laboratories Ltd., [sensitivity $55.1 \text{ U}\cdot\text{L}^{-1}$; linearity $1191.0 \text{ U}\cdot\text{L}^{-1}$]). This kinetic method utilises the *optimised standard method* for LDH by the German Society for Clinical Chemistry (1972), and is based on the principle developed by Wacker, Ulmer, and Vallee (1956); LDH catalyses pyruvate to lactate acid, with Nicotinamide Adenine Nucleotide Hydride (NADH) oxidised to NAD (Nicotinamide Adenine Nucleotide) during the process (Figure 3.13); the concentration of LDH is directly proportional to the rate of decrease in NADH.

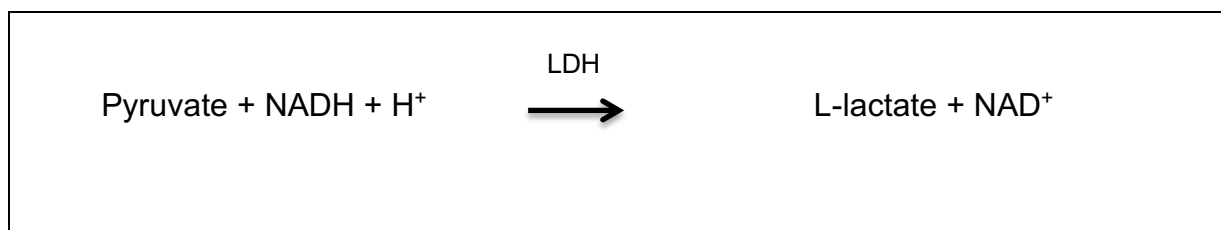


Figure 3.13. Principle of Lactate dehydrogenase (LDH). NADH = Nicotinamide Adenine Nucleotide Hydride; H⁺ = hydrogen; NAD = Nicotinamide Adenine Nucleotide. This method was used to analyse LDH present in serum in chapters 5 & 6 (Wacker et al., 1956). Reproduced from Randox LDH P - L kit insert.

In brief, a gain calibration was first performed on the RX Monza™ in flow cell mode to check the photometer. Lactate dehydrogenase reagents were prepared in accordance with the manufacturer's instructions, by reconstituting R1B reagent with 3 mL R1A reagent (Table 3.8). The RX Monza™ was then calibrated using a 2-point calibration using (i) 10 µL saline (as a standard blank); and (ii) 10 µL universal standard (LDH concentration ~667.0 U·L⁻¹ [Randox Calibration Sera Level 3, CAL2351, Randox Laboratories Ltd]). Standards were reacted in test tubes with 500 µL of reconstituted R1B reagent, aspirated into the RX Monza™ to incubate (37°C; 60 s), and absorbance read after 0.5, 1 and 2 min (340 nm), and a standard concentration curve generated (Figure 3.14). Samples were assayed following the same procedure as standards. Lactate Dehydrogenase concentration was calculated automatically from the calibration curve generated using the equation: 8095 * Δabsorbance. Samples, standards and blanks were assayed in duplicate; (analytical variance CV 6.3%; intra-assay variance CV 14.8%; inter-assay variance CV 14.0%); results expressed as U·L⁻¹ (International Units per litre; the amount of enzyme activity required to catalyse 1 µmol of substrate per minute, under specified temperature & pH), post-exercise samples corrected for plasma volume change (refer to section 3.3.4.9).

Reagent	Reagent Composition	Concentration	Amount
R1A reagent Buffer/substrate	Phosphate buffer Pyruvate	50.0 mmol·L ⁻¹ ; pH 7.5 0.6 mmol·L ⁻¹	3 mL
R1B reagent	NADH	0.18 mmol·L ⁻¹	Dry enzyme

Note. NADH = Nicotinamide Adenine Nucleotide Hydride.

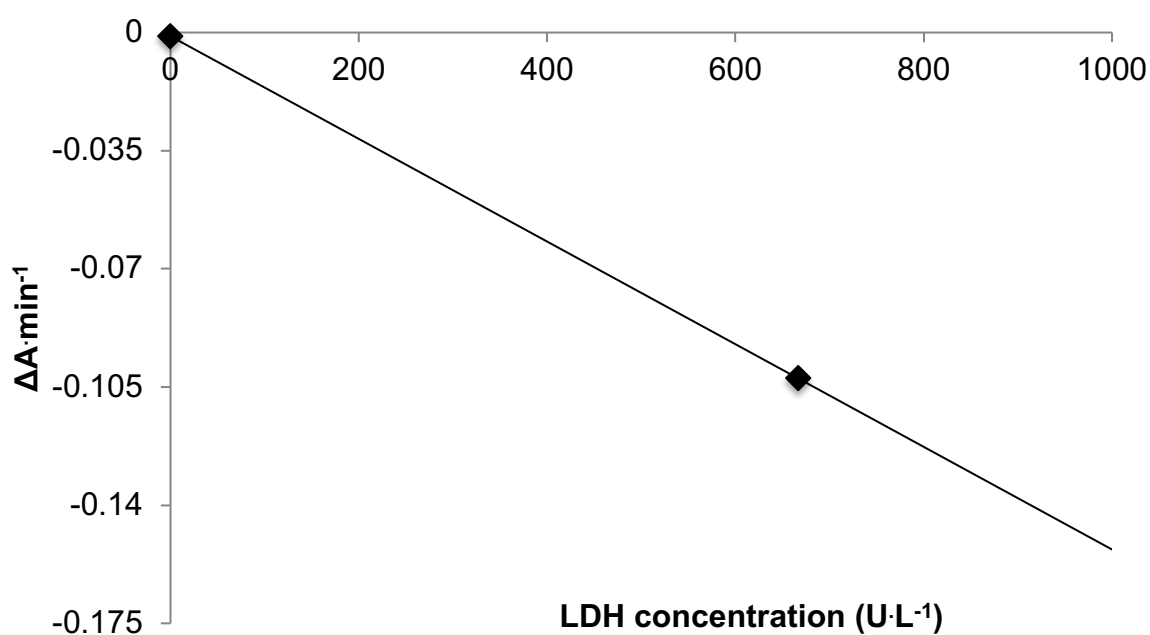


Figure 3.14. Exemplar scatterplot of Lactate Dehydrogenase (LDH) standard concentration curve. Serum LDH concentration was automatically calculated using the regression equation generated from the curve. A = absorbance.

3.3.4.5 Isolation of Nuclear Proteins & Quantification of NF- κ B p65

Subunit Activation.

Prior to detecting and quantifying NF- κ B p65 subunit activation, nuclear proteins were first isolated from PBMCs (because activated NF- κ B p65 translocates to the nucleus; refer to section 3.3.4.5.1). In summary, reagents were first prepared (Table 3.9; Nuclear Extraction Kit, ActiveMotif Inc., California: USA). The process then involved two stages: (i) cell lysis, and (ii) nuclear extraction. In brief, PBMCs were pelleted and re-suspended in 3 mL ice-cold PBS (pH 7.4) with protease inhibitor cocktail (added to preserve proteins). Cells were centrifuged (5 min; 200 g; 4°C) and the supernatant discarded. Cell pellets were re-suspended in 500 μ L hypotonic buffer and incubated on ice (15 min, to swell cell membranes). Subsequently, cells were then chemically lysed via an added detergent (25 μ L), and mechanically lysed via vortexing (10 s; highest speed), and use of a small clearance Dounce homogeniser. Cells were checked under a microscope for lysis. The cell suspension was then centrifuged (30 s; 14,000 g; 4°C), supernatant (cytoplasmic fraction) discarded, and the cell pellet (nuclear fraction) used for nuclear extraction. The nuclear fraction was re-suspended in 50 μ L complete lysis buffer, incubated on ice (30 min; on a rocking platform [150 rpm; Stuart™SSM4 Rocker, ThermoFisher Scientific, MA: USA]), vortexed (30 s; highest speed) and centrifuged (10 min; 14,000 g; 4°C). The supernatant (nuclear fraction) was stored (-80°C in pre-chilled cryo-vials) for analysis of NF- κ B p65 subunit activation. Aliquots of the nuclear fraction suspension were later used to quantify nuclear protein concentration, determined using the Bradford Assay (refer section 3.3.4.5.1).

After nuclear extraction, NF- κ B p65 subunit activation (alternatively known as NF- κ B

p65 DNA-binding activity) was determined and quantified using a commercially available ELISA (enzyme-linked immunosorbent assay) kit (TransAM™ NF-κB p65 Transcription Factor Assay Kit [ActiveMotif Inc.] range: 0.1 - 40.0 ng). The protocol is based on the principle that activated NF-κB p65 binds to an oligonucleotide consequence sequence (5'-GGGACTTTCC-3') pre-coated onto a well plate. A primary antibody is added to bind to an epitope on NF-κB p65 that is only available when NF-κB is activated and bound to DNA. The addition of a second antibody binds with NF-κB to provide a colourimetric reaction that allows activated NF-κB p65 to be measured spectrophotometrically. First, reagents were prepared (Table 3.10). Complete binding buffer (30 µL) was added to each well. Each sample or standard (20 µL pre-diluted in complete lysis buffer) was then added. For blank wells, 20 µL complete lysis buffer, and for positive controls, 20 µL Jurkat nuclear extract and wild type consensus oligonucleotide (1 µL diluted in 19 µL complete lysis buffer) were added per well. The plate was covered and incubated with mild agitation on a rocking platform (1 hr; 21°C; 100 rpm). After 1 hr, the plate was washed three times (200 µL wash buffer per well) and diluted NF-κB antibody (100 µL [diluted 1:1,000 with antibody binding buffer]) added to each well. The plate was then covered and incubated (1 hr; 21°C). After 1 hr, the plate was washed three more times (200 µL wash buffer per well) and diluted HRP-conjugated antibody (100 µL [diluted 1: 1,000 with antibody binding buffer]) added to each well, the plate covered and incubated (1 hr; 21°C). After 1 hr, the plate was then washed four more times (200 µL wash buffer per well), and developing solution (100 µL) added to each well. The plate was protected from light (tin foil) and incubated until standards were a medium-dark blue colour (~3 min). Stop solution (100 µL) was then added to each well and the absorbance of the yellow colour read using a microplate reader (450 nm;

Multiskan™). Standards and controls were assayed in duplicate, samples in single replicate on a single microplate. Activated NF-κB p65 subunit was quantified using a regression equation generated from a 5-point standard curve (generated using NF-κB recombinant protein p65: 0.625; 1.250; 2.500; 5.000; 10.000 ng). Samples corrected for nuclear protein concentration (refer to section 3.3.4.5.1), results expressed as ng·ug⁻¹. Jurkat nuclear extract was used as a QC and specificity of the assay checked with wild-type consensus oligonucleotide (a competitor for NF-κB binding).

Table 3.9

Nuclear Extraction Kit Reagent Preparation

Reagent	Components	Amount
Hypotonic Buffer	Hypotonic Buffer dH ₂ O	50.0 μL 450.0 μL
Complete Lysis Buffer	10mM DTT (prepared from 1M DTT) Lysis Buffer AM1 Phosphate Inhibitor Cocktail	5.0 μL 44.5 μL 0.5 μL

Note. DTT = Dithiothreitol.

Table 3.10 <i>NF-κB p65 Subunit Activation Reagent Preparation</i>		
Reagent	Components	Amount per plate
Complete Lysis Buffer	1M DTT Lysis Buffer AM2 Phosphate Inhibitor Cocktail	10.8 μ L 21.6 μ L 2.128 mL
Complete Binding Buffer	DTT Herring Sperm DNA Binding Buffer AM3	6.5 μ L 32.4 μ L 3.2 mL
Wash Buffer	dH ₂ O Wash buffer AM2	199.4 mL 21.6 mL
Antibody Binding Buffer	dH ₂ O Ab Binding Buffer AM2	19.44 mL 2.16 mL

Note. dH₂O = distilled water; DTT = Dithiothreitol.

3.3.4.5.1 Determination of PBMC nuclear protein concentration.

Nuclear protein content was quantified using a standardised Bradford assay (Bradford, 1976); under acidic conditions, Coomassie-dye binds with protein to form a blue chromagen, the colour change proportional to the amount of protein present. A 50 μ L aliquot of each PBMC nuclear fraction was mixed with 1,500 μ L Coomassie Plus reagent (Coomassie Plus-The Better Bradford Assay™ kit, ThermoScientific Ltd., Illinois: USA), incubated (10 min; 21°C) and absorbance read using a spectrophotometer (595 nm [Cecil 100, Cecil Instruments]). Nuclear protein

concentration was determined from a standard concentration curve (Figure 3.15) prepared using serial dilutions of 2,000 $\mu\text{g}\cdot\text{mL}^{-1}$ (0.0; 2.5; 12.5; 50.0; 75.0; 150.0; 200.0 $\mu\text{g}\cdot\text{mL}^{-1}$) Bovine Serum Albumen (BSA) in a 0.9% saline and 0.05% sodium azide solution (BSA Standard; Coomassie Plus-The Better Bradford Assay™ kit, ThermoScientific Ltd.), with complete lysis buffer as the diluent. Samples were blanked against a sample cuvette containing complete lysis buffer only. Samples and standards were assayed in duplicate. Nuclear protein concentration was expressed in $\mu\text{g}\cdot\text{mL}^{-1}$.

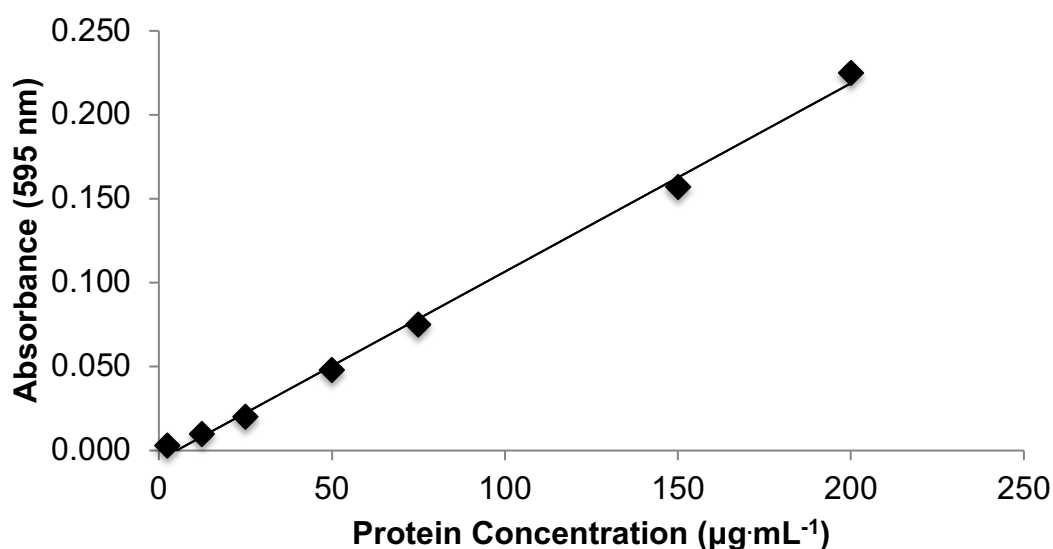


Figure 3.15. Exemplar protein concentration standard curve. Protein concentrations in nuclear extracts were quantified using the regression equation generated from the curve.

3.3.4.6 Determination & quantification of inflammatory cytokines.

Multiple inflammatory cytokines (IL-6; IL-8; IL-10; & MCP-1) were analysed in single serum samples at Randox Health Checks (London: UK) using biochip Array Technology, a chemiluminescent sandwich immunoassay (Randox Cytokine &

Growth Factors High-Sensitivity Array I [CTK HS EV 3513/3623 Randox Laboratories Ltd.] in conjunction with the Evidence Investigator™ [Randox Teoranta, Donegal: Northern Ireland] sensitivity & range: IL-6: 0.12 - 400 pg·mL⁻¹; IL-8: 0.36 - 1450 pg·mL⁻¹; IL-10: 0.37 - 450 pg·mL⁻¹; MCP-1: 3.53 - 500 pg·mL⁻¹). This method allows simultaneous detection of pro- and anti-inflammatory cytokines and was used to determine potential pre- to post-exercise alterations in these inflammatory biomarkers that would warrant attenuation with quercetin. The principle of the immunoassay is based on cytokines (if present in the serum sample) competing with horseradish peroxidase conjugate for binding sites on specific antibodies that have been coated onto a biochip. A signal reagent is added that labels the antibodies, emitting a chemiluminescent signal directly proportional to the concentration of cytokines bound to the antibodies. In summary, reagents were first prepared in accordance with the manufacturer's instructions. Next, 100 µL serum/ calibrator/ QC and 200 µL diluent reagent (per sample) were added to each biochip, immediately protected from light, incubated and agitated; firstly, on a thermoshaker (1 hr; 370 rpm [Randox Thermoshaker, Randox Teoranta]); secondly, in the fridge (16 hr; 4°C). Following incubation, the biochip was washed six times (2 min; 350 µL wash buffer per sample), 300 µL horseradish peroxidase conjugate added per sample, and the biochip incubated and agitated (thermoshaker; 1 hr; 370 rpm). The biochip was washed six more times with wash buffer (350 µL per sample) to remove unbound analytes, and working reagent added (250 µL per sample). After two minutes incubation (protected from light) the biochip was inserted into the Evidence Investigator™ (Figure 3.16) and cytokines detected and captured using digital imaging technology (2 x 60 s photos), quantified using a standard concentration curve (9-point multi-analyte calibration; $r = 1.0$). Samples were assayed in single

replicate, results presented in $\text{pg}\cdot\text{mL}^{-1}$, corrected for post-exercise plasma volume change. Precision of the assay was checked with three levels of multi-analyte QC specific for each cytokine (intra-assay variance & inter-assay CV < 10%).

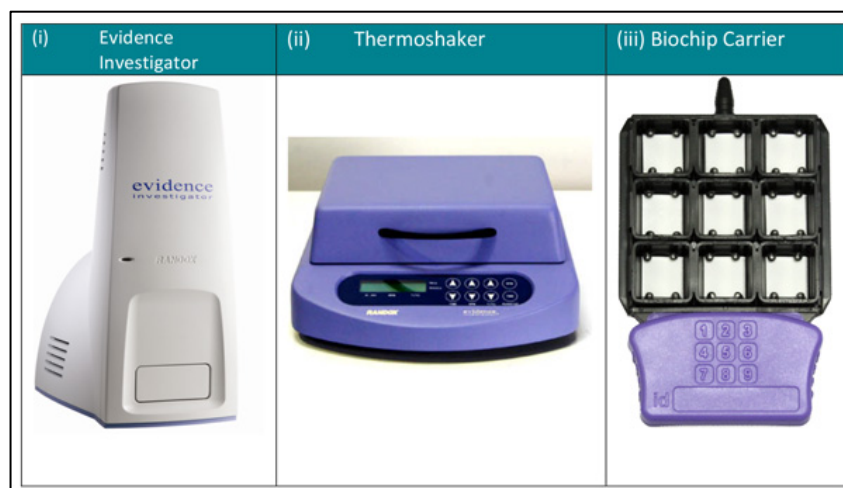


Figure 3.16. Radox Evidence Investigator™, thermoshaker, & biochip. The Radox Evidence Investigator™ was used in the determination and quantification of inflammatory cytokines.

3.3.4.6.1 IL-6 (chapter 6).

In chapter 6, IL-6 was analysed using the same method as chapter 5 (Evidence investigator™ Biochip Array Technology; refer to section 3.3.4.5). Samples were analysed in duplicate, results expressed in $\text{pg}\cdot\text{mL}^{-1}$. Precision of the assay was checked using three different levels of QC assayed in duplicate (inter-assay variance & intra-assay variance CV < 10%).

3.3.4.7 Determination of C-Reactive Protein.

3.3.4.7.1 C-Reactive Protein (chapter 5).

Serum samples were analysed for C-Reactive Protein (CRP) using the RX Monza™ in cuvette mode and a commercially available CRP immunoturbidimetric assay kit

(CRP, CP7950 Randox Laboratories Ltd., [sensitivity 1.93 mg·L⁻¹; linearity 198.00 mg·L⁻¹]). The assay is based on the principle that CRP present in the sample binds with latex particles coated with CRP-specific antibodies to form an antibody-antigen complex which results in turbidity and scattering of light, the rate of which is detected as absorbance change; the intensity proportional to the concentration of CRP present in the sample (Claus, Osmand, & Gewurz, 1976).

Reagent	Reagent Composition	Concentration	Amount
R1 Assay Buffer	Polyethylene Glycol Tris/HCL buffer Sodium Chloride	4% max 20.0 mmol·L ⁻¹ ; pH 7.4 150 mmol·L ⁻¹	500.0 µL
R2 Antibody Reagent	Anti (human) CRP		75.0 µL

Note. CRP = C-Reactive Protein; HCL = Hydrochloric.

The RX Monza™ was calibrated in accordance with the manufacturer's instructions. Reagents were ready made (Table 3.11) and a gain calibration performed in cuvette mode to check the photometer. A 6-point standard concentration curve (Figure 3.17) was generated using ready-made standards (CRP concentrations 0.0; 12.3; 24.6; 49.2; 98.4; 196.7 mg·L⁻¹; [CRP CP2479, Randox Laboratories Ltd.]). Each standard (50 µL) was reacted with 500 µL R1 assay buffer in semi-micro cuvettes and equilibrated at room temperature (3 min). R2 antibody reagent was added (75 µL), standards mixed, covered in Parafilm M® (Parafilm, Wisconsin: USA) and incubated using a dry heat block (15 min; 25°C [RX Monza™ Cuvette Incubator, Randox

Teoranta, Donegal: Ireland]). After exactly 15 min incubation, standards were remixed and absorbance read (25°C; 340 nm). Standards were blanked against a reference sample containing 50 μL saline and 500 μL R1 assay buffer. Standards were assayed in duplicate. Samples were assayed as per standards. C-Reactive Protein concentration of the samples was determined automatically using the regression equation generated from the standard concentration curve (analytical variance was calculated for 2 levels of QC: Level 1 target value 1.01 $\text{mg}\cdot\text{L}^{-1}$, CV 14.6%; Level 2 target 4.36 $\text{mg}\cdot\text{L}^{-1}$, CV 12.8%; intra-assay variance CV 34.1%; inter-assay variance CV 47.8%). Results presented in conventional units, $\text{mg}\cdot\text{L}^{-1}$. Post-exercise results corrected for plasma volume change.

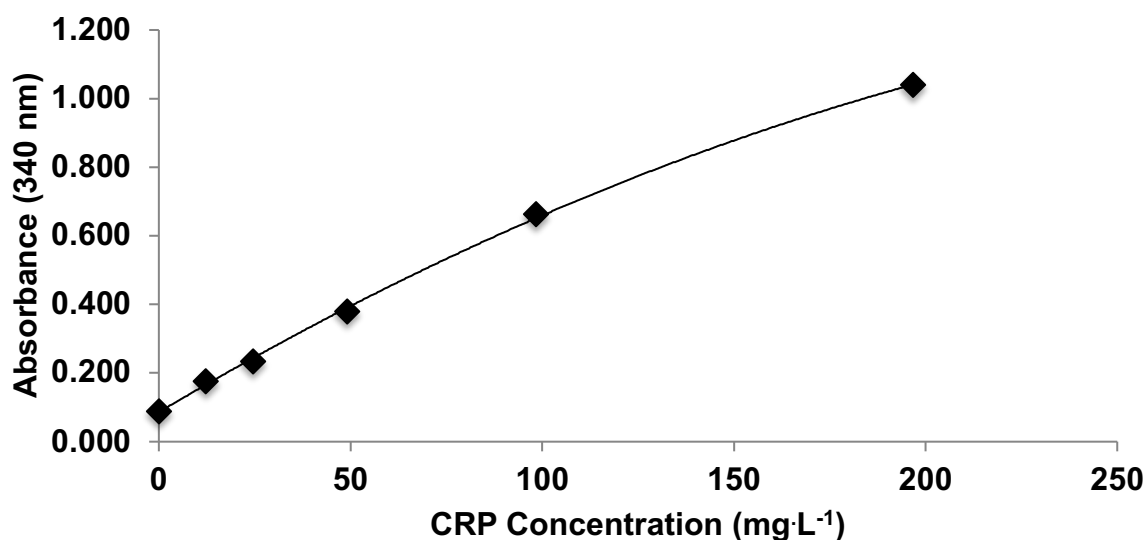


Figure 3.17. Scatterplot of C-Reactive Protein (CRP) standard concentration curve. CRP concentration of the samples was calculated automatically using the regression equation generated from the curve.

3.3.4.7.2 Full Range CRP (chapter 6).

In chapter 6, serum samples were analysed for full range CRP concentration (Full Range CRP immunoturbidmetric assay, Randox Laboratories Ltd.) at Randox Health, London. The principle of the method was as utilised in chapter 5; however, samples were assayed on an automated analyser (RX Imola analyser, Randox Teoranta [570 nm; sensitivity 0.3 mg·L⁻¹; linearity 161.0 mg·L⁻¹). Reagents were ready made (Table 3.12). Precision of the assay was checked using three different levels of QC, analysed in single replicate, which were within target range levels (inter-assay & intra-assay variance CV < 10%).

Reagent	Reagent Composition	Concentration
R1 Assay Buffer	Glycine Sodium Chloride Sodium EDTA disodium salt dehydrate Bovine serum albumin	170.0 mmol·L ⁻¹ 100.0 mmol·L ⁻¹ 50.0 mmol·L ⁻¹ 1%
R2 Antibody Reagent	Latex particles coated with antibody specific to CRP	

Note. CRP = C-Reactive Protein; EDTA = Ethylenediaminetetracetic acid.

3.3.4.8 Quantification of total leukocyte & neutrophil counts.

Total leukocyte counts and specifically, neutrophil counts were measured in K2EDTA venous whole blood samples via an automated blood cell counter (WBC DIFF System, HemoCue®, Ängelholm: Sweden; measurement range 0.3 – 30.0 x 10⁹·L⁻¹).

Samples (10 μL) were pipetted into microcuvettes (WBC DIFF microcuvettes, HemoCue®) pre-coated with cell lysing reagent (Saponin & Surfynol) and nuclear staining dye (Triton & Methylene blue), and loaded into the cell counter. Samples were analysed in single replicate within 1 min of collection. Total leukocyte and neutrophil counts were expressed as the number of cells $\times 10^9\text{L}^{-1}$ (intra-assay variance: total leukocyte counts CV 18.8%; total neutrophil counts CV 17.5%; inter-assay variance: total leukocyte counts CV 24.8%; total neutrophil counts CV 26.9%). Samples unsuitable for the counter due to errors (e.g., outside of the range; air bubbles; uneven cell distribution) were analysed manually for total leukocyte counts only.

3.3.4.9 Haematocrit, Haemoglobin & Plasma Volume Change.

Exercise-induced plasma volume change (ΔPV) was accounted for by measuring haemoglobin (Hb) and haematocrit (Hct; also known as packed cell volume) in whole blood samples. Haemoglobin was analysed using an automated haemoglobin analyser (HemoCue® HB201+, HemoCue® [sensitivity: 0 $\text{mg}\cdot\text{dL}^{-1}$; linearity: 256 $\text{mg}\cdot\text{dL}^{-1}$]) that utilises a modified azidemethemoglobin reaction (haemolysed erythrocytes release haemoglobin that is converted to methemoglobin; azidemethemoglobin is formed from the reaction of methemoglobin with azide, the absorbance of which is directly proportional to haemoglobin concentration). Whole blood samples (10 μL) were pipetted into microcuvettes (HemoCue® HB201 microcuvette, HemoCue®), checked for air bubbles and analysed immediately (within 10 min in accordance with the manufacturer's instructions), in single replicate, results expressed in $\text{mg}\cdot\text{dL}^{-1}$ (analytical variance CV 1.09%; intra-assay variance CV 1.50%). Haematocrit was determined by centrifuging $\sim 80\ \mu\text{L}$ K2EDTA whole blood

samples (Na-heparinised micro-capillary tubes plugged with Cristaseal; 5 min; 13,000 g [Hawksley 1300, Hawksley, Sussex: UK]), and measuring haematocrit concentration using a manual haematocrit reader (Hawksley); taking into account 1.5% plasma trapped within the packed cell volume of erythrocytes (Dacie & Lewis, 1963), results expressed as percentage. Samples analysed in single replicate (intra-assay variance CV 1.49%). Pilot work demonstrated no difference in precision in single replicates compared to triplicates. Haematocrit and haemoglobin measurements were used to calculate plasma volume change (ΔPV) using the equation by Dill and Costill equation (1974; Equation 3.3). The concentration of each post-exercise analyte in serum was corrected by multiplying by $100 - PV\Delta\%$ (expressed as a decimal). Post-exercise analytes assessed in whole blood were corrected using the alternative equation proposed by Matömaki et al. (2018; Equation 3.4).

(Equation 3.3)

$$\Delta PV (\%) = \left[\left(\frac{Hb_{pre}}{Hb_{post}} \right) \times \left(\frac{(100 - Hct_{post})}{(100 - Hct_{pre})} - 1 \right) \right] \times 100$$

Note. Plasma volume change equation. Hb = haemoglobin (mg·dL⁻¹); Hct = haematocrit (%); ΔPV = plasma volume change (Alis et al., 2015).

(Equation 3.4)

$$\left(\frac{BM_{post}}{BM_{pre}} \times \frac{Hb_{pre}}{Hb_{post}} \right) - 1$$

Note. BM = biomarker; Hb = haemoglobin (Matömaki et al., 2018).

3.4 Control of data.

3.4.1 Training load.

In chapter 6, participants were provided with a training log (Appendix A) and asked to log any training undertaken one-week prior to main experimental testing. To control for the acute effects of exercise, participants were asked to rest 24 hr prior to attending the laboratory. Training load (TL, expressed in AU) was calculated from session RPE (sRPE; 1 – 10 scale) and exercise duration using the equations proposed by Foster et al. (2001) and Day et al. (2004): $TL (AU) = \text{Exercise duration (min)} \times sRPE$. The lead investigator reviewed training loads to compare the training status of participants and to identify high training loads that may have confounded the study results. Compliance with the 24 hr rest period was also checked.

3.4.2 Dietary intake.

Participants were asked to adhere to their habitual dietary intake throughout the course of each study. During chapters 5 and 6, participants recorded their habitual diet using estimated food records. Pre-exercise and pre-blood sampling diet was controlled using standardised meal replacement drinks (Jeacocke & Burke, 2010).

3.4.2.1 Estimated food record.

Throughout chapters 5 and 6, participants recorded their estimated dietary intake. Participants were issued a paper-template food record (Appendix B) and asked to record their daily intake for a 3-day period (3 consecutive days; 2 weekdays, 1 weekend day), one-week prior to the main testing period, and daily throughout the testing period. Participants received verbal instructions on how to estimate portion

sizes and complete the food record and were instructed to complete the food record in as much detail as possible at the time of consumption. Participants unfamiliar with food recording techniques were trained using food models and food portion size photos (Nelson, Atkinson & Meyer, 1997). On completion of the food records, the lead investigator further clarified recorded food items with participants during a face-to-face conversation.

3.4.2.1.1 Dietary analysis.

Estimated food records were analysed using computerised dietary analysis software (DietPlan Version 6.60d4 for Mac [Forestfield Software Ltd., West Sussex: UK]). Food and drink items were entered into the database and automatically coded using McCance and Widdowson's Composition of Foods, 6th Edition (2002); items not present in the database were substituted or entered manually. Food records were analysed for average daily: (i) total energy intake; (ii) macronutrient intake (carbohydrates, including fibre; protein; fat; PUFAs; alcohol; macronutrient ratios); and (iii) micronutrient intakes. Flavonoids, flavonols and quercetin intake were also analysed (chapter 6) using the United States Department of Agriculture Nutrient database (add-on available in the software). Pilot work ($N = 6$) confirmed the validity of 3 and 4-day estimated food records to analyse quercetin intake by comparison to a 7-day weighed food record (data were within $< 10\%$ of 1 SD). To ensure consistency, all food records were analysed by the lead investigator. Food records were reviewed for (a) compliance of habitual diet; (b) nutritional status of the participants; and (c) consumption of the standardised meal replacement drink.

3.4.2.2 Standardised dietary intake.

Participants were provided with a nutritionally complete liquid supplement *Ensure Plus® Nutrition Shake* (Abbott Laboratories, OH: USA) to ingest as a standardised pre-exercise and/or pre-blood sample meal during chapters 5 and 6. The supplement was medium-high in energy (per 100 mL: energy 150 kcal; carbohydrate 22 g [60%], fat 5 g [30%], protein 5 g [14%]; Table 3.14) and was provided to participants energy-intake adjusted for body mass ($10 \text{ kcal}\cdot\text{kg}^{-1}$); based on a similar standardised meal used by Nieman's research groups to control for substrate utilisation (Konrad et al., 2011; Nieman et al., 2014). Participants were instructed to consume the drink ~2 hr prior to testing on each experimental day. To test suitability of the drink, participants were initially issued different flavours of the drink to try (all flavours equal in nutritional content) and asked to consume the preferred drink at the calculated amount, reporting any side effects.

Table 3.14

Ensure Plus® Nutritional content

Nutrient	Per 100 mL
Energy	150 kcal
Total Carbohydrate	21.17 g
Dietary Fibre	-
Sugars	21.17 g
Fat	4.81 g
Saturated fat	0.717 g
Trans fat	-
Polyunsaturated fat	3.314 g
Monounsaturated fat	1.367 g
Protein	5.5 g
Cholesterol	2 mg
Sodium	101 mg
Calcium	84 mg
Iron	1.91 mg
Potassium	6 mg
Vitamin A	529 IU
Vitamin C	12.7 mg
Vitamin D	26 IU
Vitamin E	1.2 mg
Thiamine	0.138 mg
Riboflavin	0.1 mg
Niacin	2.55 mg
Vitamin B6	0.21 mg
Vitamin B12	0.00021 mg
Folic Acid	0.026 mg
Sodium	106 mg
Potassium	170 mg
Chloride	115 mg
Phosphorous	117 mg
Magnesium	27.6 mg
Iron	1.62 mg
Zinc	1.70 mg
Selenium	0.006 mg

Note. Kcal = kilocalories; IU = International Units.

Chapter 4

Assessment of High-Intensity Training Load and Exercise-Induced Lipid

Peroxidation in Professional Soccer Players

(Study one)

4.1 Introduction

The hormetic theory of exercise-induced reactive oxygen and nitrogen species (RONS) is now well established (Powers & Radak, 2016; Radak et al., 2007, 2017). Exercise-induced RONS play an important role in the training adaptation response of skeletal muscle, acting as cell signalling molecules to induce endogenous antioxidant enzyme expression, as demonstrated by the enhanced basal antioxidant status of trained athletes (Cazzola et al., 2003; Gomez-Cabrera et al., 2008). However, excessive accumulation of RONS, beyond that of endogenous antioxidant defences, can lead to a state of oxidative stress (Powers & Jackson, 2008), with negative implications for the athlete (Finaud et al., 2006a). At the skeletal muscle level, oxidant-mediated damage to cellular components, such as DNA, lipids, and proteins, can result in muscle dysfunction (Powers et al., 2010a). For the athlete this can translate to (i) decreased force production, (ii) induced muscle damage, (iii) muscle soreness, (iv) fatigue, (v) compromised recovery, (vi) increased susceptibility to injury, and ultimately, (vii) underperformance (Becatti et al., 2017; Finaud et al., 2006a; Powers et al., 2010a; Silva et al., 2013).

Exercise-induced oxidative stress (EIOS) has been proposed to be dose-dependent, relative to the duration, frequency, and intensity of exercise (Finaud et al., 2006a; Gomez-Cabrera et al., 2009; Powers & Jackson, 2008). Soccer is a prolonged duration sport that involves high-intensity intermittent bouts (Becatti et al., 2017); professional soccer players are susceptible to EIOS due to the high metabolic and mechanical demands associated with repetitive muscle contractions and increased training loads (Bangsbo et al., 2006; Le Moal et al., 2016; Nédélec et al., 2012). Research investigating the oxidative stress response to soccer has been limited.

Studies have reported augmented oxidative stress in response to single bouts of soccer (e.g., simulated soccer, soccer training, or match play [Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Mello et al., 2017]), with measured biomarkers remaining elevated throughout acute and sustained recovery periods (24 hr [Ispirlidis et al., 2008]; 72 hr [Ascensão et al., 2008; Fatouros et al., 2010]). Oxidative stress has also been demonstrated to vary throughout periods of soccer training (da Silva Barbosa et al., 2017; Ferrer et al., 2009), and competitive soccer seasons (Becatti et al., 2017; Le Moal et al., 2016; Silva et al., 2013), and to be associated with periods of increased training load (Becatti et al., 2017; Le Moal et al., 2016) and elevated CK (Becatti et al., 2017). However, the practical significance of these changes in biomarkers of oxidative stress remains unknown (Becatti et al., 2017).

Given that intensified periods of soccer training have been associated with oxidative stress, it is interesting that there has been limited research investigating the EIOS response throughout different microcycles of the competitive season representative of different training loads. The lack of prospective studies in this area could be attributed to (i) limited access to professional athletes; (ii) difficulties quantifying the physiological demands of the sport; and (iii) the disruption associated with monitoring professional team sport athletes throughout training and competition (Lindsay & Costello, 2017). Furthermore, there is a reluctance by professional soccer clubs to disseminate data pertaining to training load (Kelly, Strudwick, Atkinson, Drust, & Gregson, 2019). An integrative approach, quantifying oxidative stress concomitantly with indicators of training load intensity could provide a biochemical insight into the cumulative effects of the weekly training load imposed on players during a

competitive in-season. The main focus of the competitive period is to facilitate recovery and maintain optimal performance (Bangsbo et al., 2006; Jeong et al., 2011; Le Moal et al., 2016). This study may provide knowledge to further aid sports scientist/coaches in the prescription of training loads that maintain redox homeostasis, minimising the negative effects associated with EIOS (as per Becatti et al., 2017; Finaud et al., 2006b; Le Moal et al., 2016). In their 2016 study, Le Moal et al. found periods of increased training load, quantified using subjective RPE, were associated with oxidative stress. To date, no study has used objective measures (such as HR & GPS variables) to explore the association between training load intensity and EIOS in professional soccer players throughout microcycles of a competitive season.

Malondialdehyde (MDA), a stable, end product of lipid peroxidation, formed primarily through the oxidation of PUFAs, is the most commonly evaluated biomarker of oxidative stress (Halliwell & Gutteridge, 2015). Typically, MDA is sampled in venous blood or muscle tissue; however, these methods are invasive and impractical for soccer field-based testing. Urinary MDA has recently been established as a valid and reliable biomarker of oxidative stress and may be a better indicator of whole body oxidative stress than plasma (Drury, Nycyk, & Cooke, 1997; Weitner, Iničč, Jablan, Gabričević, & Domijan, 2016). Monitoring urinary MDA may provide a more advantageous method of oxidative stress assessment in this cohort (Lindsay & Costello, 2017), especially given professional soccer players familiarity with the technique of urine sampling (providing regular samples for hydration & anti-doping purposes).

The primary aims of this study were (i) to quantify urinary MDA and high-intensity training load in professional soccer players throughout a competitive in-season; and (ii) to examine the association between high-intensity training load and MDA. A secondary aim was to compare urinary MDA in professional soccer players to recreational soccer players. In comparison to lesser-trained athletes, trained athletes tend to have lower basal concentrations of oxidative stress as an adaptive response to training. However, because of the demands of daily training with minimal recovery, it is possible that trained athletes may demonstrate higher basal oxidative stress than lesser-trained athletes; therefore, the difference in lipid peroxidation between trained and recreational players could be bi-directional. This novel study adds to the literature by: (a) quantifying in-season high-intensity training load using objective measures based on variables of GPS and HR; and (b) assessing urinary MDA as a biomarker of lipid peroxidation in an ecological setting.

4.2 Methods

4.2.1 Participants.

Nineteen participants volunteered to partake in the study. Recruited by convenience sampling, participants formed two groups: (i) experimental (EXP) and (ii) non-equivalent comparison (COM)⁵. A cohort of male professional soccer players, playing for an English Football League One team, formed the EXP group ($n = 10$ [forwards = 1; midfielders = 5; defenders = 4]), based upon the following inclusion criteria: (i) aged 18 - 35 years; (ii) first team outfield player (goalkeepers were excluded because of training load differences [Malone et al., 2015a]); (iii) competing in the current season; (iv) healthy and injury free; (v) having previously played at a

⁵ Due to the use of professional athletes randomised groups could not be formed.

professional level for a minimum of 12 months; and (vi) non-smoker. Initially 16 EXP group participants were recruited, however, data was excluded from analysis for participants who failed to meet the following criteria: (i) participation in all training sessions (± 2); (ii) not undertaking additional exercise that would influence the study results (e.g., pre-habilitation/rehabilitation); (iii) participation in > 70 min of match play per match; and (iv) not undertaking antioxidant supplementation. Two EXP group participants were also withdrawn from the study; one because of injury, and another player was transferred ($n = 10$). The COM group ($n = 9$) consisted of recreationally trained soccer players (as it was impractical for professional soccer players to form a control group), who met similar inclusion criteria to the EXP group ((i) male; (ii) aged 18 - 35 years; (iii) healthy & injury free; (iv) non-smoker); and trained or played no more than twice per week, without a compulsory schedule. Participants' characteristics appear in Table 4.1 ($N = 19$). Using the effect size reported by Hazar (2012; MDA; $d = 1.10$), an a priori power estimation (t-test, one tailed; $\alpha = .5$; $\beta = .2$; $1-\beta = 80\%$) conducted using G*Power software (version 3.1 [Erdfelder, Faul & Buchner, 1996]) proposed a total sample size of 22 participants (11 per group) sufficient to detect a large effect size ($[t = 1.72$; $d = 0.8]$). A sensitivity analysis confirmed the sample size of 19 achieved in the present study, adequate to detect a large effect ($d = 1.20$; $\alpha = .5$; $\beta = .2$; $1-\beta = 80\%$; effect size in accordance with Cohen, 1988).

Table 4.1

Descriptive Characteristics of Participants; M (± SD)

Variable	EXP (<i>n</i> = 10)	COM (<i>n</i> = 9)
Age (years)	23 (± 2)	26 (± 6)
Stature (cm)	181.3 (± 5.3)	176.5 (± 8.2)
Body Mass (kg)	83.5 (± 6.2)	79.2 (± 12.3)
Body Fat (%)	13.0 (± 2.2)	15.7 (± 7.8)
$\dot{V}O_2\text{max}$ (mL·kg ⁻¹ ·min ⁻¹)	57.2 (± 6.7)*	48.6 (± 7.1)
HR _{max} (beats·min ⁻¹)	203 (± 9)	187 (± 12)

Note. COM = comparison; EXP = experimental; HR_{max} = maximum heart rate; $\dot{V}O_2\text{max}$ = maximal oxygen uptake.

*Denotes significant difference between groups (Independent Samples t-test, $t = 2.707(17)$, $p = .015$, $d = 1.24$, 95% CI [1.90, 15.29]).

Study procedures were approved by the University of Hertfordshire's Life and Medical Sciences Ethics Committee (approval code: LS4/4/12P), and conducted in accordance with the ethics standards for sports science research (Harriss & Atkinson, 2011). Following verbal and written explanations of the study procedures, written informed consent was obtained. Prior to undertaking any exercise set by the lead investigator each participant completed a health screen.

4.2.2 Experimental design.

This study employed a repeated measures, prospective, cohort design to quantify the exercise-induced lipid peroxidation response to high-intensity training loads in a cohort of professional soccer players (EXP group). Throughout 3 one-week microcycles of a competitive in-season ((i) T1: early in-season [1st microcycle]; (ii) T2: mid-season [16th microcycle]; and (iii) T3: end of in-season [32nd microcycle])

training sessions were monitored using GPS and HR to allow quantification of objective indicators of external and internal high-intensity training load. Urine samples were also collected during each time point and analysed for MDA, as a relatively simple, non-invasive biomarker of lipid oxidation (Figure 4.1). Preliminary physiological measurements ((i) stature; (ii) body mass; (iii) %bf; (iv) $\dot{V}O_2\text{max}$) were assessed during preseason (a minimum of 3 weeks prior to experimental testing). Body mass was reassessed at T2 and T3.

Measurements of urinary MDA were also conducted in the COM group alongside the EXP group at T3 (+/- 2 weeks [Figure 4.1]). The purpose of the COM group was for a comparison of lipid peroxidation in athletes of a lower playing level, as it was hypothesised that participants in the EXP group would either have elevated MDA due to high training loads, potentially causing maladaptation, or would have lower MDA, as an adaptive response to training. The T3 time point was chosen because it was also the end of the competitive in-season for participants in the COM group, comparable to the method of Mukherjee & Chia (2009).

To control for the effects of (a) exogenous antioxidants and (b) psychological stress, a food frequency questionnaire that assessed total antioxidant intake, and a self-report inventory of stress symptoms, were administered to both groups. Both questionnaires have previously been validated and used in exercise studies.

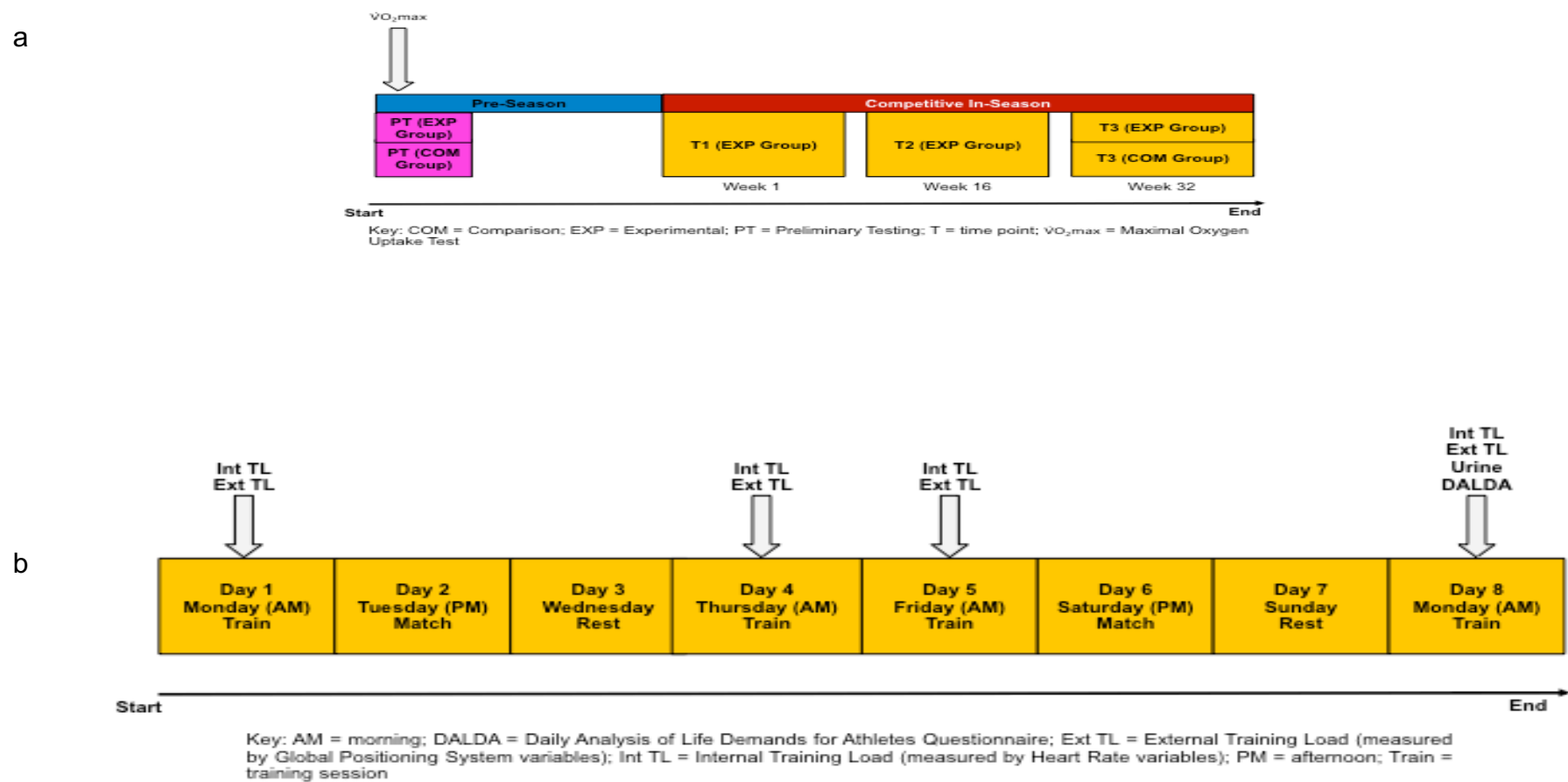


Figure 4.1. a) Schematic representation of the overall study design. b) Schematic representation of the experimental protocol during each microcycle (Experimental group only). Data were collected throughout one-week microcycles of a competitive in-season in the experimental group. Data were collected at time point 3 in the comparison group.

4.2.3 Experimental procedures.

4.2.3.1 Preliminary measurements.

Pretesting measurements were conducted between 10 am - 12 pm and 2 - 4 pm (intra-day variations have been shown to be comparable with the circadian rhythm of body temperature [Tanita UK Ltd., 1993; Winter et al., 2007]).

4.2.3.1.1 Anthropometric measures.

Stature and body mass were determined (refer to General Methods sections 3.2.1.1 & 3.2.1.2). After assessment of stature and body mass, percentage body fat (bf%) was determined using the method of eight-electrode whole-body bioelectrical impedance (Tanita Body Segmental Analyser, Model 418-BC [50 kHz; 500 μ A; range 1 - 75%], Tanita Corporation, UK: Middlesex). Refer to General Methods section 3.2.1.3.1. Body mass was also determined at T2 and T3.

4.2.3.1.2 Determination of maximal oxygen uptake & heart rate.

Maximal oxygen uptake ($\dot{V}O_{2\max}$) was calculated during the pre-season from a continuous graded exercise test performed on a motorised treadmill (Refer to General Methods section 3.2.1.4).

Maximum heart rates (HR_{\max}) established at the end-point of the $\dot{V}O_{2\max}$ test were used to assign heart rate zones for calculations of internal training load (EXP group only).

4.2.3.2 Training load assessment.

Training load was monitored throughout all training sessions during the study via GPS and HR telemetry (10 sessions [T1: 3 sessions; T2: 4 sessions; T3: 3 sessions]; 91 player recordings [mean session duration 90:51 ± 18:20 min]). Training sessions were field-based, conducted at the soccer club training ground, on an outdoor grass pitch, free from GPS obstruction (ambient conditions are displayed in Table 4.2). Training was prescribed and conducted by the soccer club coaching and management staff and was uninfluenced by the study. The lead investigator was present throughout all training sessions to ensure compliance and to manually record the training duration of each participant (EXP group only).

Table 4.2

Ambient Conditions; M (± SD)

Variable	T1	T2	T3
Temperature (°C)	15.7 (± 1.2)	9.3 (± 2.6)	5.5 (± 3.3)
Pressure (kPa)	101.8 (± 0.9)	100.1 (± 1.3)	100.8 (± 0.9)
Relative Humidity (%)	84.7 (± 13.8)	92.0 (± 2.1)	76.5 (± 6.6)

Note. kPa = kilopascal; T = time point.

The GPS units (SPI Pro, GPS systems, Canberra: Australia) were set up in accordance with the manufacturer's instructions, as previously described (Aughey, 2011; Cummins et al., 2013; Jennings et al., 2010). Fifteen minutes prior to the start of the training sessions the GPS units were switched on and satellites located (satellites detected: 11 ± 2). Participants wore the GPS units in purpose-built vests that housed the unit between the shoulder blades and incorporated a HR monitor

chest strap (Model T31 [0.2 Hz] Polar). Participants wore the GPS units and HR monitors throughout the duration of each training session (to avoid inter-unit error [pilot work demonstrated moderate reliability] each participant wore the same GPS unit throughout the study [Jennings et al., 2010]). After each training session, GPS and HR data were downloaded from each GPS unit for the analysis of training load with TeamAMS software (version 1.2, GPSports). Data were edited to the duration of each individual participant's training session as observed by the lead investigator. Competitive match data was not recorded because of restrictions set by the football club and league (2012 - 13 season), however, data was only included for analysis from participants who started and played > 70 min of each competitive match played at each time point.

4.2.3.3 Training load quantification.

At present, there are no standardised variables used to quantify training load (Akenhead & Nassis, 2016), with several variables cited in the GPS literature. To determine the relative stress imposed on each individual athlete indicators of high-intensity training load were quantified using established high-intensity zone variables based upon individual peak velocity and maximal HR (Dwyer & Gabbett, 2012; Sparks et al., 2016). The frequency of intense accelerations and decelerations was also included (detected via in-built tri-axial accelerometry within the GPS unit [reliability previously demonstrated by Waldron et al., 2011]), to account for high-intensity actions that may occur at lower velocities and would also contribute to the external load (Cummins et al., 2013; Dalen et al., 2016; Harper, Carling & Kiely, 2019; Vanrenterghem et al., 2017). The selected variables are commonly used in the literature and in practice (Bangsbo, 2006; Harper et al., 2019; Krstrup et al., 2003).

4.2.3.3.1 High-intensity external training load.

Total distance covered in the high-intensity velocity zone (HIVZ; > 61% peak velocity) was selected as the primary indicator of high-intensity external training load (HETL), expressed relative to training time ($\text{m}\cdot\text{min}^{-1}$). To reflect the cumulative load of each weekly microcycle, data were summed across training sessions and reported as the group average for the week. Individual peak velocities were determined as the peak velocity value achieved during either: (i) 30 m sprint test (refer to General Methods section 3.3.1.1.1); or (ii) GPS monitored training sessions or friendly match (Massard, Eggers & Lovell, 2017). The total number of high-intensity accelerations (HIACC) and decelerations (HIDEC) were also determined for each training session, using previously established absolute thresholds (HIACC = > $3 \text{ m}\cdot\text{s}^{-2}$ for 1 s; HIDEC = > $-3 \text{ m}\cdot\text{s}^{-2}$ for 1 s [Akenhead et al., 2013]), reported as accumulated weekly load.

4.2.3.3.2 High-intensity internal training load

High-intensity internal training load (HITL) was calculated as the percentage of the duration of the training session spent in the high-intensity HR zone (> 80% HR_{max}), expressed as % Time > 80% HR_{max} . Data reported as mean accumulated training load per microcycle. Individual HR_{max} was determined as the highest HR achieved during either: (i) $\dot{V}\text{O}_2\text{max}$ testing; (ii) 30 m sprint test; or (iii) GPS monitored training sessions or friendly match.

4.2.3.4 Urine sampling.

Urine was sampled as a non-invasive alternative to blood. Spot urine samples were collected in the morning (10 am; to minimise the effects of circadian rhythm on MDA) on day eight, which was the start of the next weekly microcycle following each experimental week (T1: T3 [EXP group]; T3 [COM group]). This sampling time point was chosen specifically to determine the impact of the training load from the previous week. Samples were collected pre-training, after a period of 24 hr rest (i.e., following a post-match rest day, to avoid the effects of acute exercise), with participants in a fasted state. Participants provided urine samples by voiding into 70 mL sterile containers (Sarstedt, Leicester: UK). Samples were immediately stored on ice, protected from light (to avoid photo-oxidation). Within two hours of collection, aliquots of each sample (1.5 mL) were stored at -80°C for quantitative measurement of urinary MDA and creatinine concentration.

Participants in the EXP group were familiar with the technique of urine collection (providing samples for hydration & drugs testing). The lead investigator explained how to collect a *clean-catch sample* (using the technique of mid-stream collection to minimise bacterial contamination) to participants unfamiliar with the technique.

4.2.3.5 Analytical methods.

Urinary analytes, MDA & creatinine, were analysed in batches at each time point by the lead investigator. Urinalyses were conducted using a Cecil 1000 spectrophotometer (Cecil Instruments, Cambridge, UK) with quartz cuvettes. (Wavelength accuracy of the spectrophotometer was checked at each time point, Appendix C). Criterion validity of the spectrophotometer was confirmed by comparing

standard samples to a criterion measure (Cecil Auris 2021 scanning Spectrophotometer, Cecil 100, Cecil Instruments, Cambridge, UK). Agreement between the methods was demonstrated using correlation coefficients ($r = .95$), paired samples t-tests ($p \geq .05$) and a Bland Altman plot (mean absorbance of the two methods plotted against differences in absorbance) with data within 95% LoA (data not shown).

4.2.3.5.1 Determination of urinary MDA.

Malondialdehyde was chosen as biomarker of lipid peroxidation as it is a common method of indirect assessment of oxidative stress within exercise literature and should be detectable in urine in the presence of oxidative damage to lipids (Lamprecht et al., 2004). Total MDA was measured using a standardised 2-Thiobarbituric Acid Reactive Substances (TBARS) spectrophotometric method (Buege & Aust, 1978), based on the principle that one molecule of MDA reacts with two molecules of 2-Thiobarbituric Acid under acidic conditions (Knoevenagel-type condensation) to yield a pink chromogen (Ohkawa et al., 1979; Yagi, 1976). In brief, 300 μ L urine samples were reacted with 300 μ L 2-Thiobarbituric Acid (pre-mixed with 10% acid solution in Dimethylsulfoxide [FR35 TBARS Cuvette Assay Kit, Oxford Biomedical Research Inc., Oxford: UK; sensitivity 1.0 μ M]), vortexed, incubated using a dry heat mantle (65°C; 45 min [DB-3, Techne]), and absorbance measured at 532 nm using a spectrophotometer (Cecil 100, Cecil Instruments). To control for background interference, samples were blanked against a reference cuvette (containing the sample reacted with 10% acid solution in Dimethylsulfoxide) and net absorbance calculated: sample absorbance optical density – sample blank absorbance optical density. Samples were assayed in single replicate. Total MDA

concentration (expressed in μM^6) was calculated using the regression equation generated from a standard concentration curve (0.0 - 20.0 μM MDA standards; Figure 4.2). Analytical variance: CV 1.21%; intra-assay variance: CV 10.98%; inter-assay variance CV 20.54%; determined from 10 replicate samples of QC, a common sample, & 10 independent samples, respectively). Samples below the sensitivity of the assay were excluded from analysis (resulting in removal of one value at each time point in the EXP group data [$n = 9$], and one value in the COM group [$n = 8$]). Individual differences in urine concentration were controlled through normalisation of MDA to urinary creatinine concentration (refer to section 4.2.3.5.2).

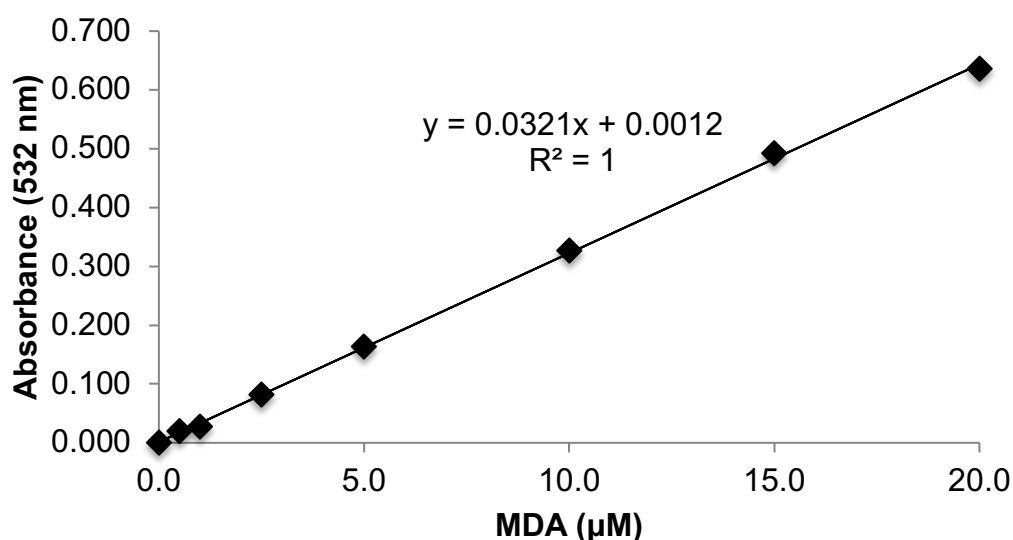


Figure 4.2. Exemplar of Malondialdehyde (MDA) standard concentration curve. nm = nanometres; μM = micromolars. Total MDA in the urine samples was calculated using the regression equation generated from the curve.

⁶ The terms TBARS and MDA are often used interchangeably. MDA standards were used to generate the standard concentration curve, hence, results reported as MDA.

4.2.3.5.2 Determination of urinary creatinine.

Urinary creatinine concentration was determined using a modified spectrophotometric method (QuantiChrom™ Creatinine Assay Kit DICT-500, Universal Biologicals Cambridge Ltd., Cambridge: UK; sensitivity 0.10 mg·dL⁻¹; linearity 300.00 mg·dL⁻¹), based on the principle of Jaffe (1886). Under alkaline conditions, picrate reacted with creatinine forms a red-coloured chromagen (peak absorbance 510 nm), the colour of which is directly proportional to creatinine concentration. To summarise, the picrate working reagent was first prepared by mixing 50 µL of reagent A (1.60% Sodium Hydroxide; < 0.05% EDTA) with 50 µL of reagent B (< 0.50% Picric Acid; 20.00% Dimethylsulfoxide; < 0.20% Polyoxyethylenesorbitan Monolaurate), and 100 µL of water (per sample). Urine samples (15 µL) were pre-diluted with dH₂O (20-fold), reacted with 1,000 µL picrate working reagent and absorbance measured (510 nm) after 1, and 5 min, and net absorbance calculated: optical density value of the sample at 5 min - optical density value of the sample at 1 min. Samples were assayed in duplicate. Urinary creatinine concentration was determined from a standard concentration curve using 50.00 mg·dL⁻¹ creatinine standard (Figure 4.3).

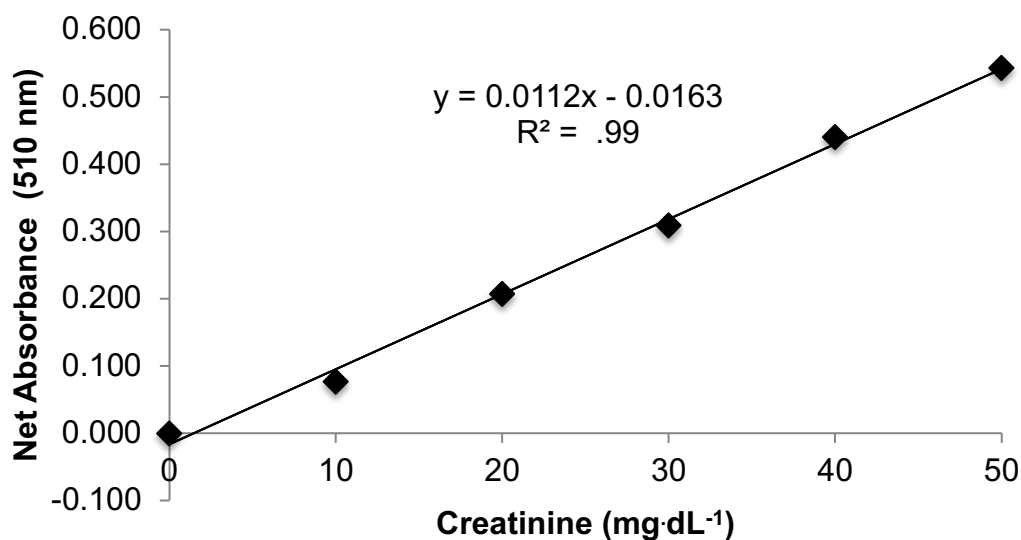


Figure 4.3. Exemplar of creatinine standard concentration curve. nm = nanometres; mg·dL⁻¹ = milligrams per deciliter. Urine creatinine concentration was calculated using the regression equation generated from the curve.

Results expressed in mg·dL⁻¹ converted to mmol·L⁻¹; conversion factor 0.08842. (Analytical measurement range: 0.01 - 26.53 mmol·L⁻¹; analytical variance: CV 3.0%; intra-assay variance: CV 3.4%; inter-assay variance: CV 8.2%, determined from 10 replicate samples of QC, a common sample, & 10 independent samples).

Urinary creatinine was used to control individual differences in the concentration of the spot urine samples, therefore, total MDA was expressed as a ratio of MDA: Creatinine (expressed in $\mu\text{M}\cdot\text{mmol}^{-1}$).

4.2.3.6 Dietary antioxidant intake.

Participants were instructed to maintain habitual dietary intake throughout the study with participants in the EXP group following dietary recommendations set by the soccer club (breakfast & lunch were provided daily & consumed in the club). To

avoid the participant burden of food records, total antioxidant intake was assessed via a self-administered antioxidant-specific food frequency questionnaire developed by Braakhuis, Hopkins, Lowe, & Rush (2011), the *Dietary Antioxidant Questionnaire* (DAQ; adapted version; test-retest reliability: ICC .971, 90% CI [.733, .997]; Appendix D, Braakhuis, 2010]). Responses to the DAQ were tick box, with participants indicating what and how much they ate by selecting the most appropriate answer for frequency and portion size (Braakhuis et al., 2011). Participants received verbal and written instructions on how to complete the DAQ with the lead investigator present to answer individual questions. Antioxidant intake was quantified using the coding provided with the questionnaire, total antioxidant intake reported in FRAP units (ferric reducing ability of plasma). Three incomplete questionnaires were discounted (EXP group, $n = 7$).

4.2.3.7 Psychological stress.

Psychological stress has been associated with oxidative stress (Liu & Mori, 1999). To ensure psychological stress was not a confounding variable, participants completed the Daily Analysis of Life Demands in Athletes (DALDA [Appendix E]) questionnaire (Rushall, 1990) at each time point, prior to training (EXP group T1: T3 [COM group at T3 only]). The DALDA is a self-report questionnaire that consists of 34 items, divided into two parts: (i) part A: sources of stress (9 items); and (ii) part B: symptoms of stress (25 items). Participants were required to rate each item from three responses: (i) a) *worse than normal*; (ii) b) *normal*; or (iii) c) *better than normal*. Participants received verbal and written instructions on how to complete the questionnaire with the lead investigator present to address queries at the time of completion. The number of part B '*worse than normal*' responses was totaled and

used to assess psychological stress (Rushall, 1990). Previous research has demonstrated increased part b 'a' scores in response to intensified periods of exercise, and the sensitivity of using the DALDA on a weekly basis (Achten et al., 2004; Robson-Ansley, Blannin & Gleeson, 2007). Participants in both groups (EXP vs. COM) reported low scores, with no change in scores over time or differences between groups (visually). Therefore, participants were not found to be experiencing symptoms of psychological stress and all participants' data was included for analysis (range 0 - 8; EXP group: T1: 2 [\pm 3]; T2: 2 [\pm 2]; T3: 2 [\pm 3]; COM group: 2 [\pm 2]).

4.2.4 Statistical analysis.

Statistical analyses were performed using the computerised statistical package SPSS (version 23.0, IBM Corp., Armonk, NY [also used in chapters 5 & 6]). Normality of the data was verified using (i) Shapiro-Wilk tests, (ii) inspection of histograms, (iii) box plots, (iv) skewness, and (v) kurtosis values. Outliers (defined as $1.5 * IQR$) were included for analysis as data were considered to be true biological observations. An Independent samples t-test was first conducted to test for differences in Total Antioxidant Intake between the groups (EXP vs. COM). A series of one-way analysis of variance with repeated measures (One-way RM ANOVA) were conducted to test for differences across time points (T1: T3) in variables of (i) high-intensity training load and (ii) MDA (EXP group only). Sphericity was verified using Mauchly's Test of Sphericity (Greenhouse-Geisser adjustment applied to data violating Sphericity, $\epsilon < .75$, or Huynh-Feldt correction, $\epsilon > .75$; Girden, 1992). Bonferroni post hoc corrections were used to detect significant differences, where applicable (Bonferroni adjustment $p \leq .017$). Friedman's tests, with post hoc Wilcoxon Signed ranks test (Bonferroni adjustment $p \leq .017$) were applied to data

that did not meet the assumption of normality (MDA normalised to creatinine data). An Independent samples t-test (uncorrected MDA) and Mann-Whitney U test (MDA normalised to creatinine) were conducted to compare MDA concentrations between EXP group and COM group at T3. Furthermore, correlations between indicators of high-intensity training load and lipid peroxidation (normalised MDA data) were analysed using Spearman's rank-order correlation; strength of correlations classified as $< .1$ = trivial; $< .3$ = small; $< .5$ = moderate; $< .7$ = large; $< .9$ = very large; < 1 = nearly perfect (Hopkins, Marshall, Batterham, & Hanin, 2009). Missing values in data sets were replaced with mean data for statistical tests where required. For all tests the significance level (α) was set at $p \leq .05$ (2dp). All results (parametric & non-parametric data) presented as mean (\pm *SD*). Effect sizes (classified according to Cohen, 1988: η^2 : $.01$ = small; $.06$ = medium; $.14$ = large; d : 0.2 = small; 0.5 = medium; 0.8 = large; r : $.1$ = small; $.3$ = medium; $.5$ = large [Coolican, 2019]) were reported alongside 95% confidence intervals (CI) where appropriate.

4.3 Results

Indicators of high-intensity training load were calculated for each one-week microcycle (T1: T3) in the cohort of professional soccer players (EXP). The dependent variable, urinary MDA, was assessed at the start of the following weekly microcycle and related to indices of high-intensity training load to assess the influence of the cumulated training load from the previous microcycle on lipid peroxidation. Comparisons of urinary MDA concentration between professional soccer players (EXP) and recreational soccer players (COM) were made at the end of a competitive in-season (T3). Results discussed hereafter.

4.3.1 Total Antioxidant Intake (EXP vs. COM).

An Independent samples t-test revealed no significant differences in Total Antioxidant Intake between groups (EXP group [$n = 7$]: $M = 81$, $SD = 34$ FRAP units vs. COM group [$n = 9$]: $M = 78$, $SD = 49$ FRAP units), $t(14) = -.139$, $p = .89$, 95% CI [-49, 43]; (results comparable to those originally reported by Braakhuis et al., 2011 for a cohort of recreational & trained rowers; 61 ± 31 FRAP units).

4.3.2 Training load (EXP group only).

Training variables for each week (T1: T3) are displayed (Table 4.3). The total number of sprints, accelerations, and decelerations followed a similar pattern, increasing across the competitive in-season.

4.3.2.1 High-intensity training load.

Indicators of high-intensity external training load (HETL) and high-intensity internal training load (HITL) are presented (Figure 4.4). Results showed that HETL and HITL followed a similar pattern, varying significantly across the competitive in-season (HETL: $F(2, 18) = 8.940$; $p = .002$; $\eta p^2 = .498$; HITL: $F(2, 18) = 8.494$; $p = .003$; $\eta p^2 = .486$). Post hoc analyses with Bonferroni correction revealed that indices of high-intensity training loads were significantly higher at T2: mid-season compared to T1: early in-season (HETL: $M = 18.56$, $SD = 7.30$ $m \cdot min^{-1}$ vs. $M = 6.71$, $SD = 2.62$ $m \cdot min^{-1}$, an increase of 11.84 $m \cdot min^{-1}$, 95% CI [4.33, 19.36], $p = .004$; respectively, HITL: $M = 60$, $SD = 34$ %Time > 80% HR_{max} vs. $M = 23$, $SD = 14$ %Time > 80% HR_{max} , an increase of 37 %Time > 80% HR_{max} , 95% CI [13, 60], $p = .004$).

Table 4.3

Training Variables & Body Mass; M (± SD)

Variable	T1	T2	T3
Duration (min)	100:16 (± 06:46)	71:32 (± 10:71)	98:20 (± 15:85)
TDC (m)	5311.1 (± 641.8)	4869.6 (± 877.6)	6710.5 (± 103.2)
Relative TDC (m·min ⁻¹)	52.9 (± 8.7)	68.5 (± 8.4)	68.2 (± 6.5)
Peak velocity (km·h ⁻¹)	27.49 (± 2.11)	27.18 (± 1.68)	26.13 (± 2.05)
HR _{max}	172 (± 23)	189 (± 18)	188 (± 15)
HR _{mean}	119 (± 12)	129 (± 16)	133 (± 11)
Total number of sprints	102 (± 38)	132 (± 29)	145 (± 40)
Total number of accelerations	296 (± 83)	384 (± 64)	419 (± 99)
Total number of decelerations	110 (± 43)	158 (± 35)	177 (± 41)
Body mass (kg)	81.6 (± 5.3)	81.2 (± 5.5)	79.7 (± 5.1)

Note. Training data presented as group mean of accumulated data for each one-week microcycle ($n = 10$). HR_{max} = maximum heart rate; HR_{mean} = mean heart rate; T = time point; TDC = total distance covered. Classifications (absolute thresholds determined by the manufacturer): sprint: > 2.8 m·s⁻² for 1 s, ending at 80% peak velocity; acceleration: > 1.6 m·s⁻² for 1.1 s; deceleration: > -2 m·s⁻² for 1.1 s. ^a Body mass remained stable over time as determined by a RM One-way ANOVA ($p \geq .05$).

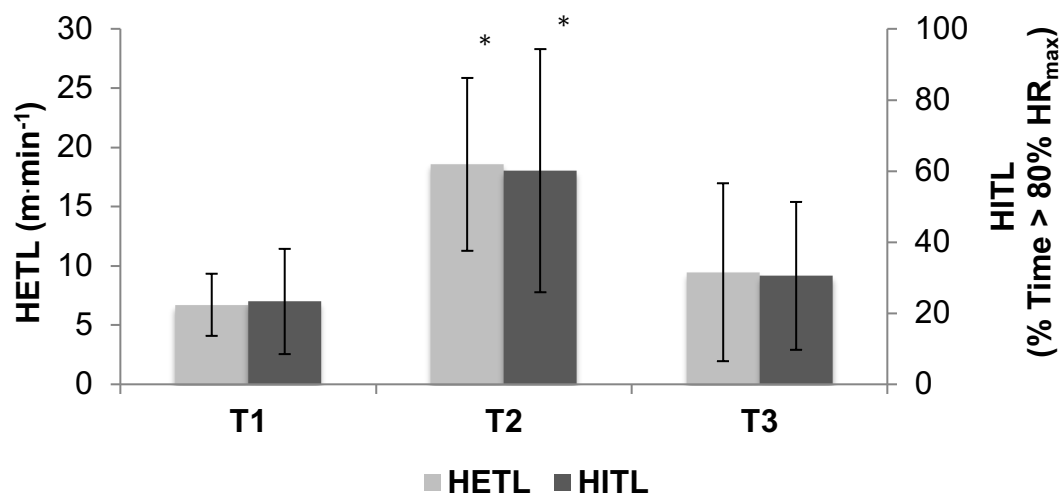


Figure 4.4. High-intensity external training load (HETL) & high-intensity internal training load (HITL) at time points (T) T1: 3 in the EXP group ($n = 10$). Data displayed as means, error bars represent *SD*. *Denotes significant difference to T1.

The frequency of high-intensity accelerations (HIACC) and decelerations (HIDEC) was consistent with HETL and HITL data, with the total number of HIACC and HIDEC observed to be highest at T2: mid-season (Figure 4.5). However, no significant differences were detected between time points across the competitive in-season (HIACC: $F(2, 18) = 1.655$; $p = .219$; $\eta^2 = .155$; HIDEC: $F(2, 18) = .1.300$; $p = .297$; $\eta^2 = .126$).

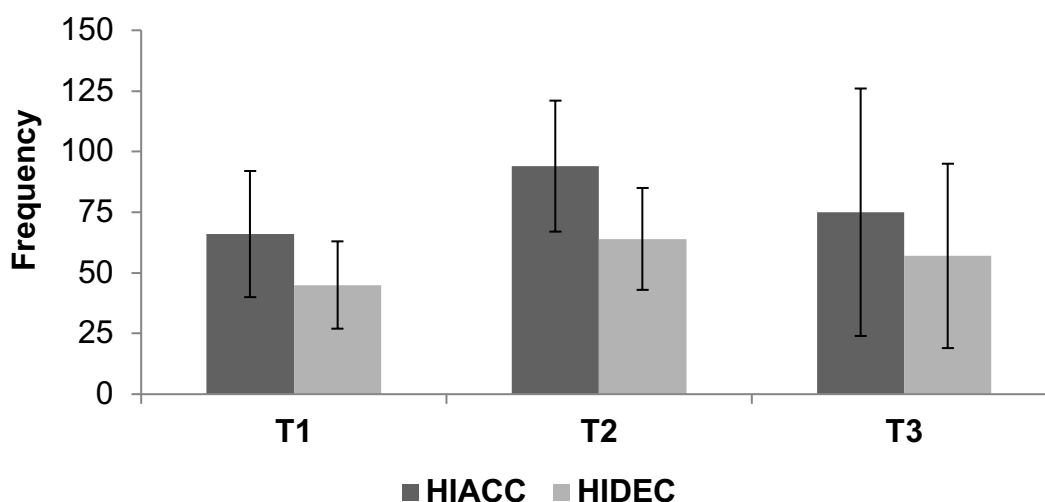


Figure 4.5. Total number of high-intensity accelerations (HIACC) & high-intensity decelerations (HIDEC) at time points (T) T1: 3 in the EXP group ($n = 10$). Data displayed as group mean of accumulated data for each one-week microcycle, error bars represent *SD*.

4.3.3 Lipid peroxidation (EXP; EXP vs. COM at T3).

Urinary MDA: Cr concentrations in the EXP group are presented as $M \pm SD$ (Figure 4.6). Urinary MDA: Cr decreased significantly over the competitive in-season, $\chi^2(2) = 6.889$; $p = .032$ (Figure 4.6). Post hoc comparisons did not detect a significance at $p \leq .017$, but there was a difference of $0.58 \mu\text{M}\cdot\text{mmol}^{-1}$ between T1 vs. T3 (T1 early in-season: $M = 0.76$, $SD = 0.90 \mu\text{M}\cdot\text{mmol}^{-1}$ vs. T3 end of in-season: $M = 0.18$, $SD = 0.12 \mu\text{M}\cdot\text{mmol}^{-1}$, $Z = -2.192$, $r = .52$, $p = .028$). There was also a significant difference between urinary MDA: Cr in the EXP group ($M = 0.18$, $SD = 0.12 \mu\text{M}\cdot\text{mmol}^{-1}$) versus the COM group ($M = 1.17$, $SD = 1.26 \mu\text{M}\cdot\text{mmol}^{-1}$) at T3, $U = .000$, $Z = -3.464$, $r = .84$, $p \leq .001$ (Figure 4.6).⁷ There were no significant differences detected in uncorrected MDA between the time points in the EXP group, or between the EXP and COM at T3, $p \geq .05$ (Table 4.4).

⁷ Median (IQR) values: T1 early in-season $Mdn = 0.51$ (IQR 0.17 – 1.07) $\mu\text{M}\cdot\text{mmol}^{-1}$; T3 end of in-season $Mdn = 0.15$ (IQR 0.08 – 0.15) $\mu\text{M}\cdot\text{mmol}^{-1}$; Mean ranks: EXP group (mean rank = 5.00); COM group (mean rank = 13.50).

Table 4.4

Uncorrected Urinary Malondialdehyde Concentrations; M (\pm SD)

Parameter	EXP ($n = 9$)			COM ($n = 8$)
	T1	T2	T3	T3
MDA uncorrected (μ M)	4.05	4.12	5.55	10.97
	(± 2.45)	(± 3.97)	(± 3.98)	(± 7.12)

Note. COM = comparison group; EXP = experimental group; MDA = Malondialdehyde; T = time point.

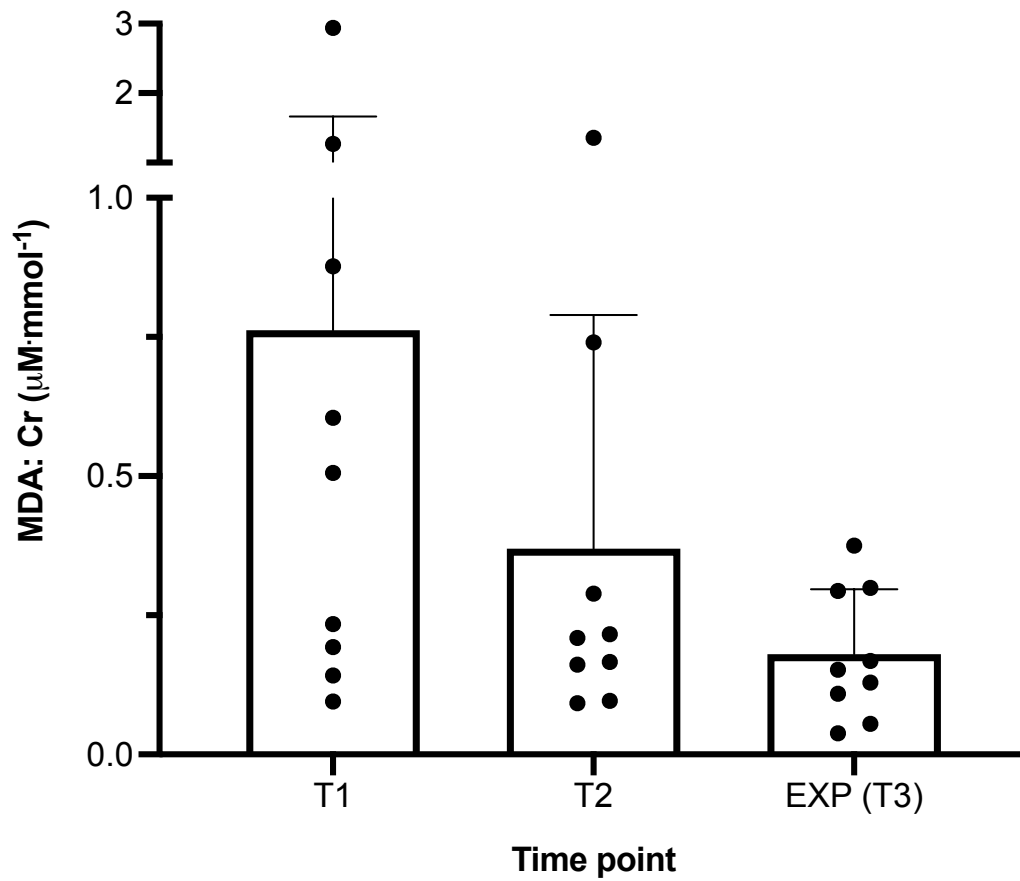


Figure 4.6. Normalised Malondialdehyde (MDA: Cr) concentration in the experimental group (EXP; $n = 9$). T = time point. Data presented as means, positive error bars represent SD. Significant difference across time points.

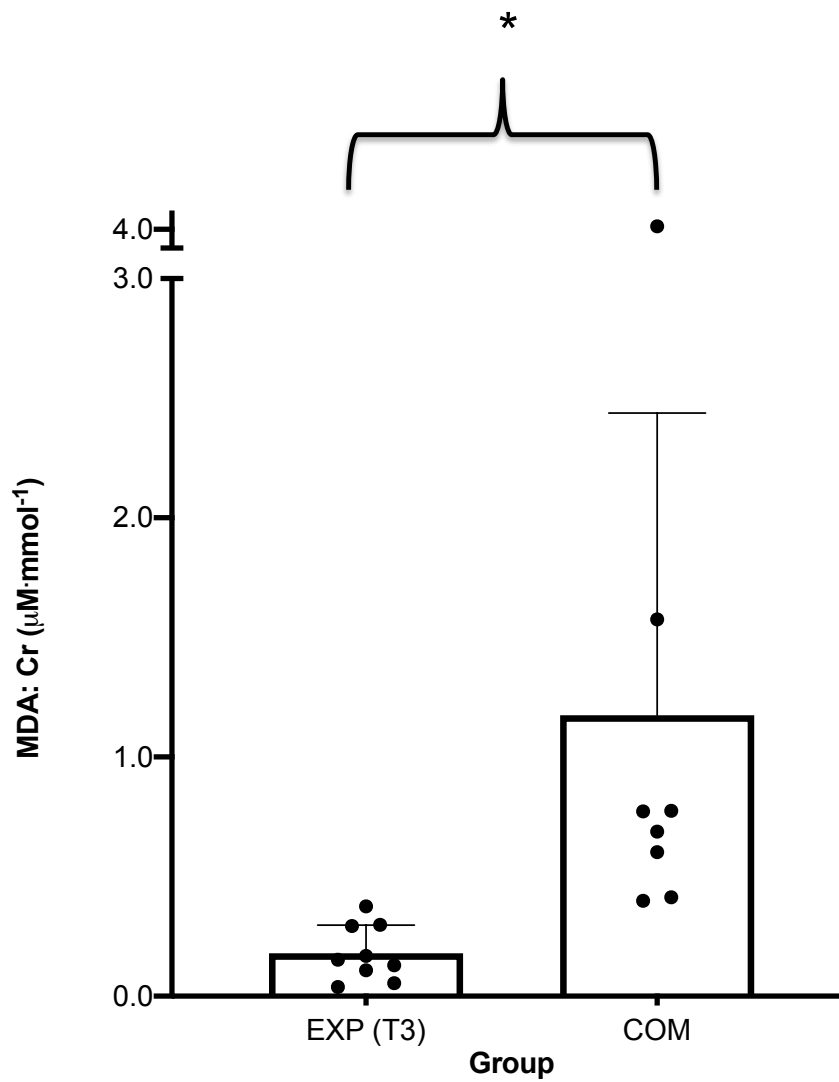


Figure 4.7. Normalised Malondialdehyde to Creatinine concentrations (MDA: Cr) in the experimental group (EXP; $n = 9$) & comparison group (COM; $n = 8$) at time point 3 (T3). Data presented as means, positive error bars represent SD. *Denotes significant difference between groups.

4.3.4 Association between high-intensity training load & lipid peroxidation.

Spearman's rank-order correlations revealed no significant correlations between variables of high-intensity training load ((i) HETL; (ii) HITL; (iii) HIACC; (iv) HIDEDEC) and lipid peroxidation (normalised MDA: Cr), $p \geq .05$ (Table 4.5).

Table 4.5

Correlations of High-Intensity Training Load & MDA

	MDA: Cr ($\mu\text{M}\cdot\text{mmol}^{-1}$)		
	r_s	p	95% CI
HETL	.063	.743	[- .305, .426]
HITL	.244	.194	[- .146, .580]
HIACC	- .156	.412	[- .459, .196]
HIDEDEC	- .269	.150	[- .567, .086]

Note. CI = confidence interval; HETL = high-intensity external training load; HIACC = high-intensity accelerations; HIDEDEC = high-intensity decelerations; HITL = high-intensity training load; MDA: Cr = Malondialdehyde: Creatinine; $n = 30$ observations for training load & oxidative stress (MDA missing values replaced with mean scores [$n = 3$]).

4.3.5 Inter-individual differences in lipid peroxidation.

Urinary MDA demonstrated high variance in both groups (Figures 4.5 & 4.6). To further explore lipid peroxidation responses, a subset of additional urine samples, provided by participants in the EXP group ($n = 4$) throughout the T1 microcycle, were analysed for MDA (as described; Appendix F).

4.4 Discussion

This study aimed to (i) quantify a biomarker of lipid peroxidation (indicative of EIOS), relative to indicators of high-intensity training load ((i) HETL; (ii) HITL; (iii) HIACC; (iv) HIDEDEC) in a cohort of professional soccer players (EXP group) throughout a competitive in-season (T1: T3), and (ii) to compare lipid peroxidation in professional soccer players to recreational soccer players (COM group) at the end of a competitive in-season (T3). This non-intervention, prospective study is innovative in that it quantified lipid peroxidation in urine, relative to objective measures of high-intensity training load (assessed using GPS & HR derived variables), in professional soccer players throughout a competitive season, thus fulfilling a gap in the literature (Le Moal et al., 2016). The findings are discussed hereafter.

4.4.1 Major findings.

The data demonstrated that urinary MDA concentrations decreased significantly throughout a competitive in-season in a group of professional soccer players (accepting hypothesis H_1), and that high-intensity training load was altered throughout the season (HETL; HITL [significant]; HIACC; HIDEDEC [non-significant]); with the greatest loads observed at the mid-season time point, and the lowest at the early in-season time point. The results also showed that professional soccer players exhibited significantly lower resting concentrations of urinary MDA at the end of the competitive in-season, in comparison to recreational players; who demonstrated urinary MDA concentrations seven-fold that of the professionals (accepting hypothesis H_2). However, the results did not demonstrate any significant associations between urinary MDA and indicators of high-intensity training load in professional soccer players (rejecting hypothesis H_3).

4.4.1.1 Urinary Malondialdehyde.

Malondialdehyde concentrations decreased significantly across the considered time points (negative 2-fold change at T1 vs. T2, & T2 vs. T3) in the professional soccer players, supporting the work of Ferrer et al. (2009), da Silva Barbosa et al. (2017) and Becatti et al. (2017), all of whom demonstrated a similar oxidative stress response over time periods in soccer players of a comparable level. The present study observed a -71% change in MDA across the season (T1 vs. T3), in similar agreement with Ferrer et al. (2009) who observed a 60% decrease in lymphocyte MDA over a three-month period of soccer training, and da Silva Barbosa et al. (2017) who reported a 21% decrease in serum MDA at the end of a competitive season, after 35 days of regular soccer training. Becatti et al. (2017), using time points similar to the present study, also demonstrated a decrease in plasma MDA over the course of a competitive season, reporting MDA concentrations greatest at T1, as in the present study. It should be noted that Becatti et al. (2017) demonstrated that MDA was increased across the season in comparison to a baseline (assessed prior to the first training session of the pre-season) and that these studies assessed MDA in blood components, in comparison to urine used in the present study. In contrast, Silva et al. (2013) observed increased MDA sampled at the middle and end of the competitive season in comparison to pre-season. The lead investigator speculates that the attenuation observed in MDA throughout the competitive in-season in the present study is evidence of an exercise-induced training adaptation and could be interpreted as such. It is proposed that exercise-induced oxidants act as cell signalling molecules that regulate gene expression for endogenous antioxidant enzymes via redox-sensitive transcription factors and pathways (which includes NF- κ B, also Nrf-2; MAPK; Gomez-Cabrera et al., 2009; Ji et al., 2004, 2007). Increased

endogenous antioxidant enzyme production would explain the decrease in MDA observed in the present study, as antioxidants exert effects through RONS scavenging, thus, potentially limiting lipid peroxidation (MDA is a secondary by-product of lipid peroxidation) to restore redox homeostasis (Powers & Radak, 2016). Support for this theory is provided by literature demonstrating that soccer players undertaking regular soccer training have enhanced antioxidant capacities in comparison to age-matched control groups (Brites et al., 1999 [sedentary controls]; Cazzola et al., 2003; [sedentary controls]; Mukherjee & Chia, 2009 [amateur players]). Evidence of an enhanced antioxidant defence system is also provided indirectly by the significantly lower basal MDA concentrations exhibited by the professional soccer players at the end of the competitive in-season (T3), in comparison to the recreational players who would have undertaken less training, despite groups being matched for total antioxidant intake. These results are in accordance with Cazzola et al. (2003) and Metin et al. (2003) who demonstrated lower plasma lipoperoxide and MDA concentrations in soccer players undertaking regular training compared to sedentary controls. It is postulated that the lower concentrations of MDA observed in the professional soccer players in the present study is further indication of training-induced adaptations. Taken together these results show that participation in regular high-level soccer training reduces oxidative stress, possibly through an enhanced antioxidant capacity (as concluded by Becatti et al., 2017; da Silva Barbosa et al., 2017; Ferrer et al., 2009). For athletes, an enhanced antioxidant capacity provides protection against the negative effects associated with EIOS and could translate to decreases in fatigue and muscle soreness, with implications for improved recovery and performance. However, the antioxidant status of participants in the present study was not measured,

subsequently, it cannot be directly concluded that the decrease in MDA over the competitive in-season was due to an increased antioxidant capacity, whereas the studies by Ferrer et al. (2009), da Silva Barbosa et al. (2017) and Becatti et al. (2017) also included analyses of antioxidants. Measurement of a single biomarker of oxidative stress (as used in the present study) may not accurately reflect redox status (Lee et al., 2017), thus, future research should consider including reductive biomarkers to accurately interpret redox status.

It should be noted that urinary MDA data observed in the present study revealed high inter-individual variability in the athletes (as demonstrated by large standard deviations in the data [Figures 4.5 & 4.6]), despite (i) each cohort being an homogeneous group (matched for sex; age; anthropometric measures; aerobic fitness; training load; dietary antioxidant intake); (ii) urinary MDA creatinine concentration correction accounting for individual differences in hydration status; and (iii) MDA quantified with a TBARS assay with enhanced sensitivity and specificity. Athletes often exhibit high variance in biomarkers (Heisterberg et al., 2013; Lee et al., 2017), however, an interesting finding was the observed high variance in MDA in the professional soccer players at the beginning of the competitive in-season (T1), in comparison to the end of the season (T3). T1 followed the first weekly microcycle of the competitive in-season, which was directly preceded by the pre-season training mesocycle. Therefore, it is proposed that the high variance observed in MDA at T1 is attributed to (i) the minimal control the soccer club had over individual fitness status and diet prior to the start of the season, and (ii) individual responses to pre-season training load and the onset of competitive match play. Furthermore, subgroup analysis (Appendix F) also revealed that three out of four of the professional soccer

players had elevated MDA at the start of the second microcycle (day 8), in comparison to the start of the previous week (day one). This data provides evidence of a cumulative oxidative stress response that may have implications for readiness to train in individual athletes. These results not only demonstrate the importance of regular and longitudinal monitoring in soccer, but the need for reliable biomarkers, sensitive to detect inter-individual changes in athletes (Lewis, Newell, Burden, Howatson, & Pedlar, 2016). Baseline concentrations of MDA prior to the start of the season were not assessed in the present study, however, normalising MDA to baseline may have accounted for inter-individual variance.

Many different biomarkers of oxidative stress have been used in soccer-based research making comparisons across studies difficult (Nédélec et al., 2012). At present, there is no recommended biomarker of oxidative stress. Sample type and method are usually dependent upon skill and instrumentation, but mechanism of action, participants, and cost should be considered (Marrocco et al., 2017). Expression of results is important, and inconsistencies exist within the literature. With reference to MDA, authors often fail to state whether total or free MDA was assayed, report different units, and do not use normalisation methods. For example, reporting results with or without correction for (i) post-exercise haemoconcentration (ii) protein content, or (iii) urine concentration. Data from the present study highlights the importance of expression of results (supporting Viitala & Newhouse, 2004), as uncorrected and corrected urinary MDA concentrations were reported, with conflicting results. Uncorrected urinary MDA concentrations demonstrated an augmented response throughout the competitive in-season in the professional soccer players, whereas, in contrast, creatinine-corrected urinary MDA was

attenuated over the course of the competitive in-season. The lack of consensus in standardising data could explain inconsistencies in previous research. Urinary MDA concentrations correlate positively with creatinine, with creatinine-correction of urinary MDA shown to reduce intra- and inter-individual variability (Martinez-Moral & Kannan, 2019). Therefore, the use of creatinine-corrected MDA in the present study strengthened the study design and it is recommended that future studies report creatinine-corrected urinary MDA concentrations to avoid inaccurate interpretation of results.

4.4.1.2 Training load.

Indices of high-intensity training load varied throughout the time points of the competitive in-season (significant HETL & HITL; non-significant HIACC & HIDECC) as expected, with reported daily and accumulative weekly data comparable to data previously observed during in-season microcycles in English soccer players of a similar standard (Anderson et al., 2016; Malone et al., 2015b). The greatest high-intensity training loads were observed at the mid-season time point (T2 [16th microcycle]), in accordance with Malone et al. (2015a). However, high-intensity training load was not associated with urinary MDA as hypothesised (rejecting hypothesis H_3). The lack of association between variables of high-intensity training load and lipid peroxidation is unclear, given the potential sources of oxidant generation (e.g., NAPH & xanthine oxidase pathways; mitochondrial respiration [Finaud et al., 2006; Magalhães et al., 2010]) associated with the metabolic stress of high-intensity exercise (Suerda et al., 2009). Furthermore, repeated acceleration and decelerations can induce mechanical muscle damage which is associated with an inflammatory response and increased oxidants as part of neutrophil burst activity

(Finaud et al., 2006). This is the first study to investigate potential associations between high-intensity training load and lipid peroxidation in soccer. The novel results are contradictory to previous research of a similar nature (Finaud et al., 2006b; Le Moal et al., 2016), which demonstrated that periods of intensified training were related to increased biomarkers of oxidative stress (rugby & soccer, respectively). Although, these studies did not quantify high-intensity training load per se, but quantified the overall intensity of training load, and analysed different oxidative stress biomarkers to that of the present study. Furthermore, Sureda et al. (2009) previously found higher intensity exercise ($> 90\%$ HR_{max} for more than 30 min) induced a greater lymphocyte MDA insult than exercise performed at lower intensities ($< 90\%$ HR_{max} for 30 min) following 60 minutes of soccer match play in trained soccer players. These results appear to suggest that urinary MDA may not be a suitable biomarker to reflect exercise intensity per se at the time points considered. The progressive reduction in lipid peroxidation observed over the competitive in-season, despite variations in high-intensity training load, demonstrates that participation in regular soccer training attenuates oxidative stress, and this adaptive response may conceivably be attributed to exposure to the volume of training, regardless of exercise intensity.

4.4.1.3 Alternative explanations.

In the present study, training load intensity was quantified using objective measures (assessed via GPS & HR) based upon (i) individual peak velocities and HR zones, and (ii) accelerometry. There are currently no set definitions, or preferred method, of quantifying training load intensities within soccer-based research (Akenhead & Nassis, 2016; Dwyer & Gabbett, 2012; Malone et al., 2015a). Even within the context

of GPS measures there are many different, often complex, variables that can be classified as *high-intensity* (Akenhead & Nassis, 2016). Research has predominantly used generic absolute speed thresholds; however, this method has been shown to misestimate training load dependent upon the sampling rate of the GPS units (e.g., 1 - 20 Hz; Casamichana, Morencos, Romero-Moraleda, & Gabbett, 2018). To understand the relative load imposed on individual athletes, the present study used individualised speed thresholds, relative to peak velocity (Dwyer & Gabbett, 2012), strengthening the study design, however, limiting comparability of the results (Barbero-Alvarez, Coutts, Granda, Barbero-Alvarez, & Castagna, 2010; Coutts & Duffield, 2010). Likewise, different thresholds defining high-intensity accelerations and decelerations exist within the literature (Dalen et al., 2016; Harper et al., 2019; Varley, Jaspers, Helsen, & Malone, 2017). It should be considered that quantification of high-intensity training load using alternative thresholds might have altered the load. A consensus on consistent methods and definitions to quantify high-intensity training load in soccer is warranted before definitive conclusions regarding high-intensity training load can be drawn (Akenhead & Nassis, 2016).

The time course of sampling a selected biomarker should also be considered (Lee et al., 2017). The response of MDA following a single soccer bout has previously been investigated (Ascensão et al., 2008; Fatouros et al., 2010; Ispirlidis et al., 2008), with MDA appearing to follow a specific time course. Plasma/serum MDA concentrations have been shown to peak 24 hours post-match and remain elevated above pre-match concentrations for up to 72 hours (Ascensão et al., 2008; Fatouros et al., 2010; Ispirlidis et al., 2008). Fatouros et al. (2010) proposed the post-exercise elevated MDA response to be related to exercise-induced muscle damage and the

inflammatory response; caused by increased neutrophil production of ROS at the site of injured muscle tissue. The time course of urinary MDA post-exercise has been demonstrated to peak at 12 hours (Subudhi, Davis, Askew, Walker, & Johnson, 2002). Therefore, the considered time points in the present study may have missed peaks in MDA concentration, which could have been linked to exercise intensity. Whilst peak and recovery data are of interest, the sampling time point used in this study was novel and has real-world application. Malondialdehyde was quantified at each time point at the start of the next subsequent training microcycle, specifically to assess the lipid peroxidation response to the loading of the previous one-week microcycle. This time point followed the maximal amount of post-match recovery permitted by the soccer club (42 hr) and was when the professional soccer players were expected to be in a state of readiness to train.

4.4.2 Strengths & limitations.

This study had high ecological validity as data was collected from a homogeneous cohort of professional soccer players (first team players of a League One club) throughout 3 one-week microcycles of a competitive in-season. Sample size and exclusion of competitive match play analyses were delimitations pre-set on the study, the latter of which would affect the estimation of high-intensity training load, providing evidence of the inherent difficulties of field-based research. Generalisability of the findings of the study can be transferred to athletes of the same level with comparable training loads. This study provides interesting data regarding training load intensity and lipid peroxidation throughout a competitive in-season in professional soccer players, adding to the literature, as there is a requirement for more prospective soccer studies (Jaspers, Brink, Probst, Frencken, & Helsen, 2017).

Further limitations are also acknowledged. Firstly, reliance on a single biomarker of lipid peroxidation to quantify oxidative stress limits the findings of the study to the sensitivity of the TBARS assay. Thus, it can only be speculated that observed decreases in MDA were due to an increase in endogenous antioxidants. An integrative approach, assessing biomarkers of antioxidant capacity or redox status (oxidant to reductive stress ratio) alongside lipid peroxidation would have provided further insight (Nikolaidis et al., 2012). Secondly, soccer-based studies have predominantly quantified MDA in blood components, making comparisons of this urine study challenging, although it has been proposed that urinary MDA may reflect changes in whole-body oxidative stress to a greater degree than plasma MDA (Drury et al., 1997; Weitner et al., 2016). Urine sampling was selected as a less invasive, convenient alternative to blood sampling (given that the players were already providing daily urine samples for hydration testing).

4.4.3 Biological significance.

In theory, monitoring the response of a biomarker of lipid peroxidation following prolonged and strenuous exercise, such as professional soccer training sessions and matches, could prove a useful tool to aid sports scientists and coaches in the management of fatigue, recovery, and training prescription; however, the biological significance of the response of such biomarkers remains debated (Becatti et al., 2017). There is currently no definitive concentration of a biomarker that indicates oxidative stress, and there are limited athlete-specific reference ranges for biomarkers (Lee et al., 2017); despite highly trained athletes often exhibiting higher oxidative stress concentrations than reference ranges (Hadžović-Džuvo et al., 2014; Lewis et al., 2016). In the present study, urinary MDA concentration was attenuated

(2-fold negative change) in professional soccer players throughout the course of the competitive in-season across the considered time points (T1: early in-season [1st microcycle]; (ii) T2: mid-season [16th microcycle]; and (iii) T3: end of in-season [32nd microcycle]). Whether this potentially adaptive cellular response manifests to a meaningful change at the level of the athlete, for example, improved skeletal muscle function, and reduced perception of effort for a given exercise intensity, should be considered.

4.4.4 Conclusion & further research.

In conclusion, high-intensity training load was altered throughout a competitive in-season in professional soccer players but was not associated with increased lipid peroxidation, which decreased throughout the season, and was demonstrated to be lower in professional soccer players at the end of a competitive season, in comparison to recreational players. At present, the relationship between oxidative stress and training load intensity remains unclear. These results suggest that regular chronic participation in professional soccer training appears to reduce lipid peroxidation and it is proposed that this may be a beneficial training adaptation to increased exercise tolerance within skeletal muscle. The use of a urinary MDA as a biomarker sensitive to detect training intensity appears unsupported, but may potentially reflect the volume of training. Exercise-induced oxidative stress has been associated with negative consequences for athletes, which may be an issue for recreational soccer players, who demonstrated greater concentrations of lipid peroxidation in the present study. Sports scientists should be aware of intra- and inter-individual differences in exercise-induced lipid peroxidation. For athletes to be in a state of readiness to train prior to the subsequent training session or match, it

may be necessary to prescribe altered training loads, or implement a recovery strategy (e.g., antioxidant supplementation) to diminish oxidative damage, counteracting the associated negative side effects in susceptible athletes. It is proposed that future research include multiple biomarkers of (i) oxidative stress, antioxidant capacity, or redox status; (ii) muscle damage; and (iii) inflammation to (a) determine potential training intensity- or recovery-dependent biomarkers, and (b) further increase understanding of the interactions and underlying mechanisms of EIOS in soccer, as was the focus of the next study.

Chapter 5

Effects of a Three-Day Period of Prolonged High-Intensity Intermittent Exercise

on Biomarkers of Oxidative Stress, Muscle Damage, and Inflammation

(Study two)

5.1 Introduction

The metabolic and mechanical demands of soccer have been well documented (Bangsbo, 1994; Heisterberg et al., 2013). Soccer has been shown to induce oxidative stress (Fatouros et al., 2010; Finaud et al., 2006a; Ispirlidis et al., 2008), with proposed sources of oxidant production within skeletal muscle including (i) NOX; (ii) increased mitochondrial oxygen consumption; (iii) ischemic-reperfusion xanthine oxidase production; and (iv) neutrophil respiratory burst activity (Finaud et al., 2006a; Henríquez-Olguín et al., 2020; Powers & Jackson, 2008; Powers et al., 2010a; Vollard et al., 2005). Exercise-induced oxidants are hermetic (Radak et al., 2007). Increased oxidants can have negative connotations for the athlete; leading to muscle damage, decreased force production, and fatigue (Ascensão et al., 2008; Powers et al., 2011; Radak et al., 2017; Reid et al., 1993). Exercise-induced muscle damage is associated with a concomitant localised and acute-phase inflammatory response as part of the repair and regeneration of skeletal muscle tissue (Fatouros & Jamurtas, 2016; Gomez-Cabrera et al., 2016; Peake et al., 2017). However, inflammation is also a proposed cause of secondary muscle damage and muscle soreness (Jamurtas, 2018). Time course studies have demonstrated a soccer-specific pattern in exercise-induced muscle damage and inflammatory responses (Ispirlidis et al., 2008; Romagnoli et al., 2016; Souglis et al., 2015), consistently reporting transient increases in biomarkers of muscle damage, cytokines, acute-phase proteins, and leukocytes for up to 72 hours following a single soccer bout, which are normalised back to baseline throughout this timeframe with passive recovery. Studies have demonstrated inflammatory responses following two consecutive matches, as performed in tournaments (Andersson et al. 2010; Malm et al., 2004), but limited research exists on inflammatory responses following

consecutive bouts of soccer, as typically performed. This data is of interest, given that (a) the high physiological load of professional athletes involves training and competing daily with minimal recovery within weekly in-season microcycles, and (b) training in an inflamed state could potentially result in cumulative fatigue. Recently, Mohr et al. (2015) simulated a typical three-match weekly microcycle, investigating biochemical responses to three experimental soccer matches separated by three, and four days of training, respectively. Biomarkers of inflammation (CRP; IL-6; IL-1 β ; leukocyte counts), muscle damage (CK), and oxidative stress (TBARS; protein carbonyls) were reported to increase after each match, paralleling decrements in performance and recovery (decreased repeated sprint ability; decreased knee joint ROM; increased muscle soreness). Interestingly, the majority of markers were observed to peak following the second match in comparison to the third match, which suggests that 3 days between matches with continued training to be an insufficient recovery period. Previous research has predominantly explored biochemical responses following single bouts of soccer-specific exercise (Mohr et al., 2015), but there is paucity in the literature investigating biochemical responses following repeated bouts of soccer-specific exercise performed on consecutive days. The underlying mechanisms of exercise-induced inflammation are complex (Cuevas et al., 2005). Several researchers have studied exercise-induced inflammation (Malm, 2001; Pederson, 2000; Pyne, 1994b; Peake et al., 2017; Vider et al., 2001) and it is proposed that the redox-sensitive transcription factor, NF- κ B, may play a key role in regulating inflammatory and immune responses following exercise (Niemen et al., 2007a). NF- κ B is activated by intracellular exercise-induced oxidants (Allen & Tressini, 2000; Aoi et al., 2004; Vider et al., 2001) and regulates gene expression of acute-phase proteins, inflammatory cytokines, immune cells, and

antioxidant enzymes to promote adaptations within skeletal muscle (Gomez-Cabrera et al., 2006; Ji et al., 2004; Niemen et al., 2007a). Exercise-induced activation of NF- κ B has been well documented in animals (Ji et al., 2004); yet, this has not translated to human exercise studies, which have shown contradictory results. For example, studies have demonstrated NF- κ B activation in response to endurance, anaerobic cycling and resistance exercise (Cuevas et al., 2005; Gomez-Cabrera et al., 2006; Vella et al., 2012, respectively), however, no NF- κ B activity in response to downhill running (Buford et al., 2009; Koenig et al., 2015). Observed differences in NF- κ B activation in these studies could be attributed to methodological differences, including different modes and intensities of exercise, sampling, and analysis techniques. No data currently exist on the NF- κ B response to prolonged high-intensity intermittent exercise, as performed in team sports, such as soccer.

Further research investigating the time course of exercise-induced oxidative stress, muscle damage, and inflammatory responses following consecutive bouts of soccer is warranted and may have implications for within microcycle recovery periods, which may be insufficient to resolve the cumulative negative effects associated with these phenomena. Biomarker monitoring of these responses may (a) aid sports scientists/coaches in the prescription of training loads that promote recovery, and (b) support the implementation of acute recovery strategies, for example, an antioxidant supplementation intervention. Therefore, the aims of this study were: (i) to explore the short-term response (42 hr) of an array of blood biomarkers indicative of (i) oxidative stress, (ii) muscle damage, and (iii) inflammation following a 3-day intensified period of prolonged high-intensity intermittent in trained athletes (using a 3-d soccer-specific intensified exercise protocol designed to simulate the

physiological demands of soccer in a controlled laboratory setting), and (ii) to attempt to elucidate underlying mechanisms of these phenomena by studying the NF- κ B classical pathway.

Biomarkers included:

- Oxidative stress: MDA and TAS.
- Muscle damage: CK and LDH.
- Inflammation: NF- κ B p65 activation, CRP, IL-6, IL-8, IL-10 and MCP-1.
- Total leukocyte and neutrophil counts.

The rationale for the selected biomarkers was based upon previous exercise literature and aimed to provide a comprehensive picture of exercise-induced oxidative stress, muscle damage, and pro- and anti-inflammatory responses. Pro-inflammatory biomarkers implicated to (i) be under the transcriptional regulation of NF- κ B (NF- κ B binds to promoter regions of DNA for genes transcribing cytokines IL-6, IL-8, & MCP-1), and (ii) induce neutrophil mobilisation were selected; the anti-inflammatory cytokine IL-10 chosen as a suppressor of NF- κ B regulated cytokines.

5.2 Methods

5.2.1 Participants.

Twenty participants volunteered to take part in the study. Recruited by convenience sampling, participants formed two groups: (i) experimental group (EXP; $n = 10$ [$n = 8$ males; $n = 2$ females⁸]); and (ii) control group (CON; $n = 10$ [$n = 8$ males; $n = 2$ females]) counterbalanced for sex, age, fitness, anthropometric data and TAS baseline concentrations (limited to TAS because of economic viability; participants' characteristics appear in Table 5.1). Participants were healthy, trained intermittent sports athletes who met the inclusion criteria ((i) aged: 18 - 40 years; (ii) $\dot{V}O_2\text{max}$: $\geq 35 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ [female]; $\geq 40 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ [male]; (iii) non-smoker; (iv) regularly training in soccer, other team sport or intermittent exercise [minimum twice per week], (v) previously trained for a minimum of 12 months; and (vi) competent in 1.5 hr high-speed treadmill running, performed on three consecutive days). Participants were asked to refrain from using (a) anti-inflammatory medication and nutritional supplements exceeding 100% Reference Nutrient Intake (RNI), and (b) an excessive intake of herbs known to affect inflammation or oxidative stress (Nieman et al., 2014) throughout the time course of the study. Participants were excluded from the study if they suffered from gastrointestinal or blood disorders. Female participants agreed to test during the luteal phase of the menstrual cycle to reduce the influence of the proposed antioxidant properties of oestrogen (Keane, Salicki, Goodall, Thomas, & Howatson, 2015). A priori power estimations (repeated measures ANOVA, between-within interaction; $\alpha = .5$; $\beta = .2$; $1-\beta = 80\%$; two tailed [G*Power version 3.1]) conducted using effect sizes reported by Souglis et al. (2015) for primary variables, CK ($f = 2.3$) and IL-6 ($f = 0.25$), proposed a total sample size of between 4 and 24

⁸ Female participants were recruited in the same female to male ratio as per Nieman et al. (2009).

participants, respectively, (2 & 12 per group) sufficient to detect large and medium effects. Post hoc power analyses (using $N = 20$; effect sizes $[\eta_p^2]$ and α calculated for each dependent variable in SPSS) confirmed adequate statistical power.

Table 5.1

Descriptive Characteristics of the Participants; M (\pm SD)

Variable	EXP ($n = 10$)	CON ($n = 10$)
Age (years)	31 (± 6)	27 (± 8)
Stature (cm)	172.6 (± 6.6)	178.2 (± 9.1)
Body Mass (kg)	67.4 (± 6.5)	73.8 (± 14.1)
Body Fat (%)	15.8 (± 3.7)	15.0 (± 3.6)
$\dot{V}O_2\text{max}$ ($\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$)	52.8 (± 5.6)	49.2 (± 5.8)
HR_{max} ($\text{beats}\cdot\text{min}^{-1}$)	187 (± 6)	186 (± 5)
TAS ($\text{mmol}\cdot\text{L}^{-1}$)	1.38 (± 0.31)	1.19 (± 0.26)

Note. CON = control; EXP = experimental; HR_{max} = maximum heart rate; TAS = Total Antioxidant Status; $\dot{V}O_2\text{max}$ = maximal oxygen uptake.

^aIndependent Samples t-tests (Mann Whitney U tests for non-parametric data) confirmed no significant differences between the groups for characteristics or baseline TAS data ($p \geq .05$).

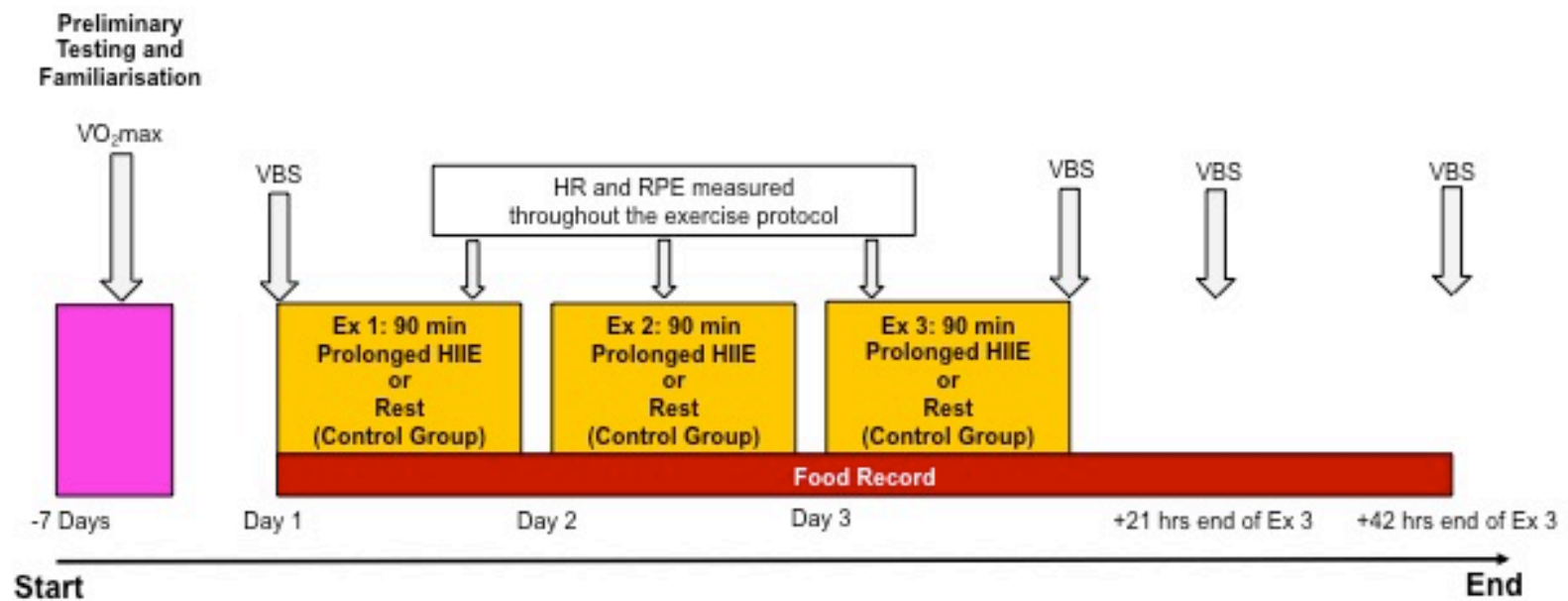
5.2.2 Experimental design.

A matched group repeated measures design was employed. Participants (EXP group) completed a 3-day exercise protocol over a time course of five days (6 laboratory visits in total). Participants first undertook preliminary testing to establish inclusion into the study ($\dot{V}O_2\text{max}$ testing); followed by randomisation into either (i) EXP or (ii) CON group (simple random allocation), and familiarisation with experimental testing procedures. Approximately one-week later, participants in the

EXP group reported to the laboratory (24 hr rested state) to perform a 3-day period of soccer-specific prolonged high-intensity intermittent exercise, performed on three consecutive days, followed by a 42 hr recovery period, reporting back to the laboratory at 21 and 42 hr post-exercise Day 3. The 42 hr recovery period was specifically chosen as it reflects the maximal post-match recovery time typically used in professional soccer (Mohr et al., 2015). This time point is when the athletes would next be expected to train and was chosen to assess athlete readiness. Blood sampling was conducted at the following time points: (i) pre-exercise Day 1 (within 15 min of the start of exercise) (T1); (ii) post-exercise Day 3 (within 15 min of cessation of exercise) (T2); (iii) 21 hr post-exercise Day 3 (T3); and (iv) 42 hr post-exercise Day 3 (T4; Figure 5.1). Peripheral venous blood samples were collected for the analysis of an array of systemic biomarkers, including (i) oxidant and reductive stress, (ii) muscle damage, (iii) pro- and anti-inflammatory cytokines, (iv) CRP and (v) total leukocyte and neutrophil counts to provide further insight into exercise-induced oxidative stress, muscle damage and inflammation. To explore underlying mechanisms, NF- κ B p65 subunit activation was assessed in PBMCs. Participants were asked to adhere to their habitual diet and record food intake throughout the experimental procedures. Dietary intake prior to resting blood samples and exercise was controlled through consumption of a standardised meal replacement drink as per Nieman et al. (2014) which was consumed ~2 hours prior to testing on each experimental day. To control for the effects of acute exercise prior to the start of the experiment, participants were asked to refrain from exercise for 24 hr prior to Day 1, and to participate in minimal training one week prior to testing, providing a training log for that week which was reviewed by the lead investigator. During the 42 hr recovery period, participants were also asked to rest and avoid the use of recovery

strategies that may influence the results of the study (e.g., compression, cold water immersion, massage; as discussed with the lead investigator). To account for the effects of psychological stress impacting biomarker data, participants completed the Perceived Stress Scale (PSS; Cohen, Kamarack, & Mermelstein, 1983) on Day 1. Participants deemed to be suffering from high stress (score > 20) were to be excluded from the study.

A control group was included in the study design to account for inter-individual variance in biomarker data across the considered time points. Participants in the CON group completed all procedures as per the EXP group, with the exception of the 3-day exercise protocol; where participants agreed to abstain from training and were observed resting seated in the laboratory for the duration of the exercise. Blood samples were taken at the same time as the EXP group over the five-day period.



Key:

Ex = Exercise; HIIE = High-Intensity Intermittent Exercise; HR = Heart Rate; RPE = Ratings of Perceived Exertion; VBS = Venous Blood Sampling; VO_{2max} = Maximal Oxygen Uptake Test

Figure 2.1. Schematic of the overall study design. Participants in the EXP group completed the 3-day intensified period of 90 min·d⁻¹ exercise, whereas participants in the CON group remained resting for the same duration.

5.2.3 Experimental procedures.

5.2.3.1 Preliminary testing.

Preliminary testing and familiarisation procedures were conducted a minimum of one-week prior to main experimental testing.

5.2.3.1.1 Anthropometric measures.

Stature, body mass and percentage body fat were assessed (refer to General Methods section 3.2.1).

5.2.3.1.2 Maximal oxygen uptake ($\dot{V}O_{2max}$).

Refer to General Methods section 3.2.1.4.

5.2.3.1.3 Familiarisation session.

Participants attended a familiarisation session with the following aims:

- (i) to practice running on the treadmill; to confirm that the treadmill speeds of the exercise protocol were achievable;
- (ii) to establish any adverse reactions to either blood sampling, or the standardised meal;
- (iii) to be trained in procedures of food recording & completion of a training diary; and
- (iv) familiarisation with the PSS

After a standardised warm-up (10 min self-paced, sub-maximal run & dynamic stretching), participants (EXP group) completed a 15 min block of the exercise protocol, experiencing the different treadmill speed changes (6.0; 12.0; 15.0; 18.0

km·h⁻¹). Participants (both groups) were issued the standardised meal replacement drink (Ensure Plus; energy intake adjusted for body weight [10 kcal·kg⁻¹]) and asked to consume the individualised amount, reporting any side effects. Participants were also familiarised with the procedure of venous blood sampling, the PSS, and trained in the use of food records.

5.2.3.2 Exercise protocol.

Following preliminary testing and familiarisation, participants reported to the laboratory for a five-day period to complete the 3-day period of soccer-specific prolonged high-intensity intermittent exercise followed by a 42 hr recovery period (ambient laboratory conditions: temperature: 22.6°C (± 1.7); pressure: 101 kPa (± 1); humidity: 32% (± 6)). Participants were asked to report to the laboratory on each day of experimental testing 2 hr after consumption of the standardised meal replacement milkshake. The exercise protocol consisted of a 90-minute intermittent run (2 x 45 min bouts, 15 min passive recovery interval [total time 105 min·d⁻¹]) at ~75% HR_{max}, performed on a motorised treadmill with automatic controller software (refer to General Methods section 3.8.1; Figure 5.2). The protocol was performed three times, on three consecutive days (at the same time each day to account for diurnal variation & to allow ~24 hr rest between bouts). Participants were fitted with a heart rate monitor (model FT1) and asked to complete a standardised warm-up (10 min, sub-maximal run; dynamic stretching), followed by the exercise protocol. Heart rate was recorded continuously throughout the protocol (1 s intervals), and RPE at 15-minute intervals (15; 30; 45; 60; 75; 90 min). Water was available ad libitum throughout the protocol (with the amount consumed by participants recorded by the lead investigator). Verbal encouragement was provided throughout the exercise

protocol. Participants recovered by walking self-paced on the treadmill until a heart rate of ≤ 120 beats \cdot min $^{-1}$ was achieved. Heart rates were exported for analysis (1 s). Treadmill speeds were verified throughout the study (refer to General Methods section 3.3.2.2).

Participants in the CON group reported to the laboratory, as per the EXP group, however, instead of undertaking the exercise protocol participants remained resting seated for the duration of the exercise (90 min \cdot d $^{-1}$; 3 d), observed by the lead investigator.

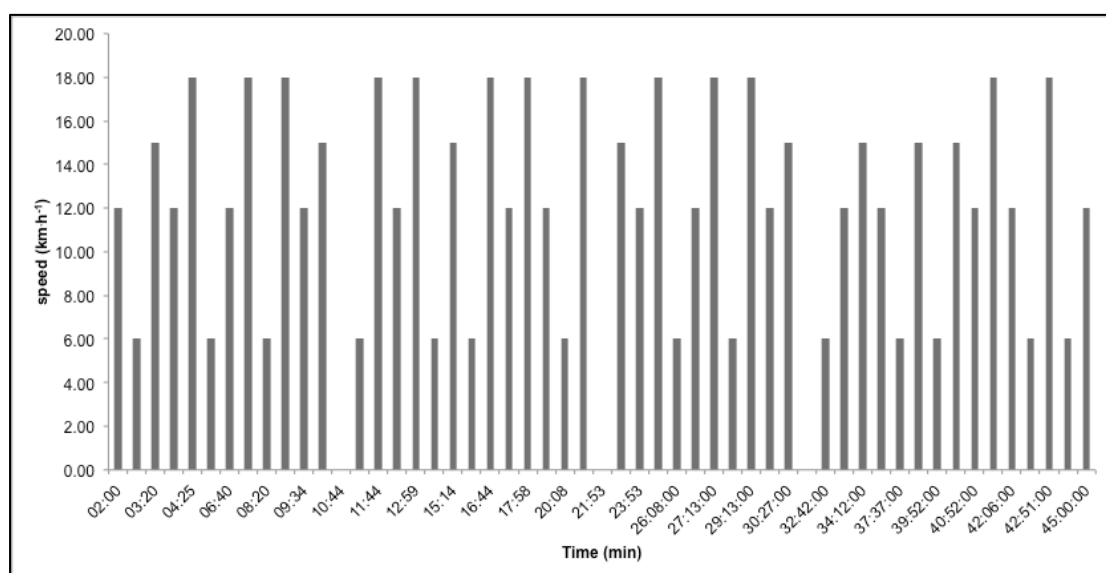


Figure 5.2. The modified Drust protocol. Participants in the EXP group performed the protocol on three consecutive days.

5.2.3.3 Perceived Psychological Stress (PSS).

To monitor perceived psychological stress participants completed the Perceived Stress Scale (PSS; Cohen et al., 1983) on Day 1, prior to the resting blood sample. Participants received verbal, and written instructions on how to complete the questionnaire, with the lead investigator present to answer questions. Participants scoring > 20 would be suffering from high stress and would be excluded from the study. All participants scored < 20.

5.2.3.4 Dietary Intake.

Participants were asked to maintain their habitual diet throughout the course of the study and to record their daily estimated food intake to monitor average daily energy intake, macronutrient energy contribution and micronutrient intake. Dietary intake prior to resting blood samples and exercise was controlled through consumption of a standardised meal replacement drink (refer to General Methods section 3.4.2.2), consumed two hours prior to testing on each experimental day. Food records were analysed and reviewed for group differences and adherence to the standardised meal replacement drink (refer to General Methods section 3.4.2.1). Independent samples t-tests (Mann Whitney U test for fat percentage & energy contribution) revealed no significant dietary differences between groups (Table 5.2). Participants in both groups met the RNI for micronutrients.

Table 5.2

Dietary Intakes of the Experimental & Control Groups M (± SD)

Variable	EXP (n = 10)	CON (n = 10)
Energy Intake (kcal·d ⁻¹)	2473 (± 370)	2472 (± 388)
Carbohydrate (%)	47 (± 6)	47 (± 4)
Fat (%)	29 (± 11)	34 (± 5)
PUFAs (g·d ⁻¹)	14 (± 3)	17 (± 5)
Protein (%)	19 (± 3)	19 (± 4)

Note. CON = control group; EXP = experimental group; g·d⁻¹ = grams per day; kcal·d⁻¹ = kilocalories per day; PUFAs = polyunsaturated fatty acids; carbohydrate, fat & protein presented as macronutrient energy contributions, expressed as percentage. No significant differences detected between groups, $p \geq .05$.

5.2.3.5 Blood collection, processing & storage.

Peripheral venous blood samples were collected at the following time points: (i) pre-exercise Day 1 (within 15 min of the start of exercise; T1); (ii) post-exercise Day 3 (within 15 min of cessation of exercise; T2); (iii) 21 hr post-exercise Day 3; T3; and (iv) 42 hr post-exercise Day 3; T4 (Figure 5.1); refer to General Methods section 3.3.3). Urinary biomarkers of oxidative stress, muscle damage, and inflammation have not been routinely assessed in athletes. Therefore, in keeping with the literature venous blood was sampled.

5.2.3.6 Analytical methods.

Blood samples were analysed for systemic markers of oxidative stress, muscle damage, and inflammation.

5.2.3.6.1 Muscle damage.

Sera samples were analysed for CK and LDH as biomarkers of muscle damage using a spectrophotometric technique. Refer to General Methods section 3.3.4.3 and 3.3.4.5.

5.2.3.6.2 Oxidant stress.

Sera samples were analysed for MDA, as a marker of lipid peroxidation and Total Antioxidant Status (TAS) as a marker of antioxidant capacity to assess oxidative stress using spectrophotometric techniques. Refer to General Methods sections 3.3.4.1 and 3.3.4.2.

5.2.3.6.3 Inflammation.

5.2.3.6.3.1 Determination of CRP.

Sera samples were analysed for CRP as a biomarker of the acute-phase response using a spectrophotometric technique. Refer to General Methods section 3.3.4.6.

5.2.3.6.3.2 Determination & quantification of inflammatory cytokines.

Multiple pro- and anti-inflammatory cytokines/chemokines (IL-6; IL-8; IL-10; & MCP-1) were analysed in sera by a member of staff at Randox Health Checks (Randox, London: UK), using the method of sandwich chemiluminescent immunoassay (Evidence investigator™ Biochip Array Technology [Randox Teoranta, Donegal: Southern Ireland] with Randox Cytokine & Growth Factors High-Sensitivity Array I [CTK HS EV 3623 Randox Laboratories Ltd., Co. Antrim: Northern Ireland]). The lead investigator was present during analyses. (Refer to General Methods section 3.3.4.5).

5.2.3.6.3.3 Determination of NF- κ B p65 subunit activation.

Peripheral Blood Mononuclear Cells (PBMCs) were analysed for NF- κ B p65 Subunit activation and normalised to nuclear protein concentration to account for individual protein concentrations. Refer to General Methods section 3.3.4.5.

5.2.3.6.3.4 Total leukocyte & neutrophil counts.

Total leukocyte and neutrophil counts were measured using an automated white blood cell differential counter (refer to General Methods section 3.3.4.8). Samples unsuitable for the analyser because of clotting were measured manually for leukocyte counts via staining and counting using a Neubauer haemocytometer ($n = 2$).

5.2.3.6.4 Plasma volume correction.

To correct for post-exercise changes in plasma volume (haemoconcentration), each analyte (assessed in serum; collected post-exercise [Day 3]) was corrected for plasma volume change using the haematocrit and haemoglobin method (Alis et al., 2015; Dill & Costill, 1974;). The concentration of each analyte was multiplied by percentage plasma volume change ($100 - PV\Delta\%$; expressed as a decimal; refer to General Methods section 3.3.4.9 for haematocrit & haemoglobin measurement & plasma volume equation). Leukocytes and neutrophil counts assessed in whole blood and were corrected using the alternative method of Matömaki et al. (2018; refer to General Methods section 3.3.4.8).

5.2.4 Statistical analysis.

Normality of data was checked using (i) Shapiro-Wilk tests, (ii) inspection of histograms, (iii) box plots, (iv) skewness, and (v) kurtosis values. Outliers (defined as $1.5 * IQR$) were included for analysis as data were considered to be true biological observations. Extreme outliers (defined as $3 * IQR$) were identified in the EXP group data set for the dependent variable CK (participant 10; refer to APPENDIX G) and data removed from the dataset⁹. Missing values in data sets were replaced with mean data. Differences in participants' characteristics and baseline TAS concentrations were checked via Independent Samples t-tests (non-parametric Mann Whitney U tests applied where data followed a non-normal distribution). Biomarker data predominantly followed non-normal distributions (excluding MDA, TAS & LDH), nevertheless, data sets were treated as parametric and analysed via mixed between-within ANOVA tests, as the assumption is that the power of *F*-tests is robust against violations of normality, even with small sample sizes as in this study ($n = 10$ per group; Ferreira, Rocha & Mequelino, 2012; Kahn & Rayner, 2003). A series of 2 x 4 mixed between-within ANOVA tests, with a between-subjects factor of group (EXP vs. CON), and a within-subjects factor of time (4 time points, T1: T4) were conducted to assess main effects for exercise and time, and interaction effects of the 3-day exercise protocol (in comparison to 3 days rest) on biomarkers of (i) oxidative stress (MDA; TAS); (ii) muscle damage (CK; LDH); and (iii) inflammation (CRP; IL-6; IL-8; IL-10; MCP-1; NF- κ B p65 activation; total leukocyte counts; total neutrophil counts) across four time points (pre-exercise [T1]; post-exercise Day 3 [T2]; 21 hr post-exercise Day 3 [T3]; and 42 hr post-exercise Day 3 [T4]). Homogeneity of variance was tested with Levene's. To investigate significant

⁹ The rationale for removal of data was that high basal CK concentrations could lead to alterations in post-exercise CK kinetics (Brancaccio, Mafulli, Politano, Lippi, & Limongelli, 2011).

interactions, follow up One-way RM ANOVA tests (Bonferroni correction applied ($.05 / 4 = p \leq .0125$)) were performed to test within-group differences over time, and Independent samples t-tests to test between-group differences at each time point (T1: T4). 95% CI and effect sizes (η_p^2) were estimated for significant main and interaction effects, classified according to Cohen (1988): $\eta_p^2 .01 =$ small; $.06 =$ medium; $.14 =$ large. For all statistical tests significance was set at $p \leq .05$ (2dp). Data expressed as mean (\pm SD).

5.3 Results

The present study assessed the short-term response of an array of biomarker responses to a 3-day period of prolonged high-intensity intermittent exercise, characteristic of soccer, in comparison to a resting control condition. Plasma and blood volume percentage changes from pre- to post-exercise were negligible ($< 1.02\%$); nevertheless, biomarker data are presented corrected for plasma or blood volume change, respectively. Results detailed below.

5.3.1 Physiological response of the 3-day exercise protocol.

The physiological load was consistent across the three days of the exercise protocol, with a downward trend in HR response (Table 5.3; Figure 5.3).

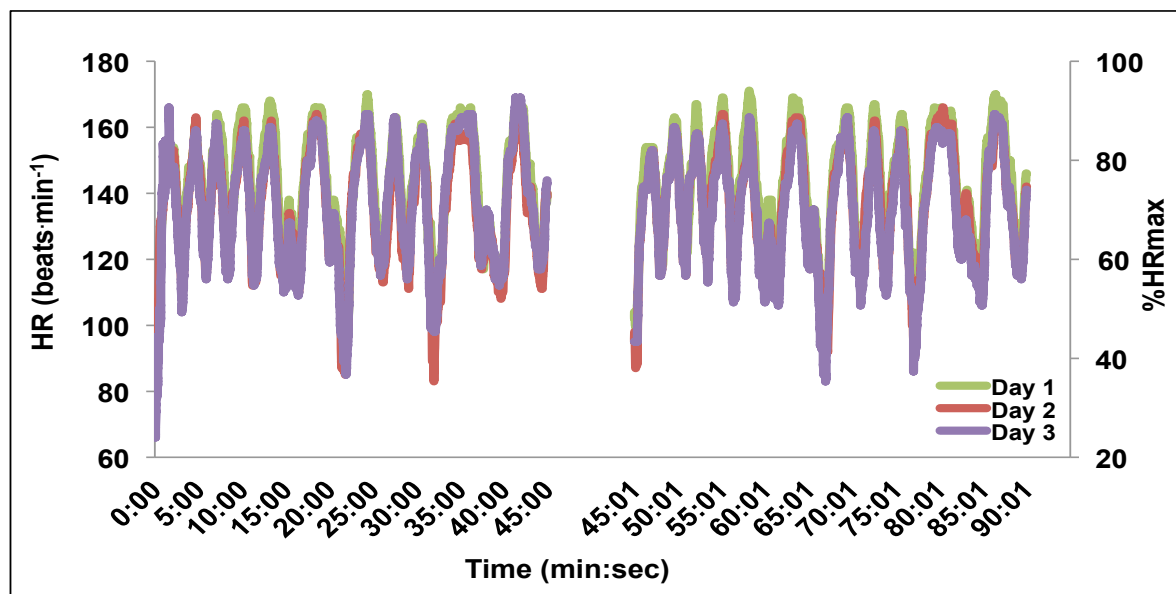


Figure 5.3. Daily heart rate response to the 3-day exercise protocol (exemplar data from one participant; HR 1 s averaging).

Table 5.3

3-Day Performance Measures (EXP); $M (\pm SD)$

Variable	Day 1	Day 2	Day 3	Mean
HR _{mean} (beats·min ⁻¹)	141 (± 12)	138 (± 11)	136 (± 16)	138 (± 11)
HR _{max} (beats·min ⁻¹)	174 (± 15)	173 (± 12)	170 (± 10)	172 (± 14)
HR _{min} (beats·min ⁻¹)	80 (± 14)	80 (± 9)	77 (± 10)	79 (± 11)
Percentage of HR _{max} (%)	75 (± 6)	74 (± 5)	73 (± 5)	74 (± 6)
RPE (AU)	12 (± 2)	12 (± 2)	12 (± 2)	12 (± 1)
Time \geq 80% HR _{max} (%)	18 (± 10)	16 (± 8)	16 (± 8)	17 (± 8)
Fluid intake (mL)	844 (± 310)	839 (± 248)	722 (± 182)	802 (± 220)

Note. Data from EXP group only ($n = 10$). Percentage of HR_{max} calculated from HR_{max} at $\dot{V}O_{2max}$. AU = arbitrary units; HR_{max} = maximum heart rate; HR_{mean} = mean heart rate; HR_{min} = minimum heart rate; RPE = Rating of Perceived Exertion. RPE presented as mean values.

^aRepeated Measures ANOVA detected no difference on variables across the three days, $p \geq .05$.

5.3.2 Biomarker responses to the 3-day exercise protocol.

The 3-day period of intensified exercise significantly altered biomarkers of muscle damage and inflammation as presented hereafter.

5.3.2.1 Exercise-induced muscle damage: CK & LDH.

Serum CK and LDH were assessed as indices of skeletal muscle damage to determine whether the stress of the 3-day exercise protocol caused EIMD. No differences were reported between baseline values (T1) for CK or LDH (CK & LDH responses are presented in Figure 5.4). There was a significant interaction between group and time for CK, $F(1.940, 34.928) = 6.283, p \leq .001, \eta_p^2 = .259$ (Greenhouse-Geisser correction), with a large effect for time, $F(1.940, 34.928) = 4.222, p = .009, \eta_p^2 = .190$. Post hoc one-way repeated measures ANOVA tests indicated significant differences in CK across time points in the EXP group, $F(1.787, 16.087) = 9.059, p \leq .001, \eta_p^2 = .502$; pairwise comparisons (Bonferroni corrected) revealed increased CK immediately post-exercise compared to baseline and the 42 hr recovery time point (T2: $M = 394.88, SD = 180.83 \text{ U}\cdot\text{L}^{-1}$, vs. T1: $M = 175.13, SD = 138.23 \text{ U}\cdot\text{L}^{-1}$, 95% CI [39.57, 281.74], $p = .009$; vs. T4: $M = 177.49, SD = 85.13 \text{ U}\cdot\text{L}^{-1}$, 95% CI [31.05, 335.37], $p = .017$), and differences between T3: $M = 268.07, SD = 84.76 \text{ U}\cdot\text{L}^{-1}$, 95% CI vs. T4: $M = 177.49, SD = 85.13, \text{U}\cdot\text{L}^{-1}$, 95% CI [21.99, 124.43], $p = .006$), in contrast to CK in the CON group, where no differences were reported across time points (Figure 5.4).

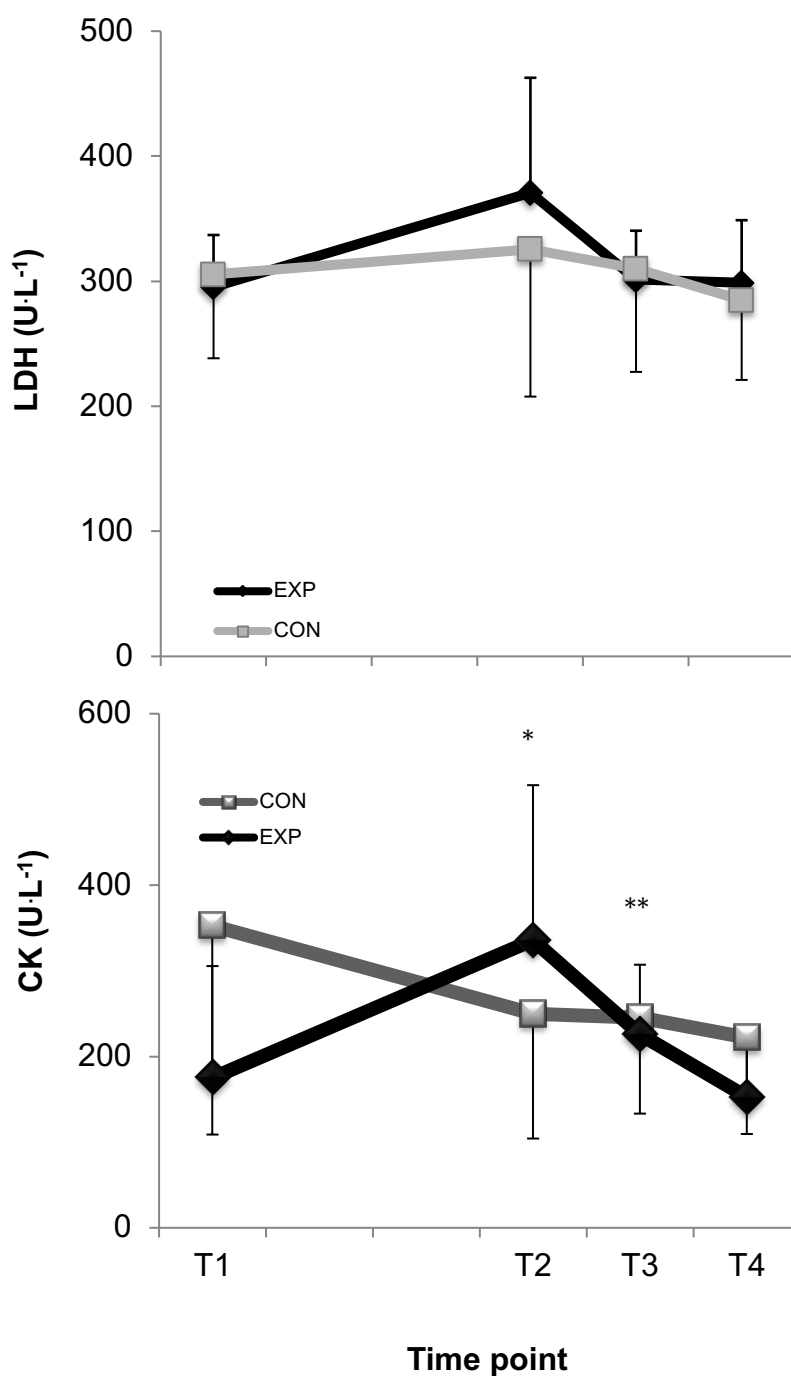


Figure 5.4. Serum Creatine Kinase (CK) and Lactate Dehydrogenase (LDH) responses in the EXP group ($n = 10$) and CON group ($n = 10$) pre (T1) and post a 3-day exercise protocol (T3) and throughout a recovery period 21 hr and 42 hr (T3; T4). Data displayed as means; error bars represent *SD*. *Denotes significant difference to T1 & T4, $p \leq .05$ ** significant difference to T4, $p \leq .05$. T = time point.

Lactate Dehydrogenase displayed similar responses to CK (Figure 5.4), however, no significant interaction was observed, $F(2.061, 37.102) = 1.369$, $p = .267$, $\eta_p^2 = .071$ (Greenhouse-Geisser correction applied), nor group effects ($F(1, 18) = 0.140$, $p = .713$, $\eta_p^2 = .008$). There was a substantial main effect for time, $F(2.061, 37.102) = 5.164$, $p = .010$, $\eta_p^2 = .223$. Pairwise comparisons (Bonferroni corrected) showed significant differences in LDH concentration, with LDH increased at T2 compared to T4 ($M = 348.15$, $SD = 105.43 \text{ U}\cdot\text{L}^{-1}$ vs. $M = 291.80$, $SD = 56.36 \text{ U}\cdot\text{L}^{-1}$, 95% CI [7.70, 105.01], $p = .018$; [pooled group data]. Within the EXP group, LDH increased pre-post-exercise by $\Delta 26\%$, compared to the CON group where LDH increased by $\Delta 7\%$ (non-significant).

5.2.2.3 Exercise-induced pro-inflammation.

5.2.2.3.1 Inflammatory Cytokines: IL-6, IL-8, & MCP-1.

Exercise-induced inflammation was assessed via an array of inflammatory cytokines (including chemokines) quantified in serum. Interleukin-6 and MCP-1 expression were altered in response to the 3-day exercise protocol (Figures 5.5 & 5.6). There was a significant interaction between group and time for IL-6, $F(2.316, 41.688) = 9.435$, $p \leq .001$, $\eta_p^2 = .344$ (Greenhouse-Geisser correction), with a substantial main effect for group, $F(1, 18) = 8.536$, $p = .009$, $\eta_p^2 = .322$. Post hoc analyses with independent samples t -tests revealed increased IL-6 immediately post the 3-day exercise protocol in the EXP group compared to the CON group, $t(18) = 5.314$, $p \leq .001$, $d = 2.37$, 95% CI [0.98, 2.26], ($M = 2.27$, $SD = 0.88$ pg·mL⁻¹ vs. $M = 0.65$, $SD = 0.40$ pg·mL⁻¹). There was also a large effect for time, $F(2.316, 41.688) = 9.925$, $p \leq .001$, $\eta_p^2 = .355$. Post hoc one-way repeated measures ANOVA tests indicated significant differences in IL-6 across time points in the EXP group, $F(3, 27) = 10.787$, $p \leq .001$, $\eta_p^2 = .545$; pairwise comparisons (Bonferroni corrected) revealed increased IL-6 immediately post-exercise compared to baseline and recovery time points (T2: $M = 2.27$, $SD = 0.88$ pg·mL⁻¹ vs. T1: $M = 1.03$, $SD = 0.84$ pg·mL⁻¹, 95% CI [0.22, 2.26], $p = .028$; vs. T3: $M = 1.01$, $SD = 1.01$ pg·mL⁻¹, 95% CI [-0.24, 2.28], $p = .018$; vs. T4: $M = 0.88$, $SD = 0.61$ pg·mL⁻¹, 95% CI [0.37, 2.41], $p = .011$), in contrast to IL-6 in the CON group, which was stable over time (Figure 5.5).

For MCP-1, there was a significant interaction between group and time, $F(3, 54) = 8.126$, $p \leq .001$, $\eta_p^2 = .311$. No main effect for group was observed, $F(1, 18) = 0.001$, $p = .978$, $\eta_p^2 \leq .001$, however, there was a large overall time effect, similar to IL-6, $F(3, 54) = 7.957$, $p \leq .001$, $\eta_p^2 = .306$. Post hoc analyses (one-way repeated

measures ANOVA with Greenhouse-Geisser correction) revealed significant differences in MCP-1 across time points in the EXP group, $F(1.519, 13.669) = 15.282$, $p \leq .001$, $\eta_p^2 = .629$, pairwise comparisons (Bonferroni corrected) indicated MCP-1 increased immediately post-exercise compared to all other time points (T2: $M = 214.88$, $SD = 81.26$ $\text{pg}\cdot\text{mL}^{-1}$ vs. T1: $M = 158.31$, $SD = 60.95$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [18.86, 94.29], $p = .004$; vs. T3: $M = 129.86$, $SD = 62.15$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [17.87, 152.], $p = .013$; vs. T4: $M = 157.15$, $SD = 54.71$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [15.95, 99.52], $p = .007$). Monocyte Chemoattractant Protein-1 remained stable over time in the CON group (Figure 5.6).

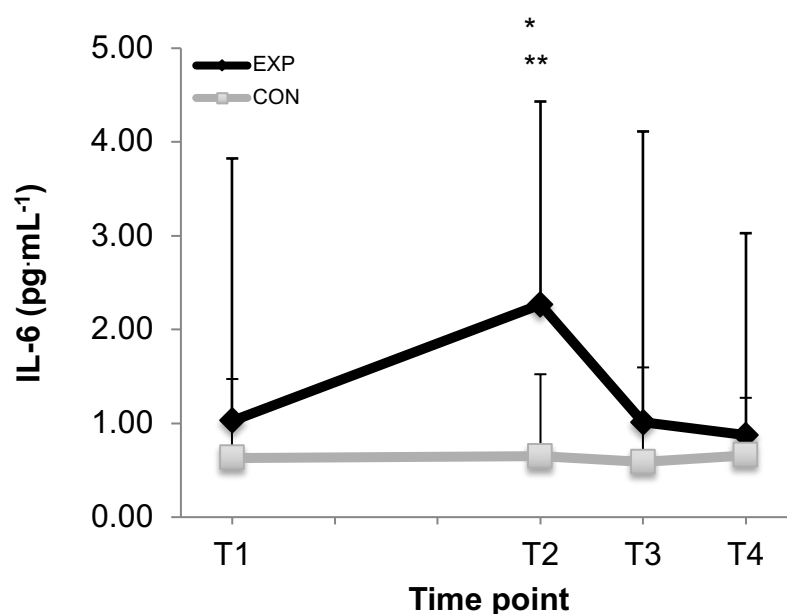


Figure 5.5. Serum Interleukin-6 (IL-6) responses in the EXP group ($n = 10$) and CON group ($n = 10$) pre (T1) and post a 3-day exercise protocol (T3) and throughout a recovery period at 21 hr and 42 hr (T3: T4). Data displayed as means; error bars represent SD. *Denotes significant difference between groups, $p \leq .05$, **denotes significant difference to T1, T3 and T4, $p \leq .001$. T = time point.

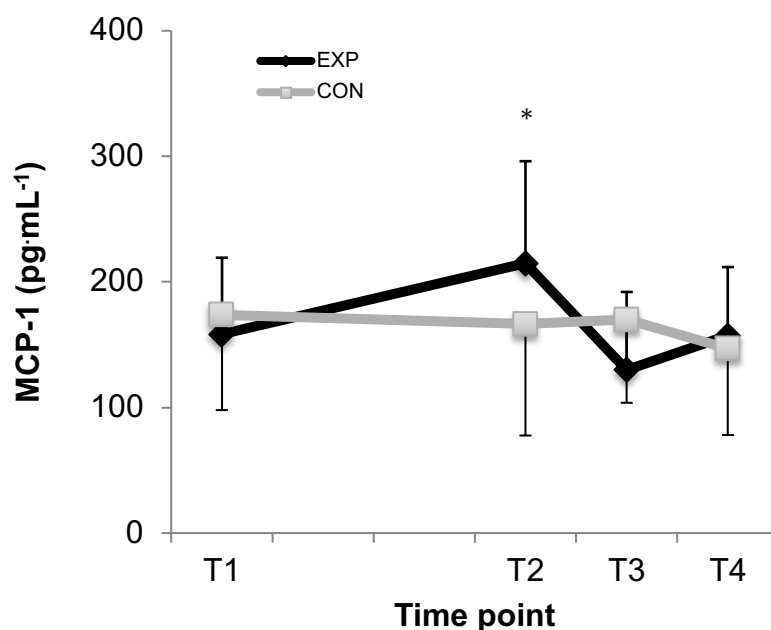


Figure 5.6. Serum Monocyte Chemoattractant Protein-1 (MCP-1) responses in the EXP group ($n = 10$) and CON group ($n = 10$) pre (T1) and post a 3-day exercise protocol (T3) and throughout a recovery period at 21 hr and 42 hr (T3: T4). Data displayed as means; error bars represent SD. *Denotes significant difference to T1, T3 and T4 in the EXP group, $p \leq .05$. T = time point.

No statistical interactions, or main effects for groups or time points were observed for IL-8 in response to the 3-day exercise protocol, $p \geq .05$ (Table 5.5).¹⁰

5.2.2.3.2 CRP.

To determine whether the 3-day exercise protocol induced a systemic inflammatory response the acute-phase protein CRP was assessed in serum. A 2 x 4 mixed between-within ANOVA revealed no significant interaction between group and time for CRP, $F(3, 54) = 2.248$, $p = .093$, $\eta_p^2 = .111$. There was a large main effect for group, $F(1, 18) = 12.611$, $p = .002$, $\eta_p^2 = .412$, with CRP concentration increased in the EXP group compared to the CON group ($M = 6.55 \text{ mg}\cdot\text{L}^{-1}$ vs. $M = 3.61 \text{ mg}\cdot\text{L}^{-1}$). There was also a moderate main effect for time, $F(3, 54) = 2.974$, $p = .040$, $\eta_p^2 =$

¹⁰ Results of the full cytokine array are presented (APPENDIX K).

.142. Pairwise comparisons (Bonferroni corrected) showed increased CRP concentration at T2, compared to T3 ($M = 6.27$, $SD = 4.15$ mg·L⁻¹ vs. $M = 5.1$, $SD = 3.45$ mg·L⁻¹, 95% CI [0.89, 2.12], $p = .035$), and T4 ($M = 6.27$, $SD = 4.15$ mg·L⁻¹ vs. $M = 4.20$, $SD = 2.02$ mg·L⁻¹, 95% CI [0.46, 3.69], $p = .015$; Figure 5.7).

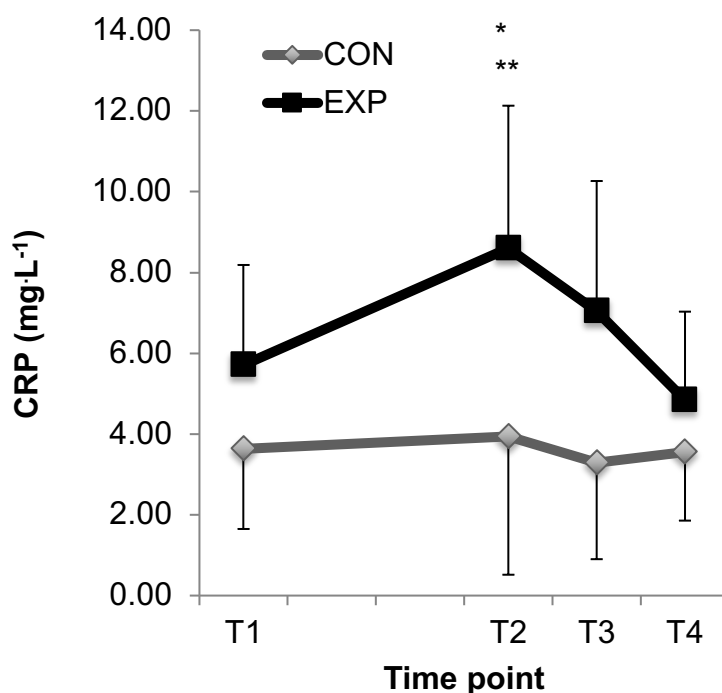


Figure 5.7. Serum C-Reactive Protein (CRP) responses in the EXP group ($n = 10$) and CON group ($n = 10$) pre (T1) and post a 3-day exercise protocol (T3) and throughout a recovery period at 21 hr and 42 hr (T3; T4). Data displayed as means; error bars represent SD . *Denotes significant difference between groups, $p \leq .05$, **denotes significant difference to T3 and T4, $p \leq .05$. T = time point.

5.2.2.3.3 Total leukocyte & neutrophil counts.

It was proposed that exercise-induced leukocytosis and neutrophilia would occur as part of the acute-phase response to exercise-induced muscle damage, therefore total leukocyte and neutrophil counts were assessed in whole blood. A significant interaction between groups and across time points was observed for leukocyte

counts, $F(3, 54) = 2.971$, $p = .040$, $\eta_p^2 = .142$. There was a main group effect, with leukocyte counts differing between the EXP and CON group, $F(1, 18) = 4.464$, $p = .049$, $\eta_p^2 = .199$. Results from post hoc independent samples t -tests indicated a significant difference between groups at T2; leukocytes were increased immediately post-exercise in the EXP group in comparison to the CON group, $t(18) = 2.520$, $p = .021$, $d = 1.11$ (EXP: $M = 7.6$, $SD = 2.8 \times 10^9 \cdot L^{-1}$ vs. CON: $M = 4.9$, $SD = 2.0 \times 10^9 \cdot L^{-1}$ 95% CI [0.5, 5.0]). The main effect for time was non-significant, $F(3, 54) = 1.493$, $p = .227$, $\eta_p^2 = .07$. (Figure 5.8). There was also a significant interaction between group and time for neutrophil counts, $F(3, 54) = 7.461$, $p \leq .001$, $\eta_p^2 = .293$, with a significant main effect for time, $F(3, 54) = 3.007$, $p = .038$, $\eta_p^2 = .143$. Post hoc one-way repeated measures ANOVA tests with Greenhouse-Geisser corrections applied, revealed significant differences in neutrophil counts across time points in both groups, $F(12.229, 1.359) = 5.584$, $p = .028$, $\eta_p^2 = .383$; $F(2.099, .888) = 4.887$, $p = .018$, $\eta_p^2 = .352$, EXP and CON, respectively. Pairwise comparisons (Bonferroni corrected) indicated increased neutrophils immediately post-exercise compared to pre-exercise in the EXP group (T1: $M = 2.7$, $SD = 1.1 \times 10^9 \cdot L^{-1}$ vs. T2: $M = 4.0$, $SD = 1.3 \times 10^9 \cdot L^{-1}$, 95% CI [0.3, 2.4], $p = .014$), with neutrophils decreased at 21 hr post-exercise during recovery (T2: $M = 4.0$, $SD = 1.3 \times 10^9 \cdot L^{-1}$ vs. T3: $M = 3.1$, $SD = 1.3 \times 10^9 \cdot L^{-1}$, 95% CI [0.2, 1.6], $p = .011$). No significant differences were detected between neutrophil counts at T4 in comparison to the other time points, $p \geq .05$, however, data revealed a biphasic neutrophil response post-exercise, with increased neutrophil counts at T4. Pairwise comparisons also revealed that neutrophil counts in the CON group were significantly higher at T1, in comparison to T3, $M = 3.7$, $SD = 1.0 \times 10^9 \cdot L^{-1}$ vs. $M = 2.5$, $SD = 0.9 \times 10^9 \cdot L^{-1}$, 95% CI [0.4, 2.1], $p = .009$, and T4 $M = 2.5$, $SD = 0.7 \times 10^9 \cdot L^{-1}$, 95% CI [0.2, 2.3], $p = .028$, and also at T2 compared to T4,

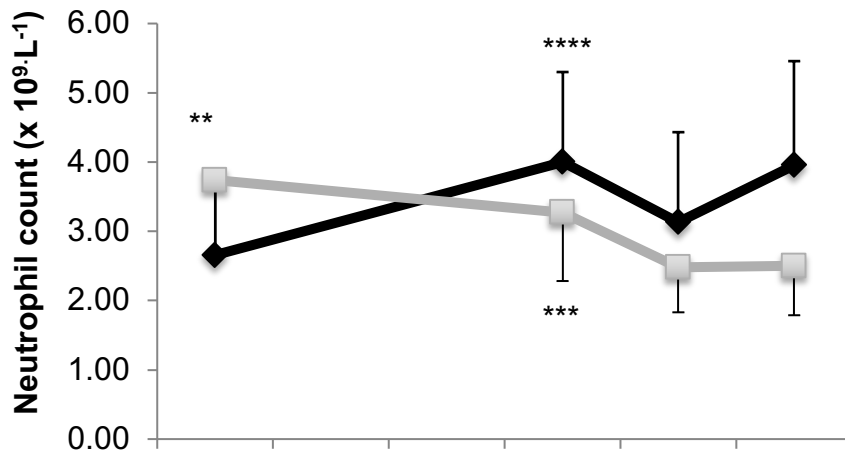
$M = 3.3$, $SD = 0.9 \times 10^9 \cdot L^{-1}$, vs. $M = 2.5$, $SD = 0.7 \times 10^9 \cdot L^{-1}$, 95% CI [0.1, 1.5], $p = .037$. The main effect for group was non-significant, $F(1, 18) = 1.464$, $p = .242$, $\eta_p^2 = .075$ (Figure 5.8). Due to (non-significant) differences in total neutrophil counts between groups at T1, total neutrophil counts are also expressed relative to total leukocyte counts (Table 5.4).

Table 5.4

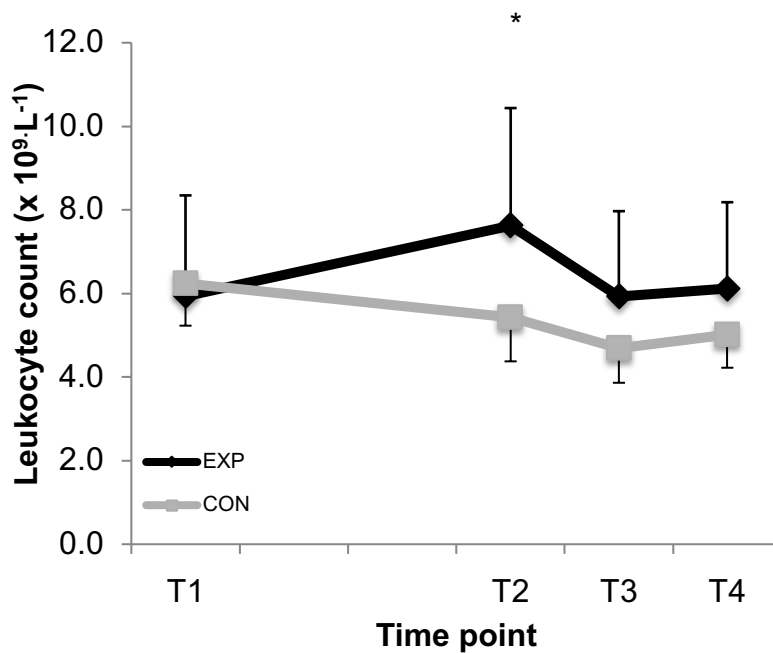
Relative Neutrophil Counts

	Total Neutrophil Count Relative to Total Leukocyte Count (%)			
	T0	T1	T2	T3
EXP ($n = 10$)	45	52	53	65
CON ($n = 10$)	60	60	53	50

Note. CON = control group; EXP = experimental group T = time point.



b.



a.

Figure 5.8. a. Whole blood leukocyte and b. neutrophil responses in the EXP group ($n = 10$) and CON group ($n = 10$) pre (T1) and post a 3-day exercise protocol (T3) and throughout a recovery period at 21 hr and 42 hr (T3; T4). Data displayed as means; error bars represent *SD*. *Denotes significant difference between groups, $p \leq .05$, **denotes significant difference to T3 and T4 in the CON group, $p \leq .05$, ***denotes significant difference to T4 in the CON group, $p \leq .05$, ****denotes significant difference to T1 and T3 in EXP group, $p \leq .05$. T = time point.

5.2.2.4 Oxidative stress: MDA & TAS.

To determine whether the 3-day exercise protocol induced oxidative stress and upregulated antioxidant capacity, serum MDA and TAS were assessed. No significant interaction between group and time were observed for MDA or TAS, $p \geq .05$ (Table 5.5).

5.2.2.5 NF- κ B p65 activation.

It was speculated that activation of the NF- κ B classical pathway by exercise-induced RONS would trigger a transient inflammatory state post-exercise; therefore, activated NF- κ B p65 subunit was determined in PBMCs, normalised to nuclear protein content. There was no significant interaction between group and time for NF- κ B p65 activation, $p \geq .05$ (Table 5.5).

5.2.2.6 Anti-inflammatory cytokine response.

To increase understanding of the balance between pro- and anti-inflammatory responses following exercise, IL-10 was quantified. No statistical interaction between group or time points were observed for IL-10 in response to the 3-day exercise protocol, $p \geq .05$ (Table 5.5).¹¹

¹¹ Results of the full cytokine array are presented (APPENDIX K).

Table 5.5

Oxidative Stress, Chemokine IL-8, Cytokine IL-10, and NF-κB p65 Responses to the 3-Day Exercise Protocol; M (± SD)

Variable	EXP (<i>n</i> = 10)				CON (<i>n</i> = 10)				<i>p</i>
	T1	T2	T3	T4	T1	T2	T3	T4	
MDA (μM)	2.71 (± 2.17)	3.40 (± 1.53)	3.66 (± 1.91)	3.11 (± 1.42)	2.26 (± 0.69)	2.61 (± 1.22)	2.44 (± 1.02)	2.26 (± 1.32)	.777
TAS (mmol·L ⁻¹)	1.38 (± 0.31)	1.64 (± 0.52)	1.60 (± 0.66)	1.54 (± 0.59)	1.21 (± 0.26)	1.31 (± 0.21)	1.27 (± 0.33)	1.20 (± 0.30)	.084
IL-8 (pg·mL ⁻¹)	7.52 (± 3.39)	9.46 (± 2.48)	7.45 (± 3.53)	8.78 (± 3.12)	7.47 (± 4.13)	9.79 (± 7.89)	10.64 (± 8.33)	7.21 (± 4.08)	.327
IL-10 (pg·mL ⁻¹)	0.90 (± 0.87)	0.84 (± 0.50)	0.82 (± 0.58)	0.86 (± 0.73)	0.40 (± 0.28)	0.43 (± 0.29)	0.50 (± 0.23)	0.37 (± 0.23)	.340
NF-κB p65 (ng·μg ⁻¹)	0.48 (± 0.71)	0.43 (± 0.51)	0.38 (± 0.25)	0.29 (± 0.17)	0.55 (± 0.59)	0.55 (± 0.78)	0.36 (± 0.18)	0.27 (± 0.14)	.877

Note. EXP = Experimental group; CON = Control group; IL = Interleukin; MDA = Malondialdehyde; NF-κB = Nuclear Factor kappa-light-chain-enhancer of activated B cells; T = time point; TAS = Total Antioxidant Status; *p* values denote Mixed ANOVA intervention group (2) x time (4) interaction effect.

5.4 Discussion

Research investigating the biochemical impact of soccer has shown that the high metabolic and mechanical demands of this type of exercise induces oxidative stress, leading to skeletal muscle damage and a subsequent inflammatory response, which is associated with muscle soreness and fatigue. During microcycles of a competitive in-season, professional soccer players train and compete on consecutive days with minimal recovery (e.g., training; tapering; match play; recovery; training), the cumulative effects of which could compromise recovery, readiness to train and performance. There is a considerable amount of literature investigating exercise-induced biochemical responses following a single bout of soccer/simulated soccer, yet, research studying these effects after consecutive days of soccer has been limited, hence, this study addresses a gap in the literature. The aims of the present study were to (i) profile an array of biomarkers indicative of oxidative stress (TAS & MDA), muscle damage (CK & LDH), and inflammation (NF- κ B p65; CRP; inflammatory cytokines; total leukocyte & neutrophil counts) following a 3-day period of soccer-specific, prolonged high-intensity intermittent exercise (90 min·d⁻¹), performed on consecutive days, to explore the sensitivity and short-term recovery time course (42 hr) of these biomarkers, and (ii) to further increase understanding of underlying mechanisms of these phenomena. This is the first study to demonstrate that a 3-day period of prolonged high-intensity intermittent exercise, characteristic of soccer, elicited an altered response in systemic biomarkers of exercise-induced muscle damage (CK) and inflammation (CRP, IL-6, & MCP-1), and induced leukocytosis and neutrophilia. Concentrations of biomarkers were elevated above pre-exercise values immediately post-exercise, and decreased throughout a 42 hr recovery period, except for total neutrophil counts which remained elevated above

pre-exercise values; in comparison to a resting control group where biomarker concentrations remained stable. Intriguingly, no response to the 3-day exercise protocol was detected in LDH, IL-8 or anti-inflammatory cytokine IL-10, biomarkers of oxidative stress (MDA & TAS), or NF- κ B p65 activation. Therefore, data partially accepted the primary experimental hypothesis. Further explorations of the findings are detailed below.

5.4.1 Main findings.

5.4.1.1 Effect of the 3-day exercise protocol on EIMD.

There was a significant transient increase observed in mean serum CK concentration immediately post-exercise in the EXP group (pre-post Δ 92%), which diminished throughout recovery (21 hr), in comparison to the CON group, where CK concentrations remained unaltered from baseline. Lactate Dehydrogenase followed a similar trend (pre-post Δ 26% LDH), however, no significant differences were detected over time or between groups. Combined these results partially accept working hypothesis H_{1b} . Systemic increases in intramuscular enzymes provide indirect evidence of skeletal muscle tissue damage at the myocyte level, as it is proposed that sarcolemma damage or increased permeability of the sarcolemma resulted in CK and LDH leakage into circulation (Brancaccio et al., 2007, 2010; Owens et al., 2019). These results are in accordance with researchers who have demonstrated elevated intramuscular enzyme concentrations in response to (a) soccer/simulated soccer bouts (Ascensão et al., 2008; Bell et al., 2016; Ispirlidis et al., 2008; Mohr et al., 2015; Souglis et al., 2015), and (b) three consecutive days of prolonged exercise (Bell et al., 2014; Nieman et al., 2014). The CK and LDH assays used in this study are not specific for isoenzymes present within skeletal muscle (i.e.,

CK-MM; CK-MB; LDH-4; LDH-5). Nevertheless, elevations in serum CK can be attributed to skeletal muscle damage as this is the major source of CK in serum (Brancaccio et al., 2007), and it is unlikely that brain or cardiac tissue were damaged in response to the exercise as the cohort were a homogeneous group of healthy athletes. To fully understand mechanisms of exercise-induced muscle damage, it is recommended that biomarkers of oxidative stress and inflammation be assessed (Brancaccio et al., 2010; Lee et al., 2017; Malm, 2001), as were included in the present study, strengthening the study design. Oxidative stress was unaltered in response to the 3-day exercise protocol (Table 5.5), therefore, there is a lack of significant evidence to suggest that cell damage was caused by metabolic stress (secondary muscle damage) and it is speculated that the predominant cause of cell damage was due to mechanical stress (primary muscle damage). The frequency of concentric and eccentric muscle actions performed within the exercise protocol during accelerations and decelerations further supports this theory (Proske & Morgan, 2001).

The magnitude of post-exercise CK observed in the present study ($335.78 \text{ U}\cdot\text{L}^{-1}$) was similar to previous research investigating CK responses following a soccer match ($\sim 400 \text{ U}\cdot\text{L}^{-1}$ [Ispirlidis et al., 2008]; $376 \text{ U}\cdot\text{L}^{-1}$ [Souglis et al., 2015]). However, no further elevations in CK were observed throughout the 42 hr recovery period, therefore, the magnitude of the peak CK response was lower than that previously observed following a single bout of soccer ($\sim 800 \text{ U}\cdot\text{L}^{-1}$ 48 hr [Ascensão et al., 2008]; $\sim 900 \text{ U}\cdot\text{L}^{-1}$ 48 hr [Ispirlidis et al., 2008]; $785 \text{ U}\cdot\text{L}^{-1}$ 24 hr [Souglis et al., 2015]) or simulated soccer ($\sim 1551 \text{ U}\cdot\text{L}^{-1}$ 24 hr [Bell et al., 2016]). This response could be attributed to (a) the intensity of the soccer-simulation protocol, (b) inter-individual

variability (training status; sex; muscle fibre type), or (c) the RBE (i.e., performing 3 consecutive days of exercise may have resulted in a protective effect, such as increasing cell membrane integrity, or producing less muscle damage on the successive bouts [McHugh, 2003]). Bell et al. (2014) reported a post-exercise CK concentration of $292.7 \text{ U}\cdot\text{L}^{-1}$ in response to a 3-day period of prolonged high-intensity intermittent cycling, suggesting that the magnitude of muscle damage is dependent upon the exercise mode; as in cycling the body mass is supported in comparison to intermittent running used in the present study. Interestingly, the post-exercise time course of CK in the present study also differed to that of Bell et al. (2016), Ispirlidis et al. (2008), and Souglis et al. (2015), where a 24 - 48 hr delay in peak concentrations was the typical response following single bouts of soccer/simulated soccer. However, the CK response in the present study (peaking immediately post-exercise) was similar to that of Nieman et al. (2014) following a 3-day period of intensified treadmill running ($2.5 \text{ h}\cdot\text{d}^{-1}$), which provides further evidence that exercise-induced muscle damage is dependent upon the mode of exercise (supporting Nieman et al., 2014). As the observed exercise-induced response did not demonstrate excessive muscle damage per se, as typically observed following soccer, damage induced by the 3-day soccer simulation will be referred to forthwith as *cellular damage*. It is also important to consider that the presence of intramuscular enzymes in serum is indicative of (a) efflux of the enzymes from myocytes and (b) clearance of the enzymes from circulation (Chatzinikolaou et al., 2014; Warren, Lowe, & Armstrong, 1999), therefore, post-exercise elevations in biomarkers of CK and LDH should be interpreted as evidence of cellular damage that may not accurately represent the magnitude of damage.

Exercise-induced muscle damage evokes a transient inflammatory response (as evidenced by increased CRP, IL6, & MCP-1 in the present study), which stimulates the repair and regeneration of damaged skeletal muscle tissue (Malm, 2001; Peake et al., 2017), but has implications for underperformance, as muscle damage and inflammation have been associated with decreased force production, fatigue, and muscle soreness, which could compromise recovery, impairing readiness to train and subsequent performances (Ascensão et al., 2008; Le Moal et al., 2016; Powers et al., 2011; Radak et al., 2017; Reid et al., 1993). The observed post-exercise elevation in CK in the present study was not sustained throughout the considered recovery period, which suggests that a 42 hr passive recovery period (representative of a typical post-match rest duration in professional soccer) sufficient to restore skeletal muscle homeostasis following a 3-day period of soccer specific exercise, but may have implications for recovery between training sessions where the recovery period is typically < 24 hours.

Athletes characteristically have elevated resting serum CK concentrations, possibly due to an increased muscle mass and higher training load (Brancaccio et al., 2010; Mougios, 2007). The concept of high and low CK responders to exercise has also been well documented (Totsuka et al., 2002; Brancaccio et al., 2007; 2010). Creatine Kinase data in this study revealed high inter-individual variability (range: 28.45 - 1503.67 U·L⁻¹; inter-individual variance 51%; Appendix G), however, it can be wholly concluded that the 92% increase in CK concentration immediately post-exercise in the EXP group was induced by the 3-day exercise protocol and was not due to biological variance. The results of this study emphasise the importance of accounting

for variance; accounting for individual differences in baseline concentrations may also be appropriate (Malm et al., 2004).

5.4.1.2 Effect of the 3-day exercise protocol on EII.

There was a significant difference between groups for mean serum CRP concentration, with a marked increase in CRP reported immediately post-exercise in the EXP group ($\Delta 82\%$ from pre-exercise values; accepting working hypothesis H_{1d}), which was attenuated over the time course of the recovery period. Whereas CRP remained unaltered over time in the CON group. This exercise-induced inflammatory response paralleled EIMD and is in line with previous research which demonstrated increased CRP in response to bouts of soccer/simulated soccer (Bell et al., 2016; Ispirlidis et al., 2008; Mohr et al., 2015; Romagnoli et al., 2016; Souglis et al., 2015), and three consecutive days of exercise (Bell et al., 2014; Nieman et al., 2014). C-Reactive Protein is an acute-phase protein secreted from hepatocytes as part of the systemic inflammatory response and is induced by IL-6 (Giudice & Gangestad, 2018; Petersen & Pedersen, 2005), which was also elevated in the present study. C-Reactive Protein is responsible for identifying and removing damaged cells and induces a mild anti-inflammatory effect (Giudice & Gangestad, 2018), but also has implications for athletes as it has been shown to parallel muscle swelling, muscle soreness and decreased range of motion (Ispirlidis et al., 2008), which could impair performance. In the present study, CRP was attenuated throughout the recovery period, which suggests that a 42 hr passive recovery period is sufficient to reduce systemic inflammation following three consecutive days of soccer-specific prolonged high-intensity intermittent exercise. It should be noted that observed serum CRP concentrations in this study were high in comparison to (a) normative data ($< 3 \text{ mg}\cdot\text{L}^{-1}$

¹ Randox CRP kit insert) and (b) CRP values previously reported in response to soccer (Ispirlidis et al., 2008; Souglis et al., 2015), yet, similar to those reported by Nieman et al. (2014) following three consecutive days of treadmill running (2.5 hr·d⁻¹). This suggests that systemic inflammation may be relative to the duration, intensity, and mode of exercise and that there may be a cumulative inflammatory response to consecutive days of exercise.

There was also a significant transient increase in serum IL-6 and MCP-1 in response to the 3-day exercise protocol in the EXP compared to the CON group where values remained stable over time, accepting working hypothesis H_{1e} (IL-8 rejected H_{1e}). Interleukin-6 expression increased two-fold immediately post-exercise, returning to pre-exercise concentrations 21 hr post-exercise, which was significantly different from the CON group where IL-6 remained stable across time points. Monocyte Chemoattractant One presented a similar response to IL-6, increasing above pre-exercise concentrations immediately post-exercise ($\Delta 36\%$), returning to baseline throughout the recovery period, compared to the CON group. Interleukin-6 and MCP-1 are inflammatory myokines secreted by contracting skeletal muscle as a localised response to muscle damage (Pedersen, 2000). Interleukin-6 stimulates the release of hepatocyte-derived CRP as part of the acute-phase response (Giudice & Gangestad, 2018; Pedersen, 2000). Interleukin-6 and MCP-1 are involved in muscle metabolism, regeneration, angiogenesis, and facilitate neutrophil infiltration into damaged muscle tissue (Pedersen, 2000), evidentiary support in the present study is provided by the observed post-exercise increases in CK and total neutrophil counts, although IL-6 can also be produced independently of muscle damage (Pedersen, 2000). Results of the present study support the work of Bell et al. (2014) and Nieman

et al. (2014) who demonstrated similar responses (Bell, IL-6 & Nieman, IL-6 & MCP-1, respectively) following three days of consecutive exercise, and Andersson et al. (2010), who demonstrated increased IL-6 and MCP-I as part of a pro-inflammatory cascade in response to two consecutive soccer matches in female soccer players. In the present study, IL-6 and MCP-1 were normalised with 24 hr passive recovery. It is documented that IL-6 has a short half-life (Pedersen, 2000), and studies investigating the IL-6 response to soccer/simulated soccer have consistently demonstrated a similar time course response to that reported within this study (Bell et al., 2014; Ispirlidis et al., 2008; Souglis et al., 2015). It is proposed that IL-6 is regulated in a downstream manner via the NF- κ B classical pathway (Kramer & Goodyear, 2007) and that hepatic-release of CRP is induced by IL-6 as part of the acute-phase inflammatory response (Giudice & Gangestad, 2018; Petersen & Pedersen, 2005). Data in the present study only partly supports this theory (refer to section 5.3.1.1. Null Findings), but supports the work of Bell et al. (2016) who found similar post-exercise responses in IL-6 and high-sensitivity CRP following a single bout of prolonged high-intensity intermittent exercise simulating soccer, and Nieman et al. (2007a) who reported post-exercise increases in myocyte and leukocyte IL-6 mRNA expression without a post-exercise increase in muscle NF- κ B p65 activity following three consecutive days of cycling. Several stimuli for post-exercise IL-6 transcription have been proposed (Fischer, 2006). Investigating the roles of other transcription factors and stressors (e.g., NFIL-6, LPS endotoxins, heat stress), AMPK and MAPK pathways, or alterations in calcium homeostasis as potential stimuli for IL-6 expression may provide further insight into underlying mechanisms of IL-6 expression following intermittent exercise.

A role of IL-6 is as an energy sensor (Petersen & Pedersen, 2005). Participants in the EXP group exercised in a post-prandial state (2 hr after consumption of the standardised meal replacement drink) with only water permitted throughout the prolonged duration of the exercise protocol (90 min·d⁻¹). Blood glucose and muscle glycogen were not assessed but it is speculated that hypoglycemia or glycogen depletion may have been the stimulus for IL-6 expression (Pedersen, 2005; Steensberg et al., 2000) observed in the present study. In the applied setting carbohydrates would have been ingested, and would have potentially suppressed IL-6 expression (Pedersen, 2005), demonstrated by carbohydrate ingestion during 2.5 hr treadmill running at 75% $\dot{V}O_2$ max blunting plasma IL-6 (trained triathletes [Nieman et al., 1998]).

An interesting finding was the modest post-exercise elevation in IL-6 (2-fold from pre-exercise concentrations in the EXP group). It has been reported that IL-6 increases exponentially with the duration of exercise, and has been shown to increase up to 100-fold following running (Pedersen et al., 2001). Post-exercise kinetics of IL-6 differ between eccentric and concentric muscle contractions and the modest increase observed in the present study is possibly due to (a) the large eccentric element of the 3-d exercise protocol and (b) the time points of sampling. Nieman et al. (2007a) found that cytokine concentrations diminished across a 3-day prolonged cycling protocol in trained athletes, indicative of a repeated bout effect. It is possible that highly trained athletes exposed to repeated days of exercise are able to rapidly adapt following each successive bout, which could be the reason for the observed magnitude of change in post-exercise IL-6 in the present study. Blood sampling was not conducted pre and post days 1 and 2 of the 3-day exercise

protocol because of increased participant burden; however, would have increased understanding of cumulative inflammatory responses to the 3-day exercise protocol.

While exercise-induced muscle damage associated inflammation is part of the repair and regeneration process that leads to adaptations (Gomez-Cabrera et al., 2016), negative consequences for the athlete include muscle soreness and performance decrements (Fatouros & Jamurtas, 2016). Interleukin-6 and MCP-1 were attenuated with a 24 hr period of passive recovery; the practical implication of this acute inflammatory state requires further research. Strategies that blunt inflammation (e.g., antioxidant supplementation) may be useful during periods of intense training where recovery time is minimal.

5.4.1.2.1 Effect of the 3-day exercise protocol on total neutrophil counts.

The data showed altered total neutrophil and leukocyte counts in response to the 3-day exercise protocol (accepting working hypothesis H_{1g}). Total neutrophil and leukocyte counts increased immediately post-exercise in comparison to pre-exercise, with a significant difference reported between the EXP versus CON group. Neutrophils were observed to increase post-exercise ($\Delta 50\%$), and displayed a biphasic response throughout the recovery period; decreasing 21 hr post-exercise, followed by an increase 42 hr post-exercise. These results support previous research that demonstrated acute neutrophilia or leukocytosis in response to a single bout of soccer (Ascensão et al., 2008; Fatouros et al., 2010; Ispiridis et al., 2008) or repeated soccer bouts (Andersson et al., 2010; Malm et al., 2004). It is proposed that neutrophils are part of the inflammatory response and are mobilised from marginal pools to infiltrate sites of skeletal muscle damage to promote the repair and

regeneration of muscle, and are regulated by IL-6 (Powers et al., 2010b). Further support for this theory is provided by the observed increases in biomarkers of muscle damage (CK) and inflammation (CRP, IL-6 & MCP-1). The underlying mechanisms of the delayed biphasic neutrophil response are unclear, but may be the consequence of performing repeated bouts of exercise or due to induced hormones (Suzuki et al., 1999; the assessment of stress hormones, i.e., catecholamines, growth hormones, cortisol would have provided support for this mechanism). Exercise-induced neutrophilia is however, a double-edged sword, as neutrophil production of ROS (e.g., O_2^- ; H_2O_2 ; OH^- ; HOCL) and proteases during the respiratory oxidative burst can cause secondary muscle damage (Pyne, 1994a). This data has applied implications as it suggests a 42 hr recovery period may be insufficient to restore alterations in immune function following three consecutive days of soccer-specific exercise. Secondary muscle damage was unsupported by the data, as there was no increase observed in oxidative stress at the time points considered or further escalation of muscle damage biomarkers.

5.4.1.3 Null findings.

Contrary to the hypotheses, no significant alterations were detected in serum biomarkers of LDH, oxidative stress (MDA; TAS), inflammatory cytokines (IL-8; IL-10), or NF- κ B p65 activity in PBMCs in response to the 3-day exercise protocol, when compared to a resting control group, rejecting working hypotheses H_{1a} , H_{1b} (CK accepted), H_{1c} , H_{1e} (IL-6 & MCP-1 accepted), and H_{1f} . Lactate Dehydrogenase concentrations in the Exp group followed a similar trend to CK, however, no significant difference was detected at the time points between the EXP and CON group. This is in contrast to Ispirlidis et al. (2008) who observed a similar response in

LDH to CK following a soccer match in trained athletes. A potential explanation for the non-significant findings could be inter-individual variance. These results do not support previous work (Ascensão et al., 2008; Fatouros et al., 2010; Ispirlidis et al., 2008; Mohr et al., 2015; Silva et al., 2013), which demonstrated increased oxidative stress (lipid peroxidation, [MDA/TBARS]) in response to bouts of soccer. Although serum TAS concentrations in the present study were similar to those previously observed (Ascensão et al., 2008). These results are also in contrast to Shing et al. (2007) who demonstrated increased plasma MDA and increased plasma TAS as a cumulative response to three days of high-intensity cycling. It appears there may be potential bias in published studies that only demonstrate an exercise-induced oxidative stress response. It is established that exercise-induced RONS can induce expression of antioxidant enzymes via inflammatory mediators such as NF- κ B as part of the adaptation process within skeletal muscle (Gomez-Cabrera et al., 2016; Powers et al., 2016), therefore, the TAS results are unsurprising given that MDA concentrations or NF- κ B activity did not significantly increase. Oxidative stress is complex and highly individual, dependent upon the trained status of the athlete and the intensity and duration of the exercise (Becatti et al., 2017). The athletes used in the present study were trained (EXP group: $\dot{V}O_{2\max}$: 52.80 ± 5.6 mL·kg⁻¹·min⁻¹) and accustomed to performing prolonged high-intensity intermittent exercise. Therefore, it is proposed that the intensity of the 3-day exercise protocol may not have been strenuous enough to induce oxidative stress, or that the athletes had enhanced antioxidant capacities to cope with the level of exercise-induced oxidants, or possibly a combination of both (Vollard et al., 2005). The TAS assay used in the present study is not specific for exogenous or endogenous (enzymatic or non-enzymatic) sources of antioxidants (Powers et al., 2010b) and assessment of individual

antioxidants may have provided more insight but would have been impractical. Dietary antioxidant intake was not assessed due to difficulties in assessment but may have provided adequate exogenous antioxidants to cope with the exercise stress.

It is proposed that the NF- κ B classical pathway can be regulated by oxidants, cytokines and muscle damage, and that NF- κ B itself regulates gene expression of acute-phase proteins, cytokines (including IL-6, IL-8, & MCP-1) and antioxidant enzymes (Gomez-Cabrera et al., 2006; Ji et al., 2004, 2007; Kramer & Goodyear, 2007; Niemen et al., 2007). The NF- κ B pathway has been implicated in energy metabolism (Kramer & Goodyear, 2007) and it was conceivable that the NF- κ B pathway would have been the mechanism for the observed increase in IL-6 in response to glucose or glycogen depletion. However, data from the present study seems to provide no causal link between contracting skeletal muscle, NF- κ B p65 activity, and IL-6 synthesis, therefore, it is speculated that observed exercise-induced inflammatory responses in the present study appear to be independent of the NF- κ B classical pathway.

Results neither support nor refute the theory of exercise-induced oxidant activation of the NF- κ B classical pathway, as there was no significant evidence that the 3-day exercise protocol was strenuous enough to induce oxidative stress in the population of trained athletes studied. Human exercise studies have shown equivocal findings in NF- κ B activity in response to exercise, with limited research investigating NF- κ B activity in response to intermittent exercise. No studies to the lead investigator's knowledge have assessed NF- κ B in response to exercise characteristic of soccer.

Results of the present study support the work of Parker et al. (2017) who reported a lack of NF- κ B activity following high-intensity intermittent cycling exercise, and also, Buford et al. (2009) and Koenig et al. (2015) who observed no increase in NF- κ B activity in response to eccentrically-biased downhill running exercise. It is possible that the lack of NF- κ B p65 activity in the present study may have been due to the intensity of the exercise protocol, as Parker et al. (2017) demonstrated NF- κ B p65 activity to be increased following supramaximal sprint exercise but not high-intensity intermittent exercise. Consequently, further research should explore the NF- κ B response following intermittent exercise using higher exercise intensities than that of the present study.

Inflammatory cytokines IL-8 and IL-10 also remained unaltered in the EXP group in response to the 3-day exercise protocol when compared to the CON group, despite the observed pro-inflammatory response. Interleukin-8 is produced locally within muscle and plays a role in the chemotactic activity of neutrophils (Pedersen et al., 2001), which were increased in response to the 3-day exercise protocol (therefore, attributed to IL-6 & MCP-1), but is also regulated by NF- κ B. The anti-inflammatory cytokine IL-10 is induced by IL-6 (hence, IL-6 being named a mixed cytokine [Pedersen & Pedersen, 2005]) and CRP, and IL-10 itself inhibits the chemokine IL-8 as part of the anti-inflammatory response to counteract pro-inflammation as part of the acute-phase response (and increase in CRP, which induces IL-10 expression (Giudice & Gangestad, 2018; Pedersen & Pedersen, 2005 Steensberg, 2003). Taken together results suggest a transient pro-inflammatory state in response to the 3-day exercise protocol that was not counter-regulated by an anti-inflammatory response.

The time points of sampling may explain the null findings. There is no optimal time points recommended for sampling exercise-induced biomarkers, oxidative stress has been shown to peak at different timings post-exercise (Michailidis et al., 2007), and peak NF- κ B activity in PBMCs has been previously reported to occur 1 - 2 hours post-exercise (Cuevas et al., 2005). Therefore, the time points chosen may have missed peaks in the data, including an anti-inflammatory response. Nevertheless, this study addressed a gap in the literature by investigating NF- κ B activity in response to prolonged high-intensity intermittent exercise. Studies investigating NF- κ B activity in response to exercise have used different sample types (PBMCs vs. skeletal muscle tissue) and have reported mRNA expression as opposed to quantifying functional NF- κ B proteins (subunit p65 binding). Standardised methods would allow greater comparability of results.

It should be noted that visual trends in redox data showed an upregulation of MDA and TAS post-exercise that remained elevated above baseline within the recovery period; however, there was insufficient significant evidence to support this conclusion.

5.4.2 Strengths & limitations.

Strengths of the study include (i) the matched groups design that controlled for the RBE of performing a 3-day exercise protocol (Ranchordas et al., 2017b), and (ii) inclusion of a non-exercising control group to account for inter-individual diurnal variation in the biomarkers across the time course of the protocol (Beedie et al., 2018). The use of a treadmill-based protocol to simulate the physiological demands of an intensified period of soccer-specific intermittent exercise in a controlled

laboratory setting, increased control of variables, such as, (i) environmental conditions, (ii) pre-exercise dietary intake, (iii) inter-individual variation in external loading, however, reduced ecological validity, somewhat limiting generalisability of the findings. Drust et al. (2000) previously acknowledged that the physiological loading of the protocol might not accurately represent the loading experienced in an applied field setting. Given the use of the treadmill permitting forward-only movements, several soccer-specific movements (e.g., (i) directional changes; (ii) jumping; (iii) sidestepping; (iv) backpedaling; (v) tackling) are omitted, alongside movements with the ball. Many of the omitted movements are typically performed at high velocities with a predominant eccentric component, potentially inducing further stress, which would contribute to the loading. The mechanical loading of the protocol has previously been criticised (Silva et al., 2018). In a recent systematic review, Silva et al. (2018) compared post-exercise biochemical, neuromuscular, and perceptual muscle soreness responses following actual soccer match play vs. treadmill-based soccer simulation protocols. Reporting differences in the magnitude of post-exercise responses in markers of muscle damage (CK), inflammation (IL-6; total neutrophil counts), and perceived muscle soreness, attributed to the differences in mechanical loading. The reduced mechanical stress of the protocol may have contributed to the observed lack of (a) oxidative stress and (b) excessive exercise-induced muscle damage following the protocol in the present study. Interestingly, the review by Silva et al. (2018) found post-exercise impairments in muscle function to be similar between match play and treadmill-based soccer simulations. Notwithstanding the limitations, the soccer-simulation protocol was used as a proof-of-concept model to represent the high-intensity intermittent nature of soccer in the controlled laboratory setting. Furthermore, the (i) duration of the protocol, (ii) heart rate response, (iii)

mean speed, and (iv) amount of time spent at high-intensity were comparable to training session data observed in professional soccer players assessed in the field during chapter 4.

This was the first study, to the lead investigator's knowledge; to provide preliminary evidence of biochemical responses following soccer-specific prolonged duration high-intensity intermittent exercise performed on three repeated days, contributing to the literature. Secondly, biomarker assessment was limited to pre- and post- the 3-day exercise protocol (because of resources & participant burden), comparable to the study design by Nieman et al. (2014). Quantifying biomarkers pre and post each exercise bout would have provided further insight into the cumulative response of performing intermittent exercise on repeated days. Pilot work incorporating these sampling time points ($N = 3$; LDH; CRP; TAS data) confirmed that the 3-day exercise protocol provoked a cumulative response, with post-exercise increases in biomarkers remaining elevated above baseline at the start of each successive bout.

5.4.3 Conclusion & further research.

To conclude, this is the first study to show that performing a 3-day period of prolonged high-intensity intermittent exercise, characteristic of soccer ($90 \text{ min}\cdot\text{d}^{-1}$) resulted in acute transient cellular damage (indirect evidence provided by increased serum CK), and associated inflammation (demonstrated by elevated serum CRP, IL-6, MCP-1, whole blood leukocyte & neutrophil counts) in intermittent trained athletes compared to a control group, but no change in LDH, oxidative stress (MDA; TAS), or NF- κ B activity. The magnitude and time course kinetics of the biomarker responses observed following the 3-day exercise protocol could potentially be attributed to

several factors, including (i) mechanical damage of the exercise protocol, (ii) substrate depletion, (iii) antioxidant status of the participants, (iv) trained status of the participants, or (v) a potential repeated bout effect.

These results provide novel data supporting linkage between repeated days soccer-specific exercise and acute cellular damage and inflammatory responses that have applied implications for recovery in soccer. The following biomarkers should potentially be considered when monitoring training load or recovery: (i) CK, (ii) LDH, (iii) CRP, (iv) IL-6, (v) MCP-1, (vi) total leukocyte counts, (vii) total neutrophil counts. The redox-sensitive transcription factor NF- κ B was studied as the underlying mechanism for exercise-induced inflammation, yet, results of this study did not demonstrate that the 3-day exercise protocol (a) induced lipid peroxidation, (b) activated NF- κ B p65, or (c) altered antioxidant capacity, as hypothesised. Therefore, causal relationships between exercise-induced oxidant activation of the NF- κ B classical pathway and antioxidant enzyme expression in this type of exercise remain unsupported by the data; further research into this mechanism is warranted. Anti-inflammatory cytokine IL-10 also remained unaltered in response to the 3-day exercise protocol at the time points considered. It is recommended that further studies expand on this research by repeating the study, using field-based soccer-specific protocols or a real-world setting with a homogeneous group of trained soccer players to establish whether the magnitude and time course of the biomarker responses transfer and whether a 42 hr recovery period is sufficient as part of an in-season training microcycle. Contextualising biomarker data to objective and subjective measures of recovery and performance would help to determine the

practical use of quantifying biomarkers throughout intensified periods (as was a focus of chapter 6).

Chapter 6

**Effect of Prolonged Quercetin Supplementation on Blood Biomarkers of
Exercise-Induced Cellular Damage and Inflammation Following a Three-Day
Period of Prolonged High-Intensity Intermittent Exercise
(Study three)**

6.1 Introduction

Chapter 5 demonstrated that a 3-day intensified period of soccer-specific exercise (3 d; 90 min·d⁻¹ prolonged high-intensity intermittent run) resulted in significant post-exercise increases in indices of cellular damage and associated inflammation. It is proposed that these exercise-induced responses may provide a stimulus for training adaptations within skeletal muscle (Gomez-Cabrera et al., 2005; Powers et al., 2016), however, in the short-term, associated negative consequences for the athlete include reduced force, increased muscle soreness, and fatigue; factors which could impair recovery (Le Moal et al., 2016; Nédélec et al., 2012; Powers, et al, 2004; Reid, 2001). Strategies that facilitate rapid recovery by blunting muscle damage and inflammation during intensified periods of training should be explored, providing a rationale for the current chapter.

Antioxidant supplementation has been promoted as a potential ergogenic aid that may be used to support periods of intensified training where recovery is the key focus (Gomez-Cabrera et al., 2008; Radak et al., 2017). The flavonoid quercetin is of interest to athletic cohorts (Nieman et al., 2007a) as it has been shown to exert pleiotropic effects, with *in vitro* and murine studies demonstrating (i) antioxidant (Boots et al., 2008), (ii) anti-inflammatory, (Nair et al., 2006), and (iii) analgesic properties (Alexander, 2006). Additionally, research by Davis' group (2009b, 2010) has linked quercetin to increased mitochondrial biogenesis and aerobic capacity. Further evidence to support the use of quercetin supplementation in athletes is provided by bioavailability studies (Jin et al., 2010), and human exercise studies that have demonstrated anti-inflammatory (Nieman et al., 2007a, 2009) and antioxidant (Askari et al., 2012; McNulty et al., 2013) effects in athletes. For example, Nieman

et al. (2007a) found prolonged quercetin supplementation (1,000 mg·d⁻¹; 3 weeks, administered before & during exercise) diminished post-exercise increases in IL-10 mRNA and IL-8 mRNA following a 3-day intensified period of cycling (3 hr·d⁻¹ at ~57% maximum power). Furthermore, MacRae & Mefferd (2006), McAnulty et al. (2013), and Nieman et al. (2010) have all shown quercetin supplementation to increase time to exhaustion in trained athletes, demonstrating the analgesic properties of quercetin. In contrast, results from other studies have been inconclusive (Cheavront et al., 2009; Cureton et al., 2009; Konrad et al., 2011; McAnulty et al., 2008; Quindry et al., 2008; Nieman et al., 2007b; O'Fallon et al., 2012; Utter et al., 2009; refer to Literature Review section 2.8.1.1.4), with contradictory results being attributed to methodological differences. The majority of studies have focused on the efficacy of quercetin supplementation in endurance exercise. At the time of the present study, only Abbey and Rankin (2011) had investigated the efficacy of quercetin supplementation in trained athletes undertaking a repeated sprint protocol characteristic of soccer (12 x 30 m), finding quercetin to be ineffective in attenuating indices of exercise-induced oxidative stress (indirect assessment via xanthine oxidase activity), inflammation, or perceived exertion. However, the exercise protocol utilised in this study was of short duration (< 6 min) and the effects of quercetin in prolonged duration intermittent exercise are presently unknown, thus providing a rationale for the current study.

It is theorised that quercetin may potentially facilitate recovery by blunting the acute phase inflammatory response demonstrated in chapter 5, attenuating associated muscle soreness and secondary muscle damage. Proposed anti-inflammatory mechanisms of quercetin include inhibition of NF-κB or COX-2 signalling pathways

(García-Mediavilla et al., 2007; Nair et al., 2006; Tejada et al., 2017). Therefore, the aim of the current study was to address a gap in the literature by investigating the efficacy of prolonged quercetin supplementation to attenuate biomarkers of cellular damage and inflammation and promote recovery following an intensified period of prolonged soccer-specific exercise (as utilised in chapter 5). Primary variables were based on outcome measures from chapter 5 and included biomarkers of cellular damage (LDH) and inflammation (CRP; IL-6; total leukocyte & neutrophil counts). Importantly, to contextualise biomarker data, measures of perceived fatigue and perceived muscle soreness were incorporated. Secondary variables included biomarkers of oxidative stress (TAS; MDA) and NF- κ B p65 activation.

6.2 Methods

6.2.1 Participants.

Twelve male participants volunteered for the study, recruited via convenience sampling and local advertisements at football clubs and the University of Hertfordshire. Participants were intermittent trained athletes who met the inclusion criteria ((i) aged 18 - 40 years; (ii) $\dot{V}O_2\text{max} \geq 40 \text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; (iii) healthy; (iv) free from musculoskeletal injury; (v) non-smoker; (vi) regularly training in a team sport or intermittent exercise, (vii) having previously trained for a minimum of 12 months, and (viii) competent in 1.5 hr per day high-speed treadmill running, performed on 3 consecutive days). Participants were asked to avoid the use of anti-inflammatory medication or nutritional supplements ($> 100\%$ RNI) throughout the study, and were excluded from the study if they had a known allergy to the ingredients of the antioxidant supplement or standardised meal replacement drink, or, if they suffered from a history of gastrointestinal or blood disorders. Participants were allocated into

two groups: (i) quercetin group (QUE [$n = 6$]) and (ii) placebo group (PLA [$n = 6$]), counterbalanced for (i) age, (ii) stature, (iii) body mass, (iv) %bf, (v) $\dot{V}O_2\text{max}$, and (v) baseline TAS concentration (Table 6.1). A priori power estimations (F tests; repeated measures ANOVA between-within interaction, $\alpha = .5$; $\beta = .2$; $1-\beta = 80\%$ [G*Power software version 3.1]) conducted using effect sizes detected by Nieman et al. (2009) for variables CK and CRP ($f = 0.83$, $f = 1.12$; CK & CRP respectively, repeated measures within group data), proposed a total sample size of 4 and 6 participants per group adequate to detect an effect. A control group was not included in the present study as data from chapter 5 provided evidentiary support of inter-individual variance in biomarker data across the 5 days of the exercise protocol. The University of Hertfordshire's Life and Medical Sciences Ethics Committee granted ethical approval for the study (protocol number LMS/PGR/UH/02431). Participants were briefed verbally, and in writing, to the requirements of the study and written informed consent obtained. Prior to participation a health screen was completed, with subsequent verbal health checks made throughout the course of the study. One participant in the PLA group dropped out due to injury (PLA: $n = 5$ [$N = 11$]).

Table 6.1

Descriptive Characteristics & Pre-Supplementation TAS Concentrations; M (\pm SD)

	QUE ($n = 6$)	PLA ($n = 5$)
Age (years)	35 (± 7)	35 (± 8)
Stature (cm)	181.5 (± 5.4)	179.6 (± 6.9)
Body Mass (kg)	75.9 (± 8.6)	74.6 (± 8.3)
Body Fat (%)	12.4 (± 6.8)	12.0 (± 6.2)
$\dot{V}O_2\text{max}$ (mL \cdot kg $^{-1}\cdot$ min $^{-1}$)	53.00 (± 5.00)	54.60 (± 7.99)
HR $_{\text{max}}$ (beats \cdot min $^{-1}$)	189 (± 10)	185 (± 11)
TAS (mmol \cdot L $^{-1}$)	1.39 (± 0.35)	1.39 (± 0.13)

Note. HR $_{\text{max}}$ = maximum heart rate; PLA = placebo group; QUE = quercetin group; TAS = Total Antioxidant Status; $\dot{V}O_2\text{max}$ = maximal oxygen uptake.

^aIndependent samples t-tests confirmed no significant differences ($p \geq .05$) between groups for anthropometric measures, aerobic fitness or TAS concentrations.

6.2.2 Experimental design.

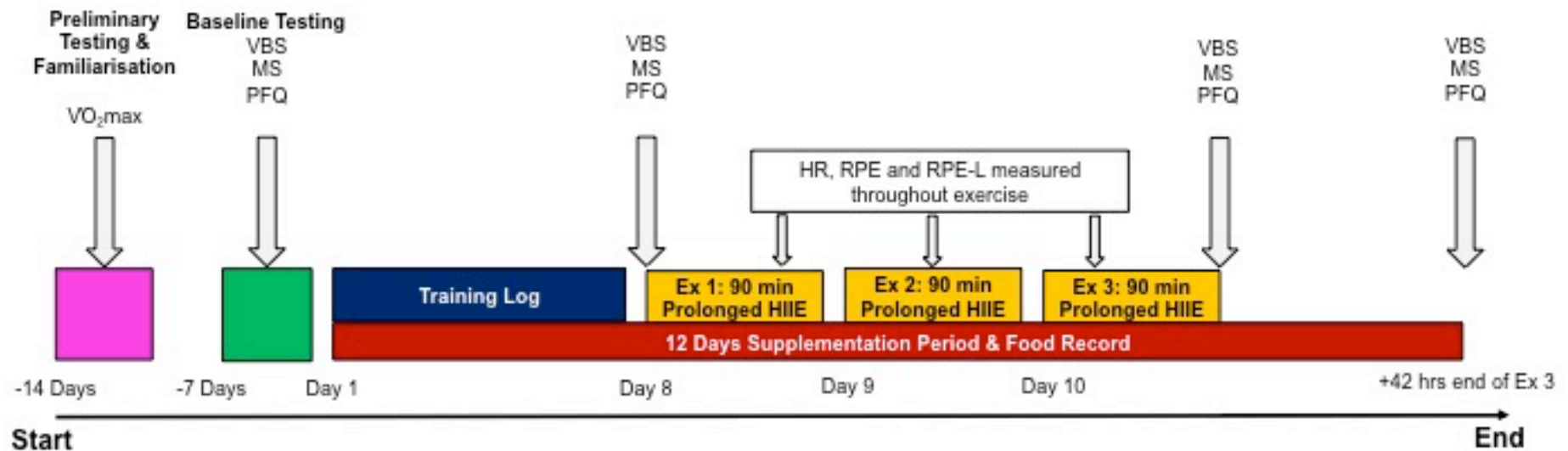
A double blind, placebo controlled, matched pairs parallel groups (non-crossover) design was employed. Participants were required to attend the laboratory on six occasions:

- (i) preliminary testing
- (ii) baseline testing; supplementation (T0)
- (iii) day 1 of exercise protocol (T1)
- (iv) day 2 of exercise protocol
- (v) day 3 of exercise protocol (T2)
- (vi) day 3 +42 hr (recovery measures) (T3)

Participants undertook initial preliminary testing to establish inclusion into the study ($\dot{V}O_2\text{max}$); followed by familiarisation with test procedures (15 min section of the

exercise protocol; recovery measures: (i) venous blood sampling; (ii) assessment of muscle soreness; (iii) perceptual fatigue questionnaire). Approximately one-week later, participants reported to the laboratory for baseline testing of recovery measures. Following baseline measurements, participants were randomised (simple randomisation) into either the (i) QUE or (ii) PLA group, counterbalanced, and issued the corresponding supplement in a double-blind manner. Participants were given verbal and written instructions on how to consume the supplement for a period of 12 days (section 6.2.2.8). Participants were also provided with standardised meal replacement drinks (refer to General Methods section 3.4.2.2), and issued food records and training logs to complete throughout the study (Appendices A & B). Participants were asked to report to the laboratory on day eight of supplementation, in a 24 hr rested state, to complete the 3-day exercise protocol (90 min·d⁻¹) over three consecutive days (on days 8 - 10 of supplementation; as described in section 6.2.2.9), returning on day 12 for recovery measures (+42 hr post-exercise day 3). Participants continued supplementation throughout the exercise protocol and recovery period. Participants completed the exercise protocol at the same time each day to account for diurnal variation. Recovery measures ((i) perceptual fatigue questionnaire; (ii) muscle soreness tests; (iii) venous blood sample) were assessed at the following time points: (i) baseline; pre-supplementation (T0); (ii) immediately pre-exercise; day 8 of supplementation, start of exercise (T1); (iii) immediately post-exercise on the third day; day 10 of supplementation (T2); and (iv) 42 hr post-exercise on the third day, day 12 of supplementation (T3; Figure 6.1). Dietary intake was controlled pre-exercise and pre-blood sampling through the use of a standardised meal replacement drink, consumed approximately 2 hr prior to testing. Food intake was recorded and analysed for energy, macro- and micro-nutrient

intakes. To account for the effects of acute exercise, participants were asked to refrain from exercising on day seven of supplementation. Participants were also asked not to partake in any other exercise except the exercise protocol on days 8 - 12, providing a 7-day training log throughout the first week of supplementation.



Key:

Ex = Exercise; HIIE = High=Intensity Intermittent Exercise; HR = Heart Rate;

MS = Muscle Soreness; PFQ = Perceptual Fatigue Questionnaire; RPE = Ratings of Perceived Exertion;

RPE-L = Ratings of Perceived Exertion Legs; VBS = Venous Blood Sampling; VO_{2max} = Maximal Oxygen Uptake Test

Figure 6.1. Schematic of the overall study design. Participants in both groups (QUE vs. PLA) completed the protocol.

6.2.3 Experimental procedures.

6.2.3.1 Preliminary testing.

Preliminary testing and familiarisation procedures were conducted a minimum of one-week prior to the start of the supplementation period.

6.2.3.1.1 Anthropometric measures.

Stature, body mass, and percentage body fat were assessed (refer to General Methods section 3.2.1).

6.2.3.1.2 Determination of maximal oxygen uptake.

Refer to General Methods section 3.2.1.4.

6.2.3.2 Dietary supplementation & standardisation.

6.2.3.3.1 Quercetin supplementation strategy.

A pre-formed commercially available supplement *Quercetin Plus*, manufactured by the company *Biocare*[®] was used as a supplement. The supplement was provided in capsule form at a dose of 900 mg·d⁻¹ quercetin (3 capsules per day); a placebo capsule was also manufactured by *Biocare*[®] specifically for this research study (ingredients Table 6.2). The dosage and duration of supplementation were comparable to Davis et al. (2010) and Nieman et al. (2010) who demonstrated performance effects. The duration of supplementation was prolonged (i.e., > 5 d [Pelletier, Lacerte, & Goulet, 2013]) and was administered 7 days before, 3 days during the exercise protocol, and throughout recovery, in line with the literature (Nieman et al., 2007a, 2009). Quercetin has *Generally Recognised as Safe* status

(Davis et al., 2009a), and currently, there is no *RNI* or *Tolerable Upper Intake Level*. High doses of antioxidants are known to be pro-oxidant (Braakhuis & Hopkins, 2015). Andres et al. (2018) recently recommended that quercetin supplementation strategies be less than 12 weeks duration, at a dosage of $< 1,000 \text{ mg}\cdot\text{d}^{-1}$ to avoid toxicity in humans; therefore, in keeping with the literature, quercetin was not dosed per kg body mass. According to the manufacturer, the form of quercetin used was quercetin dihydrate, which has increased solubility and therefore, increased bioavailability in comparison to glycoside or aglycone forms. Vitamin C (90 mg) was also incorporated into the supplement to enhance quercetin bioavailability. Quercetin scavenges free radicals via two-electron donation; the oxidation of quercetin resulting in the formation of (i) O-semiquinone and (ii) O-quinone/quinone methide (QQ), the latter of which is pro-oxidant (Boots et al., 2007). The potential for quercetin to act as an antioxidant or become pro-oxidant is known as the “quercetin paradox” (Boots et al., 2007, p. 95). O-quinone/quinone methide can form compounds with glutathione or be recycled back to the parent form of quercetin by electron donors (preferentially vitamin C; Colunga Biancatelli et al., 2020). Therefore, it is important to co-ingest quercetin with vitamin C to minimise pro-oxidant effects.

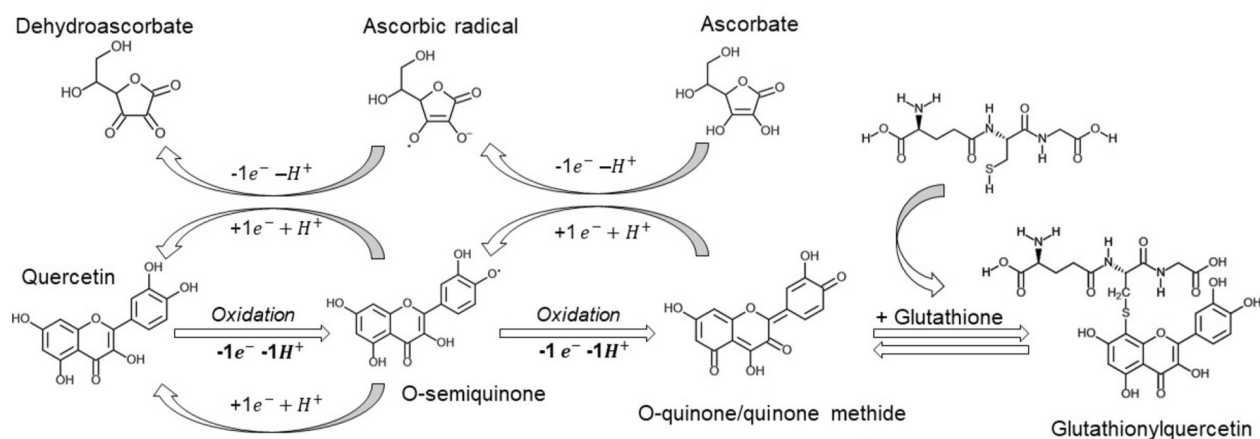


Figure 6.2. Mechanism of oxidised quercetin regeneration via vitamin C (Ascorbate). Recopied from Colunga et al. (2020), Quercetin and Vitamin C: An Experimental, Synergistic Therapy for the Prevention and Treatment of SARS-CoV-2 Related Disease (COVID-19), p. 6.

Results from a highly controlled clinical trial (Askari et al., 2012) provide further support for the co-ingestion of quercetin with vitamin C compared to an isolated quercetin supplement on biomarkers of exercise-induced oxidative stress and inflammation. It was speculated that the dose of vitamin C incorporated into the supplement would not exert an independent effect, given that much higher doses of vitamin C are required to exert an effect on exercise-induced responses (e.g., 3,000 mg·d⁻¹, Bryer & Goldfarb, 2006).

Participants were issued either (a) quercetin supplement (QUE group), or (b) quercetin placebo (PLA group). Supplements were prepped by the lead investigator and issued in an opaque envelope by another member of staff (to reduce lead investigator bias, double-blind study design). Participants received verbal and written instructions on how to consume the supplement for a period of 12 days (3 capsules per day, taken with food, i.e., breakfast, lunch & dinner [in accordance with the manufacturer's instructions]). To assist participants with adherence, capsules were provided in weekly pillboxes (Figure 6.3). The lead investigator also sent regular messages to remind participants to consume the supplement, and participants were required to return empty pillboxes at the end of the study to monitor compliance. All participants adhered to the supplementation strategy.

Table 6.2 <i>Quercetin Plus & Quercetin Placebo Ingredients List</i>	
Ingredients	
Quercetin Plus	Quercetin Placebo
Quercetin (Sophorae japonica Flower) 900 mg	
Capsule Shell (Hydroxypropyl Methylcellulose)	Capsule Shell (Hydroxypropyl Methylcellulose)
Potato	
Maltodextrin	
Bulking Agent (Cellulose)	Microcrystalline Cellulose
Nettle Extract 4:1 (Urtica dioica Leaf) 4:1 (150 mg; equivalent to 600 mg Nettle Leaf)	
Vitamin C (Ascorbic, Acid) 90 mg	
Bromelain 54 mg; providing 540 Gelatin Digesting Units	
Anti-Caking Agents (Silicon Dioxide & Magnesium Stearate)	Magnesium Stearate & Silica



Figure 6.3. Quercetin supplementation. The supplement was issued to participants in a weekly pillbox to facilitate adherence.

6.2.3.3.2 Dietary standardisation.

As per chapter 5, participants were issued a standardised meal replacement drink (*Ensure Plus*; providing 10 kcal·kg⁻¹ body mass) to consume approximately 2 hr prior to testing to minimise individual differences in nutritional status prior to exercising and blood sampling (days 8 - 12; refer to General Methods section 3.4.2.2). To monitor compliance, email reminders and verbal checks were made prior to testing. Participants were asked to continue with their habitual diet throughout the duration of the study (avoiding alcohol & other supplements). A *non-reductionist* approach was taken (i.e., participants maintained their habitual diet & were not required to avoid consumption of high quercetin content foods; Bowtell & Kelly, 2019). Participants completed a 3-day estimated food record during the first seven days of supplementation (3 consecutive days; incorporating 2 weekdays, 1 weekend day), and kept daily food records throughout the exercise protocol (5-day period, days 8 - 12 of supplementation [refer to General Methods section 3.4.2]). Food records were analysed for the following variables using Dietplan software: (i) energy intake, (ii) macronutrient energy contributions, (iii) micronutrient intake, including, total flavonoids, flavonols and quercetin (assessed using the United States Department of Agriculture Flavonoid database within Dietplan), expressed as average daily intakes. All participants complied with dietary control and supplementation. Comparison of dietary analyses between groups (using Independent samples t-tests [Mann Whitney U test for the variable fat]) reported no significant differences ($p \geq .05$) between groups for all variables (Table 6.3; data presented as means \pm SD). Participants in both groups met the RNI for micronutrients.

6.2.3.3 Training log.

Participants were instructed to keep a training log of exercise undertaken throughout the first seven days of the supplementation period (refer to General Methods section 3.4.1). Participants were then required to rest 24 hr prior to the start of the exercise protocol (day 7 of the supplementation period), and instructed to refrain from any other exercise (excluding the exercise protocol) throughout the duration of testing (days 8 - 12). Training logs were reviewed for training load and compliance with the rest period. No significant differences were reported between groups for training load (QUE: $M = 7746$, $SD = 17565$ vs. PLA: $M = 993$, $SD = 995$, $U = 17.00$, $p = .871$; Mann Whitney U test.¹² One participant presented a high training load (in comparison to other participants), but baseline blood data (lactate; glucose; Hb; Hct; leukocyte & differential counts; TAS & LDH) were within normal references ranges, as such the participant was included in the sample to maintain sample size.

¹² QUE: $Mdn = 580$ (IQR = 270 - 43574) AU vs. PLA: $Mdn = 1062$ (IQR = 250 - 2707) AU.

Table 6.3

Dietary Intake of the Quercetin & Placebo Groups M (± SD)

Variable	QUE (n = 6)	PLA (n = 5)
Energy Intake (kcal·d ⁻¹)	2777 (± 323)	2910 (± 9)
Carbohydrate (%)	51 (± 3)	50 (± 3)
Fat (%)	33 (± 4)	32 (± 4)
PUFAs (g·d ⁻¹)	14 (± 5)	15 (± 3)
Protein (%)	16 (± 2)	17 (± 1)
Total flavonoids (mg·d ⁻¹)	58 (± 50)	54 (± 41)
Flavonols (mg·d ⁻¹)	8 (± 4)	6 (± 4)
Quercetin Intake (mg·d ⁻¹)	4 (± 2)	4 (± 2)

Note. kcal·d⁻¹ = kilocalories per day; mg·d⁻¹ = milligrams per day; PLA = placebo group; PUFAs = polyunsaturated fatty acids; QUE = quercetin group; carbohydrate, fat & protein presented as macronutrient energy contributions, expressed as percentage. No significant differences detected between groups, $p \geq .05$.

6.2.3.4 Three-day exercise protocol.

The 3-day exercise protocol was the same as used in chapter 5 (refer to General Methods section 3.3.2.2). In addition, differential Ratings of Perceived Exertion-Legs (RPE-L) was also recorded at 15-minute intervals throughout the exercise protocol (i.e. 15; 30; 45; 60; 75 & 90 min) to assess perceived localised strain of the working muscles (data treated as interval, presented as means). Participants were verbally instructed to the use of RPE-L prior to undertaking the exercise protocol. Validity and reliability of differential RPE scales has previously been demonstrated Borg (1998). Participants were allowed water ad libitum throughout the duration of exercise, the quantity of which was recorded by the lead investigator.

6.2.3.5 Blood collection, processing & storage.

Venous blood samples were collected at the following time points: (i) baseline, pre-supplementation (T0); (ii) following seven days of supplementation, immediately pre-exercise day 8 (T1); (iii) immediately post-exercise day 10 (T2); and (iv) 42 hr post-exercise, day 12 (T3). Blood samples were processed to yield serum and PBMCs used in the analysis of systemic biomarkers of oxidative stress (TAS; MDA), cellular damage (LDH), and inflammation (CRP; IL-6; NF- κ B p65 activation). Total leukocyte & neutrophil counts were analysed immediately using whole blood (refer to General Methods section 3.3.4.7).

6.2.3.6 Recovery measures.

Recovery measures were assessed at each time point (T0: T3), within 15 min of the start, or cessation, of exercise (T1; T2), or following 15 min rest (T0; T3). Measurements were conducted in the following order: (i) perceived fatigue; (ii) perceived muscle soreness; (iii) blood sampling.

6.2.3.6.1 Perceived fatigue.

Subjective feelings of fatigue were assessed using the *Perceptual Fatigue Questionnaire* developed by Coutts, Kelly, McGuigan, & Cormack (2010; Appendix H). The questionnaire is intended for use in team sports athletes when athletes may be experiencing symptoms of the early stages of overreaching, and is sensitive to changes within a microcycle. The questionnaire consists of 5 items: (i) muscle soreness; (ii) fatigue; (iii) sleep quality; (iv) stress levels; and (iv) mood, with participants required to rate each item from a 5-point Likert scale (1 - 5; 0.5 increments); summation of scores provides an *Overall Well-being* score, reported in

arbitrary units (AU; higher scores equating to greater overall well-being). Participants completed the questionnaire at each time point (T0: T3) with the lead investigator present to answer questions. Test-retest reliability was determined from replicate measures assessed 42 hr post soccer match ($n = 1$) $CV < 5\%$.

6.2.3.6.2 Perceived muscle soreness.

Inflammation has been linked to muscle soreness, therefore, the amount of (i) general lower body muscle soreness in an active movement (body weight squat) and (ii) localised muscle soreness (site-specific quadriceps & hamstring) were quantified using 100 mm Visual Analogue Scales (VAS; 100 mm straight line with '0' at one end representing 'no pain', & '100' at the other end, representing 'the worst imaginable pain' [Figure 6.4]). For general lower body muscle soreness, participants performed three body weight squats to $\sim 90^\circ$, marking perceived muscle soreness on a VAS scale. Median score used for analysis, results presented in mm (test-retest reliability was determined from ten replicate measurements made on an athlete with induced DOMS, $CV < 10\%$).



Figure 6.4. Example of a 100 mm Visual Analogue Scale. The scale was used to quantify generalised and localised perceived muscle soreness.

For localised muscle soreness, the lead investigator marked sites on the quadriceps and hamstrings of the participant's dominant leg as follows:

- quadriceps: located on the belly of the muscle (rectus femoris), at the midpoint between the inguinal fold and anterior patella.
- hamstrings: located on the belly of the muscle (biceps femoris, long head), at the mid-point between the ischial tuberosity and the lateral epicondyle of the tibia.

Sites were assessed with participants in prone and supine positions. In brief, participants were instructed to relax their muscles and the flat round rubber tip (1.0 cm²) of a handheld pain algometer (Pain Test™ FTX 25 Compact Digital Algometer, Wagner Instruments, Greenwich, CT: USA) was applied perpendicular to the muscle at the marked site (constant pressure of 4.1 kgf·cm² [400 kPa] for 3 s [Lau et al., 2013]), participants then marked the amount of perceived muscle soreness on a VAS scale. Three measurements were performed at each site (1 min intervals) with data presented from the median measurement, reported in mm. Visual Analogue Scales have been widely used to assess perceived muscle soreness in athletes in muscle damage studies (Twist & Eston, 2005; Howatson's research group [e.g., Bell et al., 2016]). For reproducibility the lead investigator conducted all measurements, with sites remarked each time (test-retest reliability for the quadriceps & hamstrings sites, with & without induced DOMS, was determined from ten replicate measurements, CV < 5%; ICC > .90).

6.2.3.7 Analytical methods.

Refer to General Methods section 3.4. Biomarkers were selected based on findings from chapter 5. Serum CK analysis was excluded from the present chapter because

CK data in chapter 5 demonstrated high inter-individual variance (range: 28.45 - 1503.67 U·L⁻¹; inter-individual variance CV 51%; refer to Appendix G), with 15 out of 20 participants demonstrating high serum CK levels at baseline (> 80 U·L⁻¹; Brancaccio et al., 2011). Lactate Dehydrogenase data followed a similar post-exercise trend to CK in chapter 5, but demonstrated less inter-individual variance (CV 14%) therefore; LDH was selected as the biomarker of cellular damage for chapter 6.

6.2.3.7.1 Determination of LDH.

Sera was analysed for LDH as a biomarker of muscle damage (refer to General Methods section 3.3.4.4). To minimise variance in the data sets LDH was chosen over CK.

6.2.3.7.2 Determination of TAS.

Sera was analysed for Total Antioxidant Status (refer to General Methods section 3.3.4.2).

6.2.3.7.3 Determination of MDA.

Sera was analysed for MDA as a biomarker of oxidative stress (refer to General Methods section 3.3.4.1).

6.2.3.7.4 Determination of NF-κB p65 Subunit Activation.

Peripheral blood mononuclear cells were isolated from whole blood using density gradient centrifugation and analysed for NF-κB p65 subunit activation (refer to General Methods section 3.3.4.5).

6.2.3.7.5 Determination of CRP.

C-Reactive Protein was analysed in sera at Randox Health, London, using an immunoturbidimetric assay (refer to General Methods section 3.3.4.6.2).

6.2.3.7.6 Determination of IL-6.

Interleukin-6 was analysed in sera at Randox Health, London, using a sandwich chemiluminescent immunoassay (refer to General Methods section 3.3.4.5.1).

6.2.3.7.7 Quantification of total leukocyte and neutrophil counts.

Total leukocyte and neutrophil counts were analysed in whole blood using an automated blood cell counter (refer to General Methods section 3.3.4.7).

6.2.3.7.8 Plasma volume correction.

To account for sweat losses and fluid intake, post-exercise analyte concentrations were corrected for plasma and whole blood volume changes (refer to General Methods section 3.3.4.8). Changes were minimal because of fluid intake, with no significant differences reported between groups, $p \geq .05$, Independent samples t-test. Plasma volume change: QUE: - 3% vs. PLA: - 1%; whole blood volume: < -1% in both groups.

6.2.4 Statistical analysis.

Normality of data was checked using (i) Shapiro-Wilk tests, (ii) inspection of histograms, (iii) box plots, (iv) skewness, and (v) kurtosis values. Outliers (defined as $1.5 * IQR$) were included for analysis as data were considered to be true

observations. Missing values in data sets were replaced with mean data. Differences in participants' descriptive characteristics and baseline TAS concentrations were checked using Independent samples t-tests. Differences in variables of heart rate and RPE recorded daily throughout the 3-day exercise protocol were analysed using 2 x 3 mixed between-within ANOVA tests (between-subjects factor of group [QUE vs. PLA]; within-subjects factor of time [day 1, 2, & 3]). A series of 2 x 4 mixed between-within ANOVA tests were conducted to test main effects for group and time, and interaction effects, between the quercetin and placebo conditions (between-subjects factor of group [QUE vs. PLA]), on biomarker data, and recovery measures, across the four time points (within-subjects factor of time, baseline [T0]; pre-exercise [T1]; post-exercise Day 3 [T2]; 42 hr post-exercise Day 3 [T3]). Homogeneity of variance was tested with Levene's. Where data violated Mauchly's Test of Sphericity, Greenhouse-Geisser adjustments ($\epsilon < .75$), or Huynh-Feldt ($\epsilon > .75$), were applied (Girden, 1992). Variables CRP, TAS, MDA, LDH and NF- κ B followed non normal distributions, nevertheless, were treated with parametric tests (Ferreira et al., 2012; Khan & Rayner, 2003). Significant interactions were investigated through follow up One-way RM ANOVA tests (Bonferroni correction applied ($0.05 / 4 = p \leq .0125$) to test within-group differences over time; and Independent samples t-tests to test between-group differences at each time point (T0: T3). 95% CI and effect sizes (η_p^2) were estimated, classified according to Cohen (1988): $\eta_p^2 .01 =$ small; $.06 =$ medium; $.14 =$ large. For all statistical tests significance was set at $p \leq .05$ (2dp). Results expressed as mean (\pm SD).

6.3 Results

This study aimed to investigate the efficacy of prolonged quercetin supplementation, compared to placebo, as a recovery strategy to promote recovery following a 3-day period of soccer-specific, prolonged high-intensity intermittent exercise (3 d; 90 min·d⁻¹) in trained athletes. Primary variables included biomarkers of cellular damage (LDH) and inflammation (CRP; IL-6; total leukocyte & neutrophil counts), and recovery measures ((i) perceived fatigue; (ii) perceived muscle soreness) with secondary variables, biomarkers of oxidative stress (MDA; TAS) and NF-κB p65 activation. Results are detailed hereafter.

6.3.1 Physiological response of the 3-day exercise protocol.

Physiological load of the exercise protocol was consistent across the three days in the QUE and PLA groups, with no significant differences detected between groups for HR or RPE responses, $p \geq .05$ (physiological responses are summarised, Table 6.4).

6.3.2 Biomarkers responses.

6.3.2.1. Indices of oxidative stress.

Following seven days of quercetin supplementation mean serum TAS concentration in the QUE group was 4% greater than at baseline, in comparison to the PLA group, where serum TAS decreased by 6% (non-significant, $p \geq .05$). There were no significant interactions following seven days supplementation or after exercise for mean serum TAS or MDA concentrations, $p \geq .05$ (Table 6.6).

Table 6.4

3-Day Performance Measures; $M (\pm SD)$

Variable	QUE ($n = 6$)				PLA ($n = 5$)				p
	Day 1	Day 2	Day 3	Mean	Day 1	Day 2	Day 3	Mean	
HR _{mean} (beats·min ⁻¹)	138 (± 15)	136 (± 15)	135 (± 14)	136 (± 15)	135 (± 29)	131 (± 27)	132 (± 24)	132 (± 27)	.775
HR _{max} (beats·min ⁻¹)	179 (± 14)	176 (± 16)	168 (± 15)	174 (± 15)	166 (± 25)	160 (± 26)	160 (± 23)	162 (± 25)	.463
HR _{min} (beats·min ⁻¹)	80 (± 10)	84 (± 15)	83 (± 14)	82 (± 13)	78 (± 15)	84 (± 16)	85 (± 18)	83 (± 16)	.825
Percentage of HR _{max} (%)	73 (± 6)	72 (± 6)	71 (± 6)	72 (± 6)	73 (± 15)	71 (± 14)	71 (± 13)	72 (± 14)	.805
RPE (AU)	13 (± 2)	12 (± 2)	13 (± 2)	13 (± 2)	12 (± 1)	12 (± 2)	12 (± 1)	12 (± 1)	.679
RPE-L (AU)	13 (± 1)	13 (± 2)	13 (± 2)	13 (± 2)	11 (± 3)	12 (± 2)	12 (± 2)	12 (± 3)	.917
Time \geq 80% HR _{max} (%)	16 (± 9)	14 (± 7)	13 (± 8)	14 (± 8)	15 (± 6)	14 (± 15)	12 (± 15)	14 (± 15)	.585
Fluid intake (mL)	625 (± 458)	692 (± 465)	713 (± 502)	676 (± 475)	676 (± 396)	760 (± 777)	690 (± 553)	709 (± 575)	

Note. AU = arbitrary units; HR_{max} = maximum heart rate; HR_{min} = minimum heart rate; PLA = placebo group; QUE = quercetin group; RPE = Rating of Perceived Exertion; RPE-L = differential Rating of Perceived Exertion-Legs; p values denote Mixed ANOVA intervention group (2) x time (3) interaction effect.

6.3.2.2 Muscle damage.

There was no significant interaction between intervention groups (QUE vs. PLA) on serum LDH concentration, $F(3, 27) = 0.448$, $p = .721$, $\eta_p^2 = .047$, nor were there significant between group differences, $F(1, 9) = 0.444$, $p = .522$, $\eta_p^2 = .047$. There was a large significant main effect for time, $F(3, 27) = 11.010$, $p \leq .001$, $\eta_p^2 = .550$. Pairwise comparisons (Bonferroni corrected) revealed that the 3-day exercise protocol significantly increased LDH, with significant differences between mean LDH concentrations at T2 (post-exercise) in comparison to all other time points (T2: $M = 343.59$, $SD = 66.38$ U·L⁻¹ vs. T0: $M = 269.49$, $SD = 43.34$ U·L⁻¹, 95% CI [15.80, 132.41], $p = .012$; vs. T1: $M = 280.45$, $SD = 65.18$ U·L⁻¹, 95% CI [3.11, 123.18], $p = .038$; vs. T3: $M = 272.34$, $SD = 45.97$ U·L⁻¹, 95% CI [33.58, 108.93], $p = .001$; Figure 6.5).

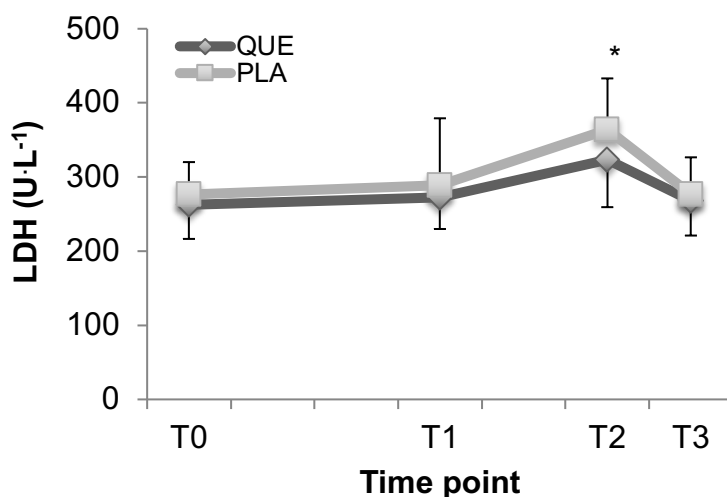


Figure 6.5. Mean serum lactate dehydrogenase (LDH) concentrations in the QUE ($n = 6$) and PLA group ($n = 5$) at baseline (T0), pre-exercise (T1), post 3-day exercise (T2), and after 42 hr recovery (T3). Data displayed as means, error bars represent SD . T = time point. *Denotes significant difference to T0, T1 & T3, Mixed ANOVA time effect, $p \leq .05$.

6.3.2.3 Inflammatory responses.

IL-6, total leukocyte & neutrophil counts, CRP & NF- κ B p65 activation.

There was no significant interaction between intervention groups and time on serum IL-6 concentration, $F(1.172, 10.547) = 0.151$, $p = .745$, $\eta_p^2 = .016$ (Greenhouse-Geisser correction applied). The main effect for group (QUE vs. PLA) was also non-significant, $F(1, 9) = 0.582$, $p = .465$, $\eta_p^2 = .061$. There was a significant main effect for time for mean IL-6 concentrations across time points (T0: T3; $F(1.172, 10.547) = 8.177$, $p = .014$, $\eta_p^2 = .476$. Mean serum IL-6 concentrations were increased following the 3-day exercise protocol in comparison to all other time points (T2: $M = 1.96$, $SD = 1.56$ $\text{pg}\cdot\text{mL}^{-1}$, vs. T0: $M = 0.68$, $SD = 0.50$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [0.35, 2.98], T1: $M = 0.66$, $SD = 0.36$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [0.45, 2.70], T3: $M = 0.63$, $SD = 0.32$ $\text{pg}\cdot\text{mL}^{-1}$, 95% CI [0.12, 2.84]), however, post hoc pairwise comparisons (Bonferroni corrected) failed to detect significant differences across the different time points (Figure 6.6).

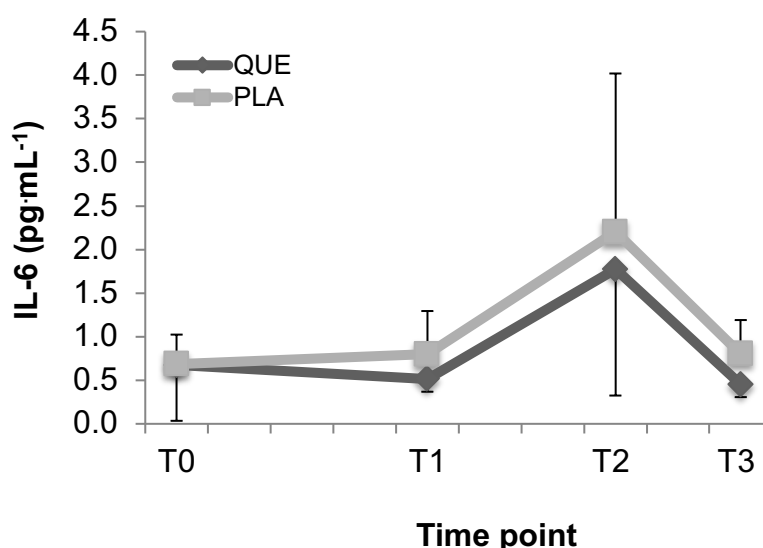


Figure 6.6. Mean serum interleukin-six (IL-6) concentration in the QUE ($n = 6$) and PLA group ($n = 5$) at baseline (T0), pre-exercise (T1), post 3-day exercise (T2), and after 42 hr recovery (T3). Data displayed as means, error bars represent SD . T = time point.

There were no statistically significant interactions between intervention groups and time points on total leukocyte or neutrophil counts assessed in whole blood, $F(3, 27) = 0.322$, $p = .809$, $\eta_p^2 = .035$, $F(3, 27) = 0.130$, $p = .941$, $\eta_p^2 = .014$ (leukocytes & neutrophils, respectively). Analyses of the main effects for group also showed no significant differences (QUE vs. PLA) for total leukocytes, $F(1, 9) = 2.702$, $p = .135$, $\eta_p^2 = .231$, and neutrophils, $F(1, 9) = 4.801$, $p = .056$, $\eta_p^2 = .348$. The main effects for time showed large significant differences in total leukocyte counts, $F(3, 27) = 4.320$, $p = .013$, $\eta_p^2 = .324$, and neutrophil counts, $F(3, 27) = 8.495$, $p < .001$, $\eta_p^2 = .486$, across time points (T0: T3). Post hoc analyses (Bonferroni corrected pairwise comparisons) revealed that mean total leukocyte counts were significantly higher following the 3-day exercise protocol (T2: $M = 6.1$, $SD = 1.5 \times 10^9 \cdot L^{-1}$) in comparison to the recovery time point (T3: $M = 4.1$, $SD = 1.1 \times 10^9 \cdot L^{-1}$; 95% CI [0.3, 3.6], $p = .021$), with no other time differences observed. Neutrophil counts were significantly lower during recovery (T3) in comparison to all other time points (T3: $M = 2.3$, $SD = 0.7 \times 10^9 \cdot L^{-1}$ vs. T0: $M = 3.1$, $SD = 0.8 \times 10^9 \cdot L^{-1}$, 95% CI [0.9, 1.6], $p = .028$; vs. T1: $M = 3.4$, $SD = 1.2 \times 10^9 \cdot L^{-1}$, 95% CI [0.1, 2.0], $p = .025$; vs. T2: $M = 4.0$, $SD = 1.3 \times 10^9 \cdot L^{-1}$, 95% CI [0.6, 2.8], $p = .004$ [Figure 6.7; due to group differences in total neutrophil counts at T0, neutrophils are also expressed relative to total leukocyte counts, Table 6.5]).

There were no significant interactions or main effects between the intervention groups and time points on serum CRP concentration or NF- κ B p65 activation in PBMCs, $p \geq .05$ (Table 6.6).

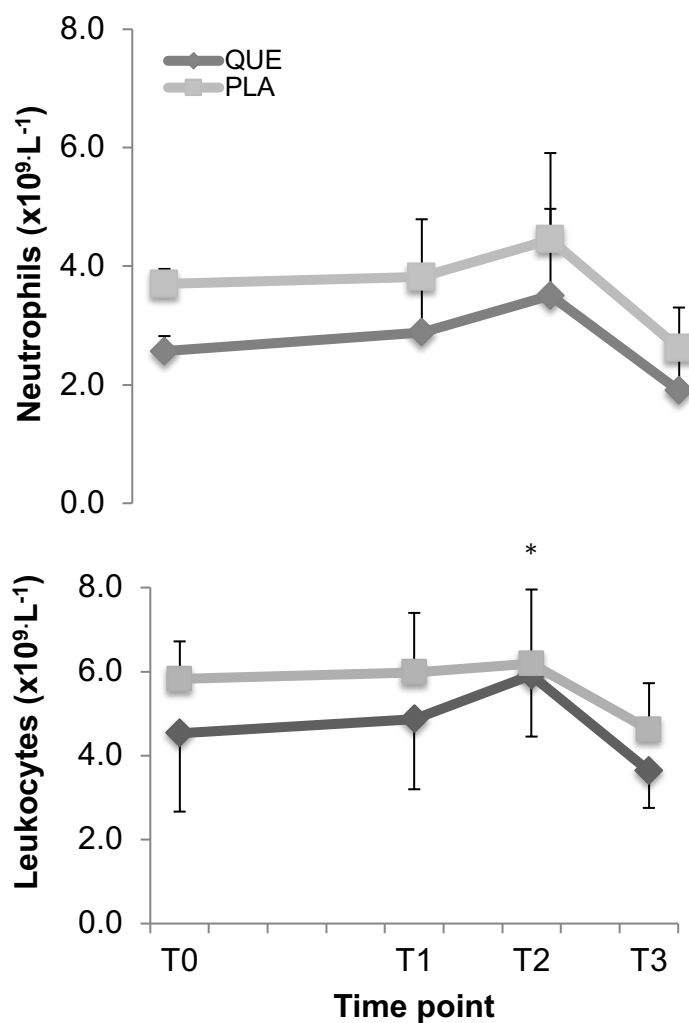


Figure 6.7. Total leukocyte and neutrophil counts in the QUE ($n = 6$) and PLA group ($n = 5$) at baseline (T0), pre-exercise (T1), post 3-day exercise (T2), and after 42 hr recovery (T3). Data displayed as means, error bars represent *SD*. T = time point. *Denotes significant difference to T3, Mixed ANOVA, time effect, $p \leq .05$, **significant difference between T3 and T0: T2, Mixed ANOVA, time effect, $p \leq .05$.

Table 6.5

Relative Neutrophil Counts

Total Neutrophil Count Relative to Total Leukocyte Count (%)				
	T0	T1	T2	T3
QUE (<i>n</i> = 6)	57	59	59	52
PLA (<i>n</i> = 5)	64	64	72	57

Note. PLA = placebo group; QUE = quercetin group; T = time point.

Table 6.6

TAS, MDA, CRP, & NF-κB p65 Responses to the 3-Day Exercise Protocol

Variable	QUE (<i>n</i> = 6)				PLA (<i>n</i> = 5)				<i>p</i>
	T0	T1	T2	T3	T0	T1	T2	T3	
TAS (mmol·L ⁻¹)	1.39 (0.35)	1.45 (0.14)	1.41 (0.14)	1.33 (0.20)	1.39 (0.20)	1.30 (0.10)	1.36 (0.02)	1.34 (0.08)	.533
MDA (μM)	1.49 (0.44)	1.35 (0.16)	1.26 (0.27)	1.49 (0.42)	1.46 (0.12)	1.35 (0.13)	1.17 (0.16)	1.33 (0.10)	.895
CRP (mg·L ⁻¹)	0.4 (0.1)	0.4 (0.2)	0.9 (0.4)	0.6 (0.4)	0.4 (0.0)	0.5 (0.1)	0.6 (0.3)	0.5 (0.3)	.676
NF-κB p65 (ng·μg ⁻¹)	0.22 (0.11)	0.38 (0.20)	0.19 (0.08)	0.25 (0.10)	0.18 (0.09)	0.29 (0.12)	0.29 (0.13)	0.32 (0.15)	.276

Note. CRP = C-Reactive Protein; MDA = Malondialdehyde; NF-κB p65 = Nuclear Factor Kappa-light-chain-enhancer of B cells p65 activation; PLA = Placebo group; QUE = Quercetin group; TAS = Total Antioxidant Status. Data presented as *M* (\pm *SD*); *p* values denote Mixed ANOVA intervention group (2) x time (4) interaction effect.

6.3.3. Functional recovery measures.

6.3.3.1 Perceived fatigue.

Subjective feelings of perceived fatigue were assessed using an overall well-being score (Perceptual Fatigue Questionnaire), with higher scores equating greater overall well-being. There was no significant interaction between intervention groups and time on overall well-being, $F(3, 27) = 1.147$, $p = .348$, $\eta_p^2 = .113$, and no main effect detected for mean group differences (QUE vs. PLA), $F(1, 9) = 1.644$, $p = .232$, $\eta_p^2 = .154$. A significant main effect was observed for time, $F(3, 27) = 3.939$, $p = .019$, $\eta_p^2 = .173$. Pairwise comparisons (Bonferroni corrected) revealed that mean overall well-being scores were significantly decreased following the 3-day exercise protocol (T2) in comparison to the recovery time point (T3; T2 $M = 16.23$, $SD = 2.09$ AU vs. T3 $M = 18.79$, $SD = 2.4$ AU, 95% CI [0.06, 5.05], $p = .044$ [Figure 6.8]).

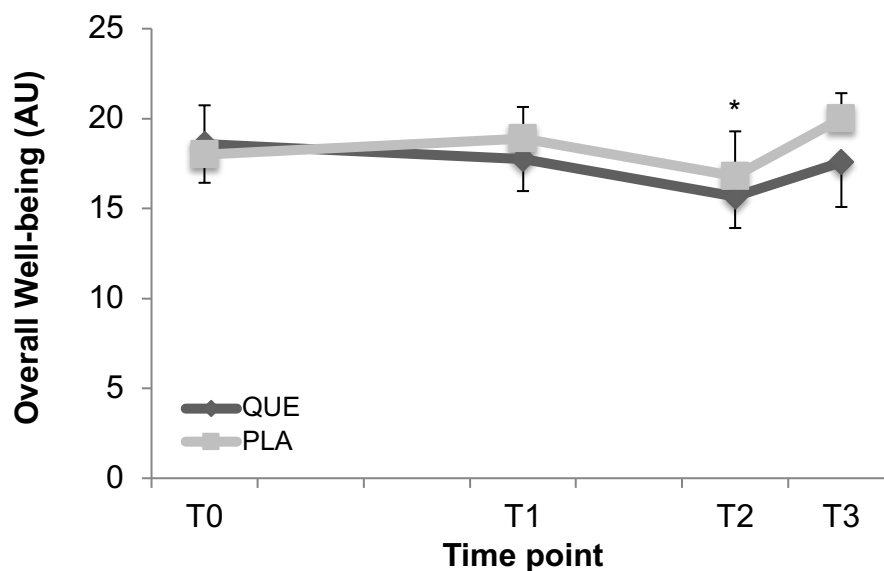


Figure 6.8. Overall well-being scores (higher scores denotes greater well-being) in the QUE ($n = 6$) and PLA group ($n = 5$) at baseline (T0), pre-exercise (T1), post 3-day exercise protocol (T2), and after 42 hr recovery (T3). Data displayed as means, error bars represent SD . T = time point. *Denotes significant difference to T3, Mixed ANOVA, time effect, $p \leq .05$.

6.3.3.2 *Perceived muscle soreness.*

General lower body muscle soreness assessed via VAS scores changed significantly across time points throughout the protocol, with a significant main effect detected for time, $F(1.564, 14.073), = 5.573, p = .022, \eta_p^2 = .382$ (Greenhouse-Geisser correction applied). Mean VAS scores increased from baseline and pre-exercise values in response to the 3-day exercise protocol, and decreased with recovery in the PLA group, but remained elevated in the QUE group (Figure 6.9). However, pairwise comparisons (Bonferroni corrected) failed to detect post hoc differences across the different time points. No significant interaction was detected between intervention groups and time, $F(1.564, 14.073), = 0.611, p = .614, \eta_p^2 = .064$, nor main effect for group, $F(1, 9), = 0.519, p = .490, \eta_p^2 = .055$. For localised perceived muscle soreness of the quadriceps & hamstrings there were no statistically significant interactions between intervention groups and time on VAS scores, $p \geq .05$. The main effects for group and time also failed to detect significant differences in mean VAS scores of the quadriceps or hamstrings between the groups (QUE vs. PLA) and across the different time points (T0: T3), $p \geq .05$ (Table 6.8).

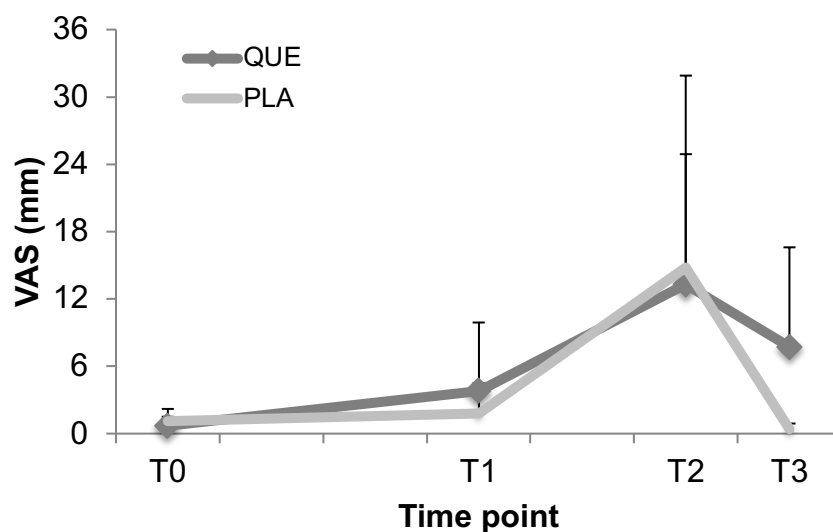


Figure 6.9. Visual Analogue Scale (VAS) scores for general lower body perceived muscle soreness in the QUE ($n = 6$) and PLA group ($n = 5$) at baseline (T0), pre-exercise (T1), post 3-day exercise (T2), and after 42 hr recovery (T3). Data displayed as means, error bars represent SD. T = time point.

Table 6.7

Localised perceived muscle soreness; M (\pm SD)

VAS score	QUE ($n = 6$)				PLA ($n = 5$)				p
	T0	T1	T2	T3	T0	T1	T2	T3	
Quadriceps (mm)	1.8 (3.5)	0.7 (0.8)	8.7 (11.7)	4.5 (5.7)	1.0 (1.7)	3.0 (5.1)	1.4 (2.1)	3.1 (6.1)	.230
Hamstrings (mm)	3.7 (5.4)	4.0 (4.7)	7.4 (4.6)	6.2 (9.4)	10.5 (9.9)	4.2 (5.0)	4.8 (9.1)	3.0 (5.6)	.239

Note. PLA = placebo group; QUE = quercetin group; VAS = Visual Analogue Scale. No significant interaction effect, or main effects for group or time; p denotes 2 x 4 Mixed ANOVA interaction effect, $p \geq .05$.

6.4 Discussion

The purpose of this study was to expand on chapter 5, by investigating the efficacy of prolonged quercetin supplementation (900 mg·d⁻¹ for 12 d [7 d loading, continuing throughout the 3 d exercise protocol & 42 hr recovery period]) to attenuate biomarkers of exercise-induced cellular damage and inflammation and promote recovery, in comparison to a placebo. Significant transient post-exercise increases in (i) serum LDH, (ii) serum IL-6, (iii) whole blood total leukocyte and neutrophil counts, (iv) general muscle soreness, and (v) perceived fatigue were observed in response to the 3-day exercise protocol. However, the 3-day exercise protocol did not elicit an altered response in (i) oxidative stress (MDA & TAS), (ii) CRP, (iii) NF-κB p65 activation, or (iv) localised muscle soreness in either group. Moreover, there was no significant effect of the quercetin intervention detected for any of the dependent variables, indicating no effect of quercetin in comparison to the placebo (rejecting the primary experimental hypothesis). Results of this study provide no significant support for the use of prolonged quercetin supplementation to promote recovery following prolonged duration, soccer-specific, high-intensity intermittent exercise performed on consecutive days in trained athletes.

6.4.1 Main findings.

A brief overview of the effects of the 3-day exercise protocol (similar to the findings of chapter 5) is first discussed. The effects of quercetin supplementation on dependent variables are then discussed (section 6.4.1.2).

6.4.1.1 Biochemical & perceptual responses to the 3-day exercise protocol.

The stress of the 3-day exercise protocol did not differ between the QUE versus PLA group, with both groups presenting similar HR responses across the three days (Table 6.3). The 3-day exercise protocol elicited acute transient post-exercise increases in cellular damage and inflammation in both groups (evidenced by elevations in (i) serum LDH, (ii) serum IL-6, (iii) whole blood total leukocyte & (iv) neutrophil counts [following the findings from chapter 5]), which diminished with a 42 hr recovery period. Corresponding with chapter 5, the 3-day exercise protocol did not induce oxidative stress in either group (evidenced by no significant temporal alterations in MDA or TAS). Serum CRP concentration and activation of the NF- κ B pathway also remained unaltered in response to the 3-day exercise protocol in both groups, which is surprising given that (i) the NF- κ B classical pathway has been implicated as a regulator of IL-6 and (ii) hepatic-release of CRP is part of the acute-phase inflammatory response induced by IL-6 (Petersen & Pedersen, 2005). General muscle soreness and perceived fatigue increased immediately post-exercise compared to pre-exercise, irrespective of group, with temporal patterns reflecting increases in cellular damage and inflammation, attributed to the pressure of swelling associated with inflammation (Chatzinikolaou et al., 2014). Localised muscle soreness of the quadriceps and hamstrings were uninfluenced by the 3-day exercise protocol in both groups, potentially due to the selected sites of palpation (sites closer to the distal myotendinous junction of the muscle may have produced more soreness [Lau, Muthalib, & Nosaka, 2013]).

The effects of the quercetin intervention on the dependent variables in comparison to the placebo are discussed hereafter.

6.4.1.2 Effect of quercetin on oxidative stress, cellular damage, & inflammation.

There was no significant effect of prolonged quercetin supplementation, compared to a placebo, on serum TAS or MDA, rejecting working hypothesis H_{1c} . Data revealed that mean serum TAS concentration did not increase significantly in the QUE group following seven days loading, compared to the PLA group, supporting the work of McNulty et al. (2008) and Quindry et al. (2008) who also found no additive effect of quercetin supplementation on antioxidant capacity (i.e., plasma FRAP & TEAC), attributed to the solubility of quercetin (Quindry et al., 2008). However, the form of quercetin used in the present study was quercetin dihydrate, which has increased solubility in comparison to glycoside or aglycone forms. The majority of quercetin supplementation studies have used glycoside or aglycone forms, making the results of this study challenging to interpret in context of those studies. Subsequent analyses of individual TAS concentrations revealed serum TAS increased in the QUE group following loading (excluding 1 participant), compared to the PLA group, where individual TAS concentrations decreased (non-significant; APPENDIX I); potentially suggesting that quercetin dihydrate supplementation increased aqueous-phase antioxidant capacity. Analysis of plasma quercetin metabolites was beyond the scope of the study but would have provided valuable information regarding the bioavailability of the quercetin supplement.

Participants in the present study were trained athletes with baseline TAS concentrations within the recommended range (1.30 - 1.77 mmol·L⁻¹ [Randox TAS kit insert]). Therefore, it is theorised that participants had well adapted endogenous antioxidant defence systems to cope with the physiological stress of the exercise (Becatti et al., 2017; Powers et al., 2010a), evidenced by no significant post-exercise alterations in serum MDA or TAS in either group, demonstrating the importance of accounting for basal oxidative stress measurements in athletes prior to supplementation, as supplementation may not be warranted. This may partly explain the lack of observed effect of quercetin on oxidative stress biomarkers in the current study as it is proposed that quercetin mediates its antioxidant properties primarily through scavenging of free radicals (Davis et al., 2009b), upon which the principle of the TAS assay is based. However, this does not explain the lack of quercetin effect in those studies demonstrating an exercise-induced oxidative stress insult (e.g., Abbey & Rankin, 2011; McAnulty et al., 2008; Nieman et al., 2007a). The results of the present study are in contrast to Askari et al. (2012), McAnulty et al. (2013) and Ramezani and Moonikh (2017) who demonstrated positive effects of quercetin on biomarkers of oxidative stress. Askari et al. (2012) and McAnulty et al. (2013) used a quercetin supplement combined with additional antioxidants, the synergistic effects of which would have increased bioavailability and bioactivity and could explain the positive results observed, while Ramezani and Moonikh (2017) used a longer duration supplementation strategy (6 weeks).

Results also showed that quercetin supplementation did not significantly attenuate post-exercise elevations in biomarkers of acute cellular damage or inflammation, compared to a placebo, rejecting working hypotheses H_{1a} and H_{1b} . It is hypothesised

that the observed increase in post-exercise serum LDH was caused by primary mechanical stress induced by the intermittent mode of the 3-day exercise protocol (based on the lack of alterations in oxidative stress data there was no clear evidence of metabolic stress). This could potentially explain the lack of observed quercetin effects. If the antioxidant properties of quercetin were effective *in vivo*, quercetin would attenuate metabolic stress by free radical scavenging with no effect on mechanical stress. It is proposed that quercetin supplementation may be more beneficial for modulating secondary muscle damage; scavenging ROS produced during the neutrophil respiratory burst as part of the inflammatory response. Total neutrophil counts were augmented immediately post-exercise and diminished with recovery in both groups, indicative of increased phagocytosis and respiratory burst activity resulting in apoptosis (Peake et al., 2017; Pyne, 1994a). However, there was no concomitant observed change in oxidative stress biomarkers to indicate neutrophil burst activity (perhaps due to the sensitivity of the selected biomarkers), thus, support for this theory is not supported by the data.

These null findings agree with several authors finding no effect of quercetin on biomarkers of muscle damage or inflammation (Abbey & Rankin, 2011; McAnulty et al., 2008; Nieman et al., 2007a; O'Fallon et al., 2012), but in contrast to Askari et al. (2012) and Nieman et al. (2009). The quercetin supplementation strategies used by Askari et al. (2012) and Nieman et al. (2009) differed in comparison to that of the present study (which used quercetin plus vitamin C [90 mg]), and those of the other studies, which used an isolated quercetin supplement. The duration of supplementation was also prolonged (56 days & 24 days, respectively). The additional antioxidants and longer supplementation period may have increased the

bioavailability and bioactivity of quercetin. Further research should explore the synergist effects of mixed quercetin supplements, dosed over longer periods than the 7-day loading used in the current study.

It should be noted that trends in the group mean data (refer to Figures 6.5, 6.6, & Table 6.5) indicated that relative increase pre-post exercise differences in several of the dependent variables (i.e., (i) LDH; (ii) IL-6; (iii) total leukocyte & neutrophil counts; (iv) NF- κ B p65 activation; (v) TAS) were lower in the QUE group versus the PLA group (non-significant). The magnitude of the intervention effect of quercetin on these biomarkers could be explained by inter-individual variability (individual responses presented in APPENDIX I). Exploring individual data demonstrates biochemical responses to be highly individualistic. However, there is a possibility that small effects of quercetin may have been present but undetected by the statistical tests. For example, group mean differences for LDH pre-post exercise were QUE 19% vs. PLA 26 %, further supported by individual responses where three out of five participants in the PLA group presented > 40% increase in serum LDH post-exercise (T2) compared to pre-exercise (T1), whereas, in comparison, five out of six participants in the QUE group presented < 20% increase. This would have provided support for the anti-inflammatory effects of quercetin through down-regulation of the NF- κ B pathway; however, this is purely speculative and unsupported by statistical tests using group mean data. Nevertheless, quercetin relative bioavailability has been demonstrated to be highly individual (Jin et al., 2010), and it is considered that marginal gains may translate to increased performance in individual athletes responding to quercetin supplementation, emphasising the importance of (a) an individualised approach to supplementation, and (b) baseline data. Further

exploration of the efficacy of quercetin supplementation in this type of exercise is potentially warranted based on non-significant trends in the data.

6.4.1.3 Effect of quercetin on recovery measures.

It was hypothesised that prolonged quercetin supplementation would promote recovery, indirectly, by reducing fatigue, through (i) dampened inflammation; (ii) reduced oxidant-mediated damage, and (iii) reduced perception of pain. In contrast to working hypothesis H_{1d} , prolonged quercetin supplementation did not influence recovery (assessed via muscle soreness & perceived fatigue), compared to a placebo. The lack of observed effects of quercetin on recovery is unsurprising given that quercetin did not attenuate biomarkers of cellular damage or inflammation (mechanisms of muscle soreness [Ranchordas et al., 2017b]), and supports previous research (Nieman et al., 2007b; O'Fallon et al., 2012) which also demonstrated no effect of quercetin on perceived muscle soreness following muscle-damaging exercise. Given that there may have been limited opportunity for the antioxidant and anti-inflammatory properties of quercetin to exert an effect, the analgesic properties of quercetin to reduce perceived exertion throughout the exercise protocol were also explored. There was no significant effect of quercetin, compared to a placebo, on perceived exertion (RPE scales) throughout the 3-day exercise protocol. These null findings corroborate the work of other studies (Cureton et al., 2009; Cheavront et al., 2009; Utter et al., 2009) that also reported no effect of quercetin supplementation on perceived exertion. In contrast, MacRae and Mefferd (2006) found prolonged quercetin supplementation increased power output for a given RPE during cycling time trials in elite athletes, however, the quercetin dosing strategy was much longer (6 weeks) than that of the present study and was administered in a mixed antioxidant

supplement. A suggested reason for the observed lack of effect may be because quercetin does not act as an adenosine A₁-receptor antagonist in vivo (Cheuvront et al., 2009). Perceived measurements are subjective which may partly explain the results. However, subjective measures are important to gain further understanding of an athlete's perception of the exercise stress, as this may differ from objective measures. Participants in the present study were blinded to intervention group until after completion of the study, where only one participant in each group correctly identified group allocation. Therefore, it is unlikely that participant scores were biased because of demand characteristics and that the lack of observed differences between the QUE versus PLA groups could be attributed to the placebo effect. These results provide further indication that the properties of quercetin demonstrated in vitro and in rodent studies do not appear to translate to trained athletes.

Combined, these results suggest that quercetin does not attenuate cellular damage or inflammation or promote recovery following a 3-day period of prolonged high-intensity intermittent exercise, characteristic of soccer in trained athletes. Null findings observed in the current study could be attributed to several factors. The cohorts of participants in the present study were highly trained athletes; studies demonstrating beneficial effects of quercetin supplementation have predominantly used lesser trained or untrained participants (Askari et al., 2012; Bazzuchi et al., 2019; Davis et al., 2010; Nieman et al., 2010; Ramezani & Moonikh, 2017), the results of which may not translate to a highly trained cohort (Tejada et al., 2017). Although daily quercetin intake was reported to be low in both groups ($4 \pm 2 \text{ mg}\cdot\text{d}^{-1}$), antioxidant capacity was within the recommended range in both groups. It is speculated that the athletes had the antioxidant capacity to cope with the stress of

the exercise protocol (also reflected by MDA, HR, & RPE data [Tables 6.5 & 6.3]), limiting the opportunity for quercetin to exert an effect as oxidative stress remained unaltered in response to the exercise protocol. Following ingestion, quercetin is metabolised and converted to quercetin metabolites (Justino et al., 2004), which could also explain the null interaction findings in this study, as quercetin metabolites may not accumulate in sufficient quantities in the compartments tested (i.e., serum; PBMCs). Interestingly, Nieman et al. (2007a) demonstrated an effect of quercetin on exercise-induced inflammatory biomarkers in leukocytes but not plasma or skeletal muscle tissue. At present, there is no optimal dosing strategy recommended for quercetin supplementation. The 7-day loading strategy used in this study was based on previous research; however, studies demonstrating positive effects have used loading strategies longer than that of the present study (range 14 - 48 d; Askari et al., 2012; Bazzuchi et al., 2019; MacRae & Mefferd, 2006; Nieman et al., 2007a, 2009, 2010; Ramezani & Moonikh, 2017). Quercetin has been reported to peak in plasma ~2 hr after ingestion (Davis et al., 2009b; Egert et al., 2008). The 900 mg·d⁻¹ dosages administered in the present study were taken in three 300 mg doses at mealtimes, with participants instructed to consume one 300 mg dose of the supplement with the pre-exercise standardised meal replacement drink, two hours before each exercise bout; therefore, it is theorised that plasma quercetin concentration should have been at peak concentration during the exercise protocol (as proposed by Abbey & Rankin, 2011; Braakhuis & Hopkins, 2015). The quercetin supplement contained vitamin C (90 mg), incorporated to increase the bioavailability of quercetin by recycling the oxidised form back to the parent form (Boots et al., 2008). Vitamin C was not included in the placebo. To determine whether vitamin C exerted an independent effect, a third group of participants, supplemented with isolated vitamin C would have

been required, but was not included because of restricted availability of participants, however, the null findings between the QUE versus PLA group in the present study provide evidence that vitamin C did not exert an independent effect. The bioavailability of the quercetin supplement dosing strategy used in the present study was unknown and the duration may not have been long enough to exert an effect. It is recommended that further research assess the bioavailability of the quercetin dosing strategy prior to testing bioefficacy.

6.4.2 Strengths & limitations.

As per chapter 5, the present study employed a parallel groups design to limit (a) the RBE of the exercise protocol, and (b) learning effects; which may partly explain the positive results demonstrated in previous quercetin exercise studies using a crossover study design. Further strengths of the study include (i) the use of a *non-reductionist approach* (i.e., participants' maintained their habitual diet & dietary sources of quercetin were not restricted; Bowtell & Kelly, 2019), which increased ecologically validity, and (ii) the inclusion of subjective recovery measures to contextualise biomarker data. A key limitation of the present study was sample size ($N = 11$ [$n = 6$; $n = 5$]), which is a common issue in research using trained athletes (Malone et al., 2015b). Evidence-based a priori power estimations (using data from Nieman et al., 2009) proposed the current sample size adequate to detect effects for selected primary variables. However, post hoc power estimations revealed the results of the study to be underpowered (i.e., $1-\beta < 80\%$ for all dependent variables [excluding NF- κ B p65]). Therefore, potential Type II errors may have led to the magnitude of differences in dependent variables between the quercetin intervention and placebo being undetected. To account for this, individual responses were

presented, as small effects may still be beneficial to individual athletes, the concept of *marginal gains*, *ceteris paribus*. The findings of the present study are also limited by testing the efficacy of quercetin supplementation to promote recovery using a laboratory-based soccer simulation protocol. A lack of exercise-induced oxidative stress and NF- κ B p65 activation following the 3-day exercise protocol may have limited the potential of quercetin to exert an effect through antioxidant or anti-inflammatory mechanisms. Nevertheless, these preliminary findings contribute to the literature. At the time the study was conducted, this was the first study to investigate the efficacy of quercetin supplementation to promote recovery following prolonged duration soccer-specific exercise (Ramezani and Moonikh, 2017 was published after data collection of chapter 6).

6.4.3 Conclusion & further research.

In summary, this is the first study, to the lead investigator's knowledge, to examine the efficacy of quercetin supplementation as a recovery strategy following soccer-specific prolonged duration high-intensity intermittent exercise performed on repeated days; adding to the current debate regarding the use of antioxidant supplementation in athletes during intensified periods of exercise. This innovative study demonstrated that a 12-day period of quercetin supplementation (900 mg·d⁻¹; 7 d loading, continuing throughout exercise & recovery) did not significantly attenuate post-exercise increases in biomarkers of cellular damage or inflammation, and had no significant effect on perceived fatigue or muscle soreness, compared to a placebo, rejecting the primary experimental hypothesis. Thus, results from the present study provide no significant evidence to support the use of quercetin supplementation as a recovery strategy in cohorts of trained athletes, confirming that

the effects of quercetin demonstrated *in vitro* and in murine models should not be generalised to trained athletes at present.

Further research expanding on this study using a larger sample size is warranted, as non-significant trends in the group data, alongside individual responses indicated potential beneficial effects of quercetin supplementation. To determine whether the findings of the present study transfer to the practical setting, it is recommended that follow up studies explore the efficacy of quercetin supplementation to promote recovery using field based protocols that have been shown to elicit a greater degree of metabolic and mechanical stress than the soccer simulation treadmill protocol that was used in chapters 5 and 6. The present study sought to determine the underlying mechanisms of quercetin by investigating the NF- κ B classical pathway. As per chapter 5, exercise-induced activation of NF- κ B p65 was unsupported by the data. Thus, another direction for further research is to continue exploring the potential mechanisms of quercetin supplementation *in vivo*.

General Discussion

This thesis set out to further explore the related concepts of exercise-induced oxidative stress, muscle damage, and inflammation, specifically focusing on periods of prolonged high-intensity intermittent exercise, the type of exercise performed in soccer. Research has predominantly investigated these responses following single bouts of soccer, demonstrating negative consequences for the athlete; such as, muscle soreness and fatigue which may compromise recovery (Fatouros et al., 2010; Finaud et al. 2006a; Ispirlidis et al., 2008). During weekly microcycles of a competitive soccer in-season, athletes train and compete daily with minimal recovery periods, yet research investigating the physiological demands of such intensified periods of soccer has been limited, providing a rationale for this thesis. Facilitating recovery between sessions is important to ensure that athletes are in a state of readiness before the next training session or competitive match. Quercetin supplementation has been shown to promote recovery following a 3-day intense period of prolonged steady-state exercise (Nieman et al., 2009) but research has yet to investigate the efficacy of quercetin supplementation on recovery following intensified periods of prolonged intermittent exercise. Therefore, the overall aims of this thesis were to (i) profile an array of biomarkers indicative of exercise-induced oxidative stress, muscle damage, and inflammation following intensified periods of soccer and simulated soccer, and to (ii) examine the efficacy of prolonged quercetin supplementation to modulate these exercise-induced responses and promote recovery. Three studies were conducted (chapters 4, 5 & 6), with biomarkers as primary dependent variables common to all three studies. Chapter 4 had four main objectives: (i) to quantify urinary MDA as a biomarker of lipid peroxidation in a cohort of professional soccer players throughout 3 one-week microcycles of a competitive

in-season ((i) T1: early in-season [1st microcycle]; (ii) T2: mid-season [16th microcycle]; (iii) T3: end of in-season [32nd microcycle]); (ii) to monitor weekly training sessions via GPS and HR based methods to quantify external and internal high-intensity training load; (iii) to explore associations between variables of high-intensity training load and urinary MDA concentrations; and (iv) to compare urinary MDA concentrations at the end of a competitive in-season with those of recreational soccer players. The main objectives of chapter 5 were (i) to profile blood biomarkers of oxidative stress (MDA; TAS), exercise-induced muscle damage (CK; LDH), and inflammation (NF- κ B p65 activation, CRP, IL-6, IL-8, IL-10, MCP-1, total leukocyte & neutrophil counts) pre and post (42 h recovery period) a 3-day period of soccer-specific intensified exercise in trained athletes, and (ii) to compare blood biomarker data to a resting control group. Finally, the objective of chapter 6 was to investigate the efficacy of prolonged quercetin supplementation (900 mg·d⁻¹ for 12 d [7 d loading, continuing throughout the same 3 d exercise protocol & 42 hr recovery period used in chapter 5]), to attenuate biomarkers of cellular damage (LDH), oxidative stress (TAS; MDA), and inflammation (NF- κ B p65 activation CRP; IL-6; total leukocyte & neutrophil counts), and promote recovery (assessed via perceived fatigue & perceived muscle soreness), compared to a placebo, in trained athletes. Increasing understanding in this area could (i) aid sports scientists and coaches in the prescription of balanced training loads and recover periods during intensified periods of exercise, such as microcycles of the soccer in-season, and (ii) provide evidence-based support for the use of quercetin supplementation as a recovery strategy in this type of exercise.

The key findings, contributions to knowledge, strengths, limitations, practical implications, and future directions for further research are discussed hereafter. Acceptance or rejections of null hypotheses are presented in APPENDIX J.

7.1 Synthesis of Key Findings

7.1.1 Exercise-induced oxidative stress.

In chapter 4, a progressive reduction in urinary MDA was observed in professional soccer players over a competitive in-season (Figure 4.6). This finding corroborates previous research that also demonstrated a progressive reduction in MDA over time with participation in high-level soccer training (Becatti et al., 2017; da Silva Barbosa et al., 2017; Ferrer et al., 2009). The increased level of lipid peroxidation observed in the EXP group at the start of the competitive in-season, in comparison to the mid-point and end of the in-season, is an interesting finding and is supported by Becatti et al. (2017) who also found oxidative stress (TBARS; PC) to be highest at the start of a competitive in-season. These findings may have negative implications for professional athletes, such as an increased risk of injury at the onset of competitive match play and may be as a result of the volume of the pre-season training load. It is understood that exercise-induced oxidants act as cell signalling molecules to activate redox-sensitive transcription factors (e.g., NF- κ B; Nrf-2) involved in the expression of endogenous antioxidant enzymes (Gomez-Cabrera et al., 2009; Ji et al., 2007, 2009). An enhanced antioxidant capacity stimulated by exercise-induced ROS could potentially explain the decrease in urinary MDA observed over time in the cohort of professional soccer players; and has previously been observed in similar cohorts of professional soccer players following periods of soccer training (da Silva Barbosa et

al., 2017; Ferrer et al., 2009). Antioxidant capacity was not assessed in chapter 4 and would have provided further insight into the observed decrease in urinary MDA.

Chapter 4 also observed lipid peroxidation to be lower in a cohort of professional soccer players, at the end of a competitive season, in comparison to a cohort of recreational soccer players (Figure 4.7), supporting the work of Cazzola et al. (2003) and Metin et al. (2003). Aerobic fitness, per se, may be a factor related to oxidative stress, as increased aerobic capacity has previously been associated with lower basal oxidative stress (futsal players; Lima et al., 2018). An enhanced antioxidant capacity as an effect of chronic participation in training would further explain the lower urinary MDA concentrations observed in the professional soccer players compared to the recreational players who would have undertaken less training, and is supported by the significantly higher $\dot{V}O_{2\max}$ scores in the professional soccer players (EXP: 57.2 ± 6.7 mL·kg⁻¹·min⁻¹ vs. COM: 48.6 ± 7.1 mL·kg⁻¹·min⁻¹). Athletes often present lower basal oxidative stress values but increased levels post-exercise, however, data is often compared to normal reference ranges (as per Hadžović-Džuvo et al., 2014), therefore, this data supports the need to determine reference values specifically for athletic populations.

Whilst chapter 4 showed a progressive reduction in lipid peroxidation with chronic participation in soccer training, there was no association between the volume of high-intensity exercise undertaken and lipid peroxidation. This was unexpected given that EIOS has been theorised to be intensity and duration dependent (Powers et al., 2016), and previous research has associated EIOS with periods of increased training load in soccer (Le Moal et al., 2016).

There appears to be a lack of association between high-intensity soccer-specific exercise and oxidative stress. Chapters 5 and 6 found that a 3-day intensified period of soccer-specific exercise (90 min·d⁻¹; 12% high-intensity \geq 80% HR_{max}) did not induce an oxidative stress insult (post-exercise serum MDA & TAS concentrations remained unaltered) at the time points considered. These findings are supported by the lack of activation of the redox-sensitive transcription factor NF- κ B, however, are unexpected, given the observed increase in cellular damage and inflammatory response and the duration of the exercise protocol is likely to have increased mobilisation of fatty acids, increasing the potential for lipid peroxidation. These results are in contrast to previous work demonstrating oxidative stress in response to bouts of soccer or simulated soccer (Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Mohr et al., 2015; Silva et al., 2013). However, research exploring the oxidative stress response to soccer has been equivocal (refer to Table 2.3). A reason for these differences may be methodological. There are currently no recommended time points for sampling the oxidative stress response after exercise (Michailidis et al., 2007). The main time point of interest in chapters 5 and 6 was when the athletes would be expected to be ready to train (post-exercise + 42 hr), in comparison to the other studies which have used commonly assessed time points, post-exercise + 24 and + 48 hr time points. Alterations in MDA and TAS may have been undetected at the sampling time points considered.

Redox homeostasis is dependent upon several factors, including the training status of the athlete (Becatti et al., 2017). The athletes studied in the experimental chapters

of this thesis were all *trained* (defined by $\dot{V}O_2\text{max}$; training history; current participation in exercise), accustomed to prolonged intermittent exercise, and reported no dietary deficiencies. The lack of redox response to the 3-day exercise protocol (chapters 5 & 6) may be because the athletes were well adapted to cope with the exercise. However, this does not explain studies demonstrating oxidative stress in trained athletes (Fatouros et al., 2010; Ferrer et al., 2009; Ispirlidis et al., 2008; Le Moal et al., 2016). Exposure to chronic training may have resulted in either (a) an enhanced antioxidant capacity (e.g., expression of MnSOD; iNOS; GPX; CAT) or (b) decreased production of exercise-induced oxidants, or (c) both (Finaud et al., 2006a; Vollard et al., 2005). Antioxidant enzymes were not assessed, however, TAS and NF- κ B data provide no support for this theory, as neither were altered in response to the exercise. It should also be considered that biochemical responses following consecutive days of exercise differs to the response of a single bout. Blood sampling pre and post each daily exercise bout would have provided evidence to support this theory.

The use of a single biomarker of lipid peroxidation in chapter 4 provided limited information regarding oxidative stress, which lead to chapter 5 profiling an array of biomarkers, indicative of oxidant stress, muscle damage, and inflammation, which then narrowed the choice of biomarkers selected for chapter 6.

7.1.2 Exercise-induced muscle damage & associated inflammation.

There was a transient increase in cellular damage (but not excessive muscle damage, per se), following the 3-day intensified period of exercise, evidenced by systemic post-exercise increases in serum CK (chapter 5; Figure 5.4) and LDH

Figures 6.5). These findings support previous research investigating CK and LDH responses following a single bout of soccer (Ascensão et al., 2008; Ispirlidis et al., 2008; Souglis et al., 2015), simulated soccer (Bell et al., 2016), and a 3-day period of intense exercise (Nieman et al., 2014). It is postulated that this primary cellular damage response was caused by mechanical stress associated with the high eccentric load of performing repeated accelerations and decelerations throughout the exercise protocol. Structural damage to the sarcolemma would have resulted in increased membrane permeability and the leakage of CK and LDH into the cytosol (Fatouros & Jamurtas, 2016). This is further supported by the lack of alterations in biomarkers of oxidative stress at the considered time points.

Muscle damage has been associated with an inflammatory response as part of the repair and regeneration process of damaged tissue (Owens et al., 2019; Peake et al., 2017; Pyne, 1994b). Post-exercise increases in biomarkers of inflammation paralleled the cellular damage response, with (i) serum CRP; (ii) serum IL-6; (iii) serum MCP-1; and (iv) whole blood total leukocyte and neutrophils counts elevated immediately post-exercise in response to the 3-day exercise protocol (Figures 5.5: 5.6; 5.7; 5.8). The observed pro-inflammatory cascade supports the role of inflammation in skeletal muscle remodeling (Suzuki, 2018), as CRP, IL-6 and MCP-1 are part of the acute phase response that facilitates neutrophil infiltration into damaged skeletal muscle tissue (Suzuki, 2018) and corroborates the work of previous studies investigating muscle damage and inflammatory responses to soccer-specific exercise (Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et al., 2008; Mohr et al., 2015) or 3-day period of intensified exercise (Nieman et al. 2014).

Interesting, there were no observed exercise-induced alterations in the chemokine, IL-8, or the anti-inflammatory cytokine, IL-10, at the time points considered, suggesting evidence of a pro-inflammatory response that was not counter-regulated by an anti-inflammatory response.

In chapters 5 and 6, cellular damage was observed to increase immediately post-exercise, with CK and LDH concentrations comparable to previous literature exploring muscle damage following single bouts of soccer-specific exercise (~ 400 U·L⁻¹; Ascensão et al., 2008; Bell et al., 2016; Ispirlidis et al., 2008; Souglis et al., 2015). However, the post-exercise time course and magnitude of cellular damage differed to the typical delayed response, where CK and LDH have been shown to peak ($\sim 800 - 1000$ U·L⁻¹) and remain elevated 24 - 96 hr post-exercise (Ascensão et al., 2008; Bell et al., 2016; Ispirlidis et al., 2008; Souglis et al., 2015), attributed to secondary muscle damage caused by ROS production during the neutrophil respiratory burst (Powers et al., 2010). In chapters 5 and 6, cellular damage was resolved within a 24 - 42 hr recovery period, similar to the time course observed by Nieman et al. (2014) following 3 days of intensified treadmill running. The similarity in the cellular damage response exhibited in chapters 5 and 6, to that observed by Nieman et al. (2014), suggests that the time course and magnitude of muscle damage may be exercise mode dependent. The lack of alterations in biomarkers of oxidative stress provides further support for no increase in secondary muscle damage, despite a further increase in neutrophils throughout recovery in chapter 5.

It should be considered that the different response in biomarkers of exercise-induced muscle damage and inflammation following repeated days of soccer-specific

exercise compared to a single bout is possibly indicative of a RBE across the repeated days. Pilot work sampling blood biomarkers pre and post each daily bout of the 3-day protocol revealed a cumulative effect of the 3-day exercise protocol as expected. Interestingly, relative increases in biomarker data were shown to be lower on the second and third day in comparison to the initial bout. It is postulated that the attenuation of biochemical responses following the second and third bouts could be evidence of a RBE effect; given the high number of high velocity eccentric contractions of the protocol (notwithstanding the cohorts being trained athletes). Further support for this theory is reflected in the observed concentrations of biomarkers post-exercise day 3, which were lower than typically expected for this type of exercise. At present, mechanisms of the RBE remain unclear. Proposed mechanisms include (i) mechanical, (ii) neural, and (iii) cellular adaptations that may work independently or synergistically (Hilda, Chen, & Nosaka, 2017; McHugh, 2003; for a review of mechanisms readers are referred to McHugh, 2003). Based on the evidence, it is posited that the observed post-exercise acute inflammatory response (increased CRP, IL-6, MCP-1, total leukocyte & neutrophil counts) may have induced a protective effect in the successive exercise bouts, or blunted further inflammation preventing subsequent secondary muscle damage (McHugh, 2003; Pizza, Koh, McGregor, & Brooks, 2002) as part of the adaptive response that leads to skeletal muscle remodeling. Further work is required to support this theory as a reduction in inflammation following the second and third bouts may also be indicative of less cellular damage in the initial bout (McHugh, 2003).

There are no set values commonly used to quantify the concentration of exercise-induced muscle damage or inflammation that poses a definitive risk to athletes.

Authors have attempted to define reference ranges for CK. Mougios (2007) proposed specific reference values for CK in athletes, and Brancaccio et al. (2010) have defined *normal* resting serum CK to be $< 80 \text{ U}\cdot\text{L}^{-1}$, with hyperCKaemia defined as a 4-fold increase from baseline. To address what constitutes a meaningful physiological change in a biomarker for the athlete (as opposed to a statistical change) researchers have used methods that account for analytical and intra-individual variance (Davison et al., 2012; Lewis et al., 2016), providing practical information for sports scientists/coaches. Normalising data to baseline values, or converting data to Z scores may also reduce inter-individual variance providing clearer exercise-induced responses, although this would require acquiring a true baseline value for athletes (Brancaccio et al., 2010), or building up a database of individual typical responses (McLean et al., 2010). Contextualising biomarker data to performance measures or subjective measures of fatigue may also be beneficial for sports scientists/coaches and for the athlete (Lee et al., 2017), as was a focus of chapter 6.

Post-exercise increases in biomarkers of cellular damage (LDH) and inflammation (IL-6; total neutrophil counts) reflected increased perceived fatigue and general muscle soreness scores at the considered time points (Figures 6.8 & 6.9). This was expected as muscle damage and inflammation are proposed underlying mechanisms of muscle soreness (Brancaccio et al., 2010; Howatson & Van Someren, 2008). This data supports previous research assessing ratings of muscle soreness concomitant with biomarkers of muscle damage and inflammation following soccer-specific exercise (Ascensão et al., 2008; Bell et al., 2016; Fatouros et al., 2010; Ispirlidis et

al., 2008; Mohr et al., 2015) and provides support for the use of these biomarkers as recovery dependent markers.

It should be noted that there appears to be an inconsistency in the exercise-induced response of serum CRP and whole blood total neutrophil counts between chapters 5 and 6 of this thesis (Figures 5.7, 5.8 & 6.7; Table 6.6). In chapter 5, serum CRP was increased post-exercise, supporting the work of authors demonstrating increased CRP in response to soccer-specific exercise (Bell et al., 2016; Ispirlidis et al., 2008; Mohr et al., 2015; Souglis et al., 2015). Interleukin-6 stimulates the release of CRP as part of the acute-phase response, with CRP responsible for identifying and removing damaged cells (Giudice & Gangestad, 2018; Petersen & Pedersen, 2005); therefore, this response was expected as IL-6 was also observed to increase. However, concentrations of CRP were considered high ($> 3.0 \text{ mg}\cdot\text{L}^{-1}$ [Randox CRP kit insert]) throughout all time points, in comparison to other researchers (Bell et al., 2016; Ispirlidis et al., 2008; Mohr et al., 2015; Souglis et al., 2015) who reported concentrations to be $< 3.0 \text{ mg}\cdot\text{L}^{-1}$. In chapter 6, there was no exercise effect on serum CRP (regardless of group) and concentrations were within the normal range ($< 1.0 \text{ mg}\cdot\text{L}^{-1}$). This difference could be attributed to inter-individual variance in the different cohorts of athletes. A methodological difference could also explain observed differences in serum CRP, as CRP analyses were conducted using a manual analyser in chapter 5, whereas an automated analyser was used in chapter 6 (because of the difficulty in performing the time-critical CRP assay manually). Whole blood total neutrophil counts also differed; remaining elevated above pre-exercise counts 42 hr post-exercise in chapter 5, but decreasing to below baseline levels 42 hr post-exercise in chapter 6 (regardless of group). It is theorised that the observed

increase in neutrophils 42 hr post-exercise may have been due to cortisol-induced demargination of neutrophils from bone marrow or MCP-1 mobilisation of neutrophils into the bloodstream in response to the exercise-induced cellular damage (Peake et al., 2017; Pyne, 1994a). The attenuated neutrophil response 42 hr post-exercise in chapter 6 could be explained by increased phagocytosis leading to cell apoptosis (Peake et al., 2017; Pyne, 1994a). Different cohorts of trained athletes were used in chapters 5 and 6 and the different response in neutrophil counts could be due to inter-individual variation.

Data from this thesis shows increased cellular damage and inflammation following a 3-day intensified period of soccer-specific exercise in trained athletes. The use of a recovery strategy that promotes recovery to minimise residual effects of cellular damage and inflammation may be warranted to preserve the quality of subsequent performances. However, the practical application of these results should be treated with caution, given that the magnitude and time course of muscle damage and inflammation may differ in field-based soccer.

7.1.3 Mechanisms: NF- κ B classical pathway.

This thesis specifically sought to investigate the NF- κ B classical pathway by assessing NF- κ B p65 activation in PBMCs in response to the 3-day period of soccer-specific intensified exercise (chapters 5 & 6). The NF- κ B classical pathway is a regulator of inflammatory responses (involved in the expression of acute-phase proteins, cytokines, & antioxidant enzymes; Gomez-Cabrera et al., 2006; Ji et al., 2004, 2007; Niemen et al., 2007a; Vella et al., 2012), and can be activated by exercise-induced responses (e.g., (i) oxidative stress; (ii) muscle damage; (iii)

ischaemic-reperfusion; (iv) pro-inflammatory cytokines; Cuevas et al., 2005; Ji et al., 2004, Ji et al., 2007; Kramer & Goodyear, 2007; Nieman et al., 2007; Vider et al., 2001). Furthermore, quercetin has been shown to suppress NF- κ B activation (Nair et al., 2006), providing the rationale for investigating the NF- κ B classical pathway in this thesis. The results of chapters 5 and 6 showed (i) no activation of NF- κ B p65 in PBMCs in response to the 3-day exercise protocol, despite an increase in pro-inflammatory proteins (i.e., serum CRP; serum IL-6; serum MCP-1), and (ii) no effect of quercetin on NF- κ B activity. These findings support Nieman et al. (2007a) who also demonstrated no effect of exercise (3-day cycling protocol; 57% W_{max}) or quercetin supplementation (1000 mg·d⁻¹; 21 d loading) on NF- κ B activity in skeletal muscle despite an increase in inflammatory cytokines in trained athletes. These findings suggest that the regulation of inflammatory responses is independent of NF- κ B p65 activation. Assessing cytokine expression in PBMCs (as opposed to serum studied in chapters 5 & 6) may have provided more insight into the NF- κ B classical pathway. Human exercise studies have shown equivocal results in the NF- κ B response to exercise, with the results of this thesis also supporting the work of Buford et al. (2009) and Koenig et al. (2015) who demonstrated no effect of downhill running (45 min, 60% $\dot{V}O_{2max}$; 60 min, 75% $\dot{V}O_{2max}$, respectively) on NF- κ B activity in recreationally trained athletes. Interestingly, Parker et al. (2017) demonstrated increased NF- κ B p65 activity following all-out maximal sprint cycling exercise (4 x 30 s), but not after repeated sprints performed at 75% maximal power (5 x 4 min), or steady-state cycling performed at 50% maximal power (30 min), despite the shorter duration of the sprint exercise. It appears that the 3-day exercise protocol (90 min·d⁻¹, performed at an average intensity of 74% HR_{max} ; 12% high-intensity [$\geq 80\%$ HR_{max}]) adopted in chapters 5 and 6 of this thesis was insufficient to

cause an oxidative stress insult and activate NF- κ B p65, possibly due to intensity of the exercise or the trained status of participants. However, the findings from chapter 4 showed no correlation was observed between high-intensity exercise and oxidative stress. Further research exploring exercise intensity and activation of the NF- κ B classical pathway is warranted. The underlying mechanisms of exercise-induced inflammation are complex. Novel data from chapters 5 and 6 of this thesis does not appear to provide any causal links between NF- κ B p65 activation and (i) soccer-specific exercise, (ii) exercise-induced cellular damage, (iii) pro-inflammatory cytokines, or (iv) elevated total neutrophil counts in trained athletes at the considered time points.

7.1.4 Quercetin supplementation strategy.

The results of chapter 6 did not support the hypothesis and provide no direct support for the use of quercetin supplementation as a recovery strategy in trained athletes undertaking an intensified period of soccer-specific exercise. Quercetin was ineffective in influencing any of the dependent variables that were elevated in response to the 3-day exercise protocol (i.e., cellular damage; inflammation; muscle soreness; fatigue), in comparison to a placebo. These findings support the work of other researchers demonstrating no effect of quercetin biomarkers of exercise-induced oxidative stress, muscle damage, or inflammation (Abbey & Rankin, 2011; Cureton et al., 2009; McAnulty et al., 2008; O'Fallon et al., 2012; as discussed in section 6.4.1), who used a similar dosage strategy to that of chapter 6, but contradict other research that demonstrated a beneficial effect of quercetin on post-exercise inflammatory biomarkers (Nieman et al., 2009), oxidative stress and antioxidant capacity (Ramezani & Moonikh, 2017), although these studies used longer dosing

strategies (6 weeks & 24 days, respectively). Nieman et al., (2009) also used a mixed quercetin supplement that contained additional antioxidants (30 mg EGCG green tea extract & 100 mg N_3 -PUFA). Interestingly, McAnulty et al. (2013) demonstrated that a 7 day loading strategy with a lower dose of quercetin (than that of chapter 6, 900 mg·d⁻¹) combined with resveratrol (225 mg·d⁻¹ of quercetin for 6 days, 450 mg·d⁻¹ on day 7) to be effective in reducing exercise-induced oxidative stress (F_2 -isoprostanes), but found no effect on inflammation (CRP) or antioxidant capacity (FRAP; ORAC; TAC) following a 1 hr treadmill run at 80% $\dot{V}O_{2max}$; providing further support for the use of mixed antioxidant supplementation and the free radical scavenging properties of quercetin, but not the anti-inflammatory properties of quercetin. The quercetin strategy used in chapter 6 (900 mg·d⁻¹ quercetin, 7 d loading) was evidence-based (Davis et al., 2010), and visual trends in group mean data (Figures 6.5, 6.6, 6.8) suggest that quercetin supplementation was potentially more effective in reducing cellular damage, inflammation, and perceived fatigue, in comparison to a placebo (non-significant findings; similar to visual trends in CK observed by O'Fallon et al., 2012). The 3-day exercise protocol did not induce an oxidative insult (demonstrated by no alterations in serum MDA or TAS) or activation the NF- κ B classical pathway; therefore, null findings in chapter 6 could be explained by the limited opportunity for quercetin to exert antioxidant or anti-inflammatory effects via inhibition of the NF- κ B classical pathway, supporting Nieman et al., (2007a). Quercetin metabolites in plasma were not assessed but have previously been demonstrated to accumulate in plasma following oral administration with 1000 mg·d⁻¹ (4 weeks, Jin et al., 2010; 14 d, Nieman et al., 2009). The 7 d loading strategy used in chapter 6 may not have been long enough for quercetin metabolites to accumulate in plasma and exert an effect. The findings from chapter 6

support previous authors who concluded that the efficacy of quercetin is likely to be “training-state dependent” (Pelletier et al., 2013, p. 79), with minimal effects in trained athletes (Braakhuis & Hopkins, 2015; Kressler et al., 2011; Pelletier et al., 2013).

7.2 Contributions to Literature

This thesis has contributed novel and valuable findings to the literature regarding the physiological demands of undertaking intensified periods of soccer/simulated soccer, and adds to the current debate regarding the use of supplementary antioxidants in athletes, addressing several gaps in the literature.

There is paucity in the literature of longitudinal studies investigating cohorts of professional soccer players, particularly adult players in an ecological setting, with the majority of studies cross-sectional in design. This is due to limited access to professional athletes because of the implications of research impacting schedules and influencing performance. Often soccer-based studies attempt to upscale results determined in youth players to adult players, however, this may be inapplicable because of maturation status. Therefore, chapter 4 contributed to the literature by providing prospective data from first-team professional soccer players from an English League One soccer club. Likewise, chapters 5 and 6 of the thesis also used trained athletes. Exercise intervention studies often use untrained cohorts where the impact of interventions may be more pronounced than in trained athletes because of adaptation and results may not upscale.

Two main overarching themes of this thesis were (i) biomarker monitoring as dependent variables in each study, and (ii) responses following repeated days of exercise. The innovative use of quantifying a biomarker of oxidative stress in urine in chapter 4 provided valuable novel findings regarding lipid peroxidation in professional soccer players at the start of a competitive in-season, which may have potential implications for player readiness. Chapter 4 also added to the literature by demonstrating the importance of creatinine correction for spot urine samples.

Chapter 5 profiled an array of biomarkers, indicative of (i) oxidative stress, (ii) muscle damage, and (iii) inflammation following a period of intensified period of soccer-specific exercise contributing to the literature. The novel findings provide data regarding the magnitude and time course of biomarker responses following an intensified period of simulated soccer, which appears to slightly differ from the response following a single bout of soccer. Furthermore, the NF- κ B classical pathway was studied to (a) increase understanding of the underlying mechanisms associated with exercise-induced oxidative stress, muscle damage, and inflammation in intermittent exercise, and (b) to determine if quercetin mediates its anti-inflammatory effects via the NF- κ B pathway in vivo. It was demonstrated that the 3-day exercise protocol increased IL-6 expression but that this upregulation was independent of the NF- κ B classical pathway or other cytokines, thus, contributing to the literature.

A novelty of each experimental chapter in the thesis was the selected time points of sampling. Studies predominantly focus on time points (i) immediately post-exercise, (ii) + 24 hr, (iii) + 48 hr, and (iv) + 72 hr throughout recovery. The main time point of

interest in this thesis was 42 hr post-exercise, which is the standard time point when professional soccer players would be expected to participate in training following a competitive match, thus, this thesis provides ecological data regarding player readiness.

Chapters 4, 5, and 6 all addressed a gap in the literature regarding biochemical responses to performing repeated days exercise. This data is important as, in an ecological setting, athletes would be required to perform on consecutive days, as observed in chapter 4, forming the basis of the study design in chapters 5 and 6.

The use and timing of antioxidant supplementation in athletes is currently being debated (Gomez-Cabrera et al., 2012). Interestingly, chapter 4 demonstrated that lipid peroxidation was reduced across a competitive season in professional soccer players, and chapters 5 and 6 demonstrated no oxidative stress response to a 3-day period of soccer-specific intensified exercise. This thesis is one of only a few studies to have investigated the efficacy of quercetin supplementation in intermittent exercise characteristic of soccer, alongside Abbey & Rankin (2011), Ramezani & Moonikh, (2017), and Ranchordas et al. (2017b). Study 6 of the thesis showed no beneficial effects of quercetin, in comparison to a placebo; which is in contrast to Ramezani & Moonikh (2017) but supports the findings of Abbey and Rankin (2011). The findings contribute to the literature regarding the use of quercetin supplementation in athletes. A novelty of the study was the non-reductionist approach, which contributes to the literature, as there is a requirement for more studies using this approach (Bowtell & Kelley, 2019).

In chapters 4 and 6 of the thesis, biomarker data was contextualised to indices of training load and recovery variables. Chapter 4 was the first study to investigate lipid peroxidation relative to objective measures of high-intensity training load, addressing a gap in the literature as stated by Le Moal et al. (2016). Moreover, findings from chapter 6 demonstrated that despite biomarker data returning to baseline throughout the considered recovery period, subjective measures of perceived fatigue and general muscle soreness remained elevated above pre-exercise values. These findings have potential implications for player readiness and subsequent quality of training and performance following a 3-day intensified period of soccer.

7.3 Strengths & Limitations

Strengths but also potential limitations exist in each of the studies in this thesis, as previously addressed in each corresponding chapter. This section addresses general strengths and limitations impacting the findings of the thesis as a whole.

The use of trained athletes in the studies was a strength of the thesis, given that trained athletes may respond differently to an successive exercise bouts in comparison to untrained cohorts. Whilst no definitive definition of *trained athletes* exists within the literature, a cohort of professional soccer players (first-team players representing the same League One soccer club) formed the experimental group in chapter 4, and in chapters 5 and 6, trained athletes accustomed to prolonged duration high-intensity intermittent exercise (predominantly soccer/team sport players; defined by aerobic fitness & training participation) were recruited because of difficulties accessing professional soccer players.

Female athletes were underrepresented in the cohorts of the three studies, with only chapter 5 including a mixed-sex sample ($N = 20$; 16 males, 4 females). However, female athletes were not excluded intentionally, but rather because of a lack of recruitment. The majority of studies investigating biochemical responses in soccer type exercise have extensively used male only cohorts; attributed to avoiding confounding effects from the potential antioxidant properties of oestrogen (e.g., increased cell membrane protection; Tiidus, 2000). To control for these effects, female athletes in chapter 5 were tested during the luteal phase of the menstrual cycle (Keane et al., 2015). Results revealed no differences in female exercise-induced biochemical responses compared to males (similar to Souglis et al., 2015); as a consequence, the female athletes were included in the cohorts to increase sample size. Given the increase in participation in women's soccer, and lack of research in female athletes in this area (Andersson et al., 2008), it is proposed that future studies include female athletes. However, the timing of menses and use of hormone medication should be considered.

The high degree of analytical control was a strength of each study, with the lead investigator using standardised collection, storage, and analysis procedures, including correction for urinary creatinine concentration and post-exercise plasma volume change. However, findings of the thesis are limited to the selected biomarkers. Oxidative stress was quantified indirectly via TBARS (chapters 4, 5 & 6) and TAS assays (chapters 5 & 6). Advantages of these assays include the relatively simple spectrophotometric techniques and cost effectiveness, making the assays popular within the literature (Amorati & Valgimigli, 2015; Bartosz, 2010; Fraga, Oteiza, Galleano, 2014). However, both assays have been criticised (Amorati &

Valgimigli, 2015; Bartosz, 2010; Cobley, Close, Bailey, & Davison, 2017), with Cobley et al. (2017) recently recommending the discontinued use of both assays. With regards to the TBARS assay, MDA can be overestimated because of a lack of specificity (Amorati & Valgimigli, 2015). Thiobarbituric acid can react with other aldehydes, or substrates present in the sample that are extraneous to lipid peroxidation (Amorati & Vagimuli, 2015; Cobley et al., 2017). Furthermore, artificial generation of MDA can occur due to the high temperature (~100°C) acidic conditions of the protocol causing further lipid decomposition (Cobley et al., 2017). To compensate for these limitations, TBARS assays with enhanced specificity and a lower temperature reaction (65°C) were used throughout the experimental chapters of this thesis. Including multiple biomarkers indicative of lipid, DNA, and protein oxidation would have helped confirm the presence of exercise-induced oxidative stress in each experimental chapter of this thesis and is recommended for future studies (Cobley et al., 2017; Powers & Jackson, 2008).

The TAS assay involves artificial generation of ABTS^{®*} radical cations at supra-physiological levels, and the ability of antioxidants present within the sample to quench ABTS^{®*} by electron transfer (Miller et al., 1993). A criticism of the TAS assay is that the non-physiological mechanism and supra-physiological levels of free radical generation may not reflect in vivo scavenging mechanisms (Amorati & Valgimigli, 2015; Cobley et al., 2017). In chapters 5 and 6, TAS was assayed in serum; yet, serum TAS excludes intracellular antioxidants and the presence of redox enzymes is limited (Bartosz, 2010; Cobley et al., 2017). Therefore, the nomenclature *Total Antioxidant Status* is misleading as TAS is not a true measure of in vivo antioxidant capacity (Bartosz, 2010). As a consequence, oxidative stress may have

been underestimated in chapters 5 and 6. Nevertheless, TAS was used to aid interpretation of oxidative stress alongside lipid peroxidation data.

In chapters 5 and 6 of the thesis exercise-induced muscle damage was assessed indirectly via serum CK and LDH analyses. A limitation of these assays is specificity, as the assays quantify total CK and LDH, and are not specific for CK or LDH isoenzymes present within skeletal muscle (i.e., CK-MM; CK-MB; LDH-4; LDH-5). However, the CK isoform in serum is predominantly CK-MM (Brancaccio et al., 2007), and the cohorts were homogeneous groups of healthy athletes, therefore, observed post-exercise elevations in CK (significant) and LDH (non-significant) could be attributed to muscle damage induced by the 3-day exercise protocol. It should also be considered that the systemic presence of CK and LDH post-exercise indicates an efflux of these intracellular enzymes into the bloodstream due to compromised sarcolemma integrity, but also reflects the rate of clearance of these enzymes from circulation (Warren et al., 1999; Chatzinikolaou et al., 2014). Thus, concentrations of CK and LDH are highly individual (Brancaccio et al., 2010), and may not accurately reflect the magnitude of muscle damage and level of muscle dysfunction (Warren et al., 1999).

Finally, and of importance, each study relied on the athletes to self-report (e.g., provision of urine samples; dietary intake; training load; supplement ingestion); these responses could have been confounded or open to response bias.

7.4 Practical Implications

There are several valuable findings from the three studies in this thesis that may have potential implications for professional soccer players who regularly perform back-to-back sessions with minimal recovery periods, for example, throughout microcycles of a competitive season.

- 1) Exercise-induced lipid peroxidation was observed to be highest following the first week of the competitive season (which was the onset of competitive matches and was preceded by pre-season training, where the volume of training would have been increased as a stimulus for training adaptations). This data is of interest because increased oxidants may stimulate training adaptations, however, potential resulting muscle dysfunction could lead to underperformance; given that the volume and intensity of the pre-season training load has previously been associated with a reduction in antioxidant efficiency (Finaud et al., 2006b) and increased risk of injury (systematic review by Jones, Griffiths, & Mellalieu, 2017). Routine monitoring of urine samples is common practice in professional soccer. The addition of monitoring urine samples for MDA could further aid sports scientists/coaches in the prescription of altered training loads/recovery periods to minimise the impact of the volume of the pre-season training load on oxidative stress, offsetting negative effects that could impair performance and increase injury risk at the onset of competitive matches. Furthermore, this data shows the potential health benefits of undertaking regular soccer training, as urinary MDA concentrations were reduced throughout the competitive season.
- 2) Post-exercise increases in acute cellular damage and inflammation were observed to peak immediately following an intensified period of soccer-

specific exercise, returning to baseline within a 42 hr passive recovery period (Figure 7.1). Sports scientists/coaches should consider the implication of the time course of these exercise-induced responses if athletes are to meet the demands of the next successive training session or match with minimal impact from previous sessions. Facilitating rapid recovery between sessions is essential if the next successive training/competition session is within a 42 hr period. Exploring strategies that reduce the magnitude of the insult may be warranted. Implementation of a recovery strategy during the conceptual region (Owens et al., 2019) should also be considered.

- 3) Chapter 6 provided insufficient evidence to support the use of prolonged quercetin supplementation (dosing strategy: 900 mg·d⁻¹, 7 d loading) to promote recovery following an intensified period of soccer-specific exercise that induced acute cellular damage, inflammation, muscle soreness, and fatigue in trained athletes with adequate habitual dietary intakes. From an applied perspective, sports scientists/nutritionists should consider implementation of nutritional interventions that are evidence-based only. Professional soccer players undertaking intensified periods of exercise (such as during in-season microcycles or tournaments) may not benefit from quercetin supplementation. However, it is speculated that athletes with micronutrient deficiencies or compromised redox homeostasis may respond differently to a quercetin supplementation strategy. Given the current debate regarding antioxidant supplementation blunting beneficial training adaptations, it is further recommended that sports scientists/nutritionists consider periodised nutritional strategies (Owens et al., 2019; Ranchordas et al., 2017a).

- 4) In chapter 6, post-exercise responses of biomarkers of cellular damage and inflammation paralleled the response of subjective measures of perceived fatigue and general lower body muscle soreness (assessed via the Perceptual Fatigue Questionnaire [McLean et al., 2010] & pain algometry [although the strength of association was not assessed]). Residual fatigue and muscle soreness could impact the quality of subsequent training sessions or competitive match play. Assessing subjective measures of recovery in conjunction with biomarker monitoring could increase understanding of the athlete's perception of fatigue. Where biomarker monitoring is not a viable option, this thesis appears to provide evidence that these subjective methods could be used as cheaper, less-invasive methods of recovery assessment; further supporting those authors already acknowledging the use of perceptual questionnaires as an alternative (Johnston et al., 2013).
- 5) Data in this thesis revealed exercise-induced responses to be highly individual. Regular monitoring is recommended in order to develop normalised data for individual athletes, allowing prescription of training loads and recovery periods that optimise individual performance (Silva et al., 2018). The practicality of implementing individualised strategies in the group setting of soccer is not without difficulties; however, is now common practice (as evidenced by the lead investigator). Sports scientists should also consider the time and cost-benefit of administering group strategies that may result in marginal gains only in individual athletes.
- 6) In chapter 4, urinary MDA concentrations in a cohort of recreational soccer players were shown to be seven-fold that of professional soccer players at the end of a competitive in-season. Recreational athletes often have limited

access to recovery strategies, nonetheless these athletes may benefit from recovery strategies that alleviate the negative effects associated with exercise-induced lipid peroxidation.

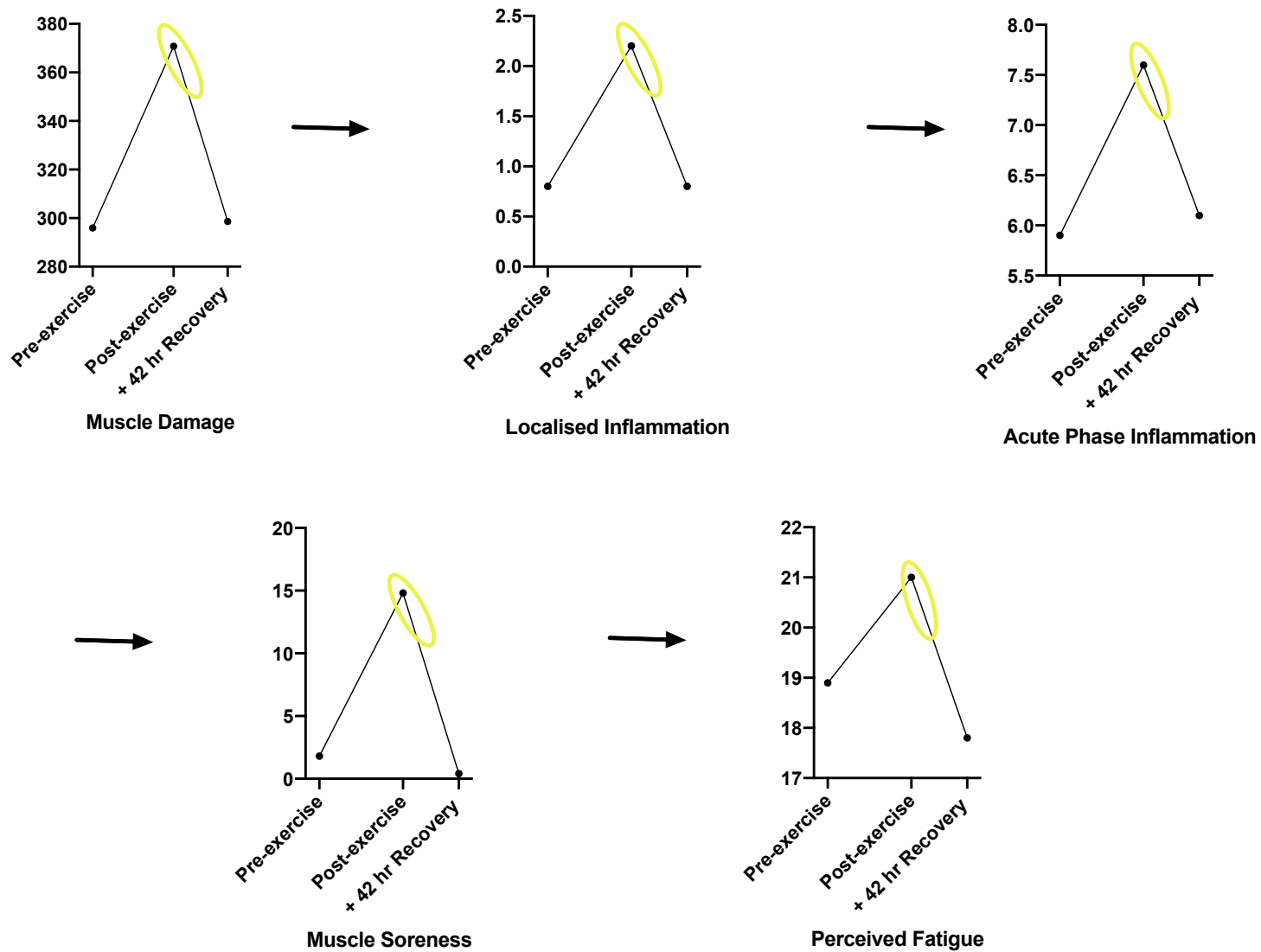


Figure 7.1. Responses to the 3-day period of intensified simulated soccer. Variables followed the same pattern. Interventions should consider targeting the time point immediately post-exercise (yellow oval). Adapted from Owens et al. (2019) p. 80.

7.5 Conclusion & Further Research

In conclusion, this thesis investigated physiological responses to intensified periods of soccer-specific prolonged duration high-intensity intermittent exercise. The thesis demonstrated that (i) the magnitude of lipid peroxidation is reduced significantly throughout a competitive in-season in professional soccer players, regardless of the volume of high-intensity training, and that (ii) professional soccer players exhibited significantly lower lipid peroxidation, at the end of the season, in comparison to recreational players (chapter 4). Collectively, these results provide evidence that regular soccer training increased exercise tolerance specific to oxidants. The thesis also showed that a 3-day intensified period of soccer-specific exercise induced a degree of cellular damage and an acute-phase inflammatory response in trained athletes, that was concomitant with fatigue and muscle soreness, but inflammatory responses were independent of the NF- κ B classical pathway (chapters 5 & 6). Oxidative stress remained unaltered following the 3-day intensified period of soccer-specific exercise, suggesting that the exercise protocol induced limited mechanical, rather than metabolic damage (chapters 5 & 6). A 42 hr passive recovery period appeared adequate to resolve these responses back to near pre-exercise values (chapters 5 & 6). For athletes that are required to train or compete within 42 hr, implementation of an evidence-based recovery strategy modulating these responses to reduce fatigue and muscle soreness may be warranted. The use of quercetin supplementation as a recovery strategy following an intensified period of exercise simulating soccer was not supported when compared to a placebo condition (chapter 6).

Expanding on the studies in this thesis, future research should translate the results of chapters 5 and 6 back into a field-based setting of professional soccer, to corroborate the magnitude and time course of the observed exercise-induced responses, given that the overall training load is likely to be increased by further mechanical and metabolic stress induced in the field setting. Assessment of daily monitoring throughout training microcycles may offer more insight into this area, demonstrating cumulative responses and/or a RBE; however, would not be without time and cost implications. In chapter 6, post-exercise responses of several dependent variables (i.e., (i) LDH; (ii) IL-6; (iii) total leukocyte & neutrophil counts (iv) NF- κ B p65; (v) TAS) were visually observed to be lower in the quercetin group compared to the placebo group, therefore, further research into the efficacy of quercetin supplementation to promote recovery in exercise characteristic of soccer using increased sample sizes, is potentially warranted. Supplementing quercetin immediately post-exercise where inflammation was observed to peak may also be of interest. Future studies should also investigate other underlying mechanisms of exercise-induced muscle damage and inflammation in soccer as targets to potentially modulate, as links to the NF- κ B classical pathway were unsupported in this thesis.

Suggestions for additional future research have also arisen. Firstly, there is a requirement for studies to focus on comparisons of exercise-induced responses in biomarkers assessed in skeletal muscle tissue concomitant with other sample types (i.e., blood components & urine), to determine whether exercise-induced responses in biomarkers in these sample types accurately reflect skeletal muscle tissue, as this has been questioned (Powers et al., 2010b), and often muscle biopsies are

impractical in exercise research, given that the procedure itself can cause localised inflammation and tissue damage (Malm, 2001). Secondly, in this thesis, biomarker responses were compared (i) across training periods, and (ii) pre-post-exercise, however, there is a requirement to identify thresholds of elevated biomarker concentrations that define the concepts that are (i) exercise-induced oxidative stress, (ii) exercise-induced muscle damage, and (iii) exercise-induced inflammation (Lee et al., 2017) to contextualise biomarker data and relate it to concurrent measures of performance and recovery (as per Buford et al., 2009). This data may need to be established for different sports, and on a group and individual basis because of the high inter-individual variance demonstrated in athletes (explained further by Heisterberg et al., 2013 & Lee et al., 2017).

Results from studies in this thesis can be transferred to professional and recreational soccer, and is specifically relevant to those responsible for setting training loads and recovery strategies. This thesis used ecologically valid and controlled approaches to profile exercise-induced oxidative stress, muscle damage, and associated inflammatory responses to intense periods of soccer-specific exercise. Results showed reduced oxidative stress across a competitive soccer season, which was observed to be greatest following the first week of the competitive in-season. Results also showed increased cellular damage, concomitant with inflammation, muscle soreness, and fatigue following a 3-day intensified period of exercise simulating soccer. Optimisation of recovery to facilitate athlete readiness for performance may be achieved through nutritional interventions, however, this thesis showed limited effects of prolonged quercetin supplementation in trained athletes during exercise

simulating soccer. Hence, practitioners should carefully consider the use of quercetin supplementation in this cohort at present.

References

- Abbey, E.L., & Rankin, J.W. (2011). Effect of quercetin supplementation on repeated-sprint performance, xanthine oxidase activity, and inflammation. *International Journal of Sport Nutrition and Exercise Metabolism*, 21(2), 91-96.
10.1123/ijsnem/21.2.91
- Akenhead, R. Hayes, P.R., Thompson, K.G., & French, D. (2013). Diminutions of acceleration and deceleration output during professional football match play. *Journal of Science and Medicine in Sport*, 16(6), 556-61.
10.1016/j.jsams.2012.12.005
- Akenhead, R. & Nassis, G. (2016). Training load and player monitoring in high-level football: Current practice and perceptions. *International Journal of Sports Physiology and Performance*, 11, 587-593.
10.1123/ijsp.2015-0331
- Alexander, S.P.H. (2006). Flavonoids as antagonists at A₁ adenosine receptors. *Phytotherapy Research*, 20, 1009-1012.
10.1002/ptr.197
- Alis, R., Sanchis-Gomar, F., Primo-Carrau, C., Lozano-Calve, S., Dipalo, M., Aloe, R., & Lippi, G. (2015). Hemoconcentration induced by exercise: Revising the Dill and Costill equation. *Scandinavian Journal of Medicine and Science in Sports*, 25: e630-e637.
10.1111/sms.12393
- Allen, R.G. & Tressini, M. (2000). Oxidative stress and gene regulation. *Free Radical Biology & Medicine*, 28(3), 463-499.
10.1016/S0891-5849(99)00242-7
- Amorati, R. & Valgimigli, L. (2015) Advantages and limitations of common testing

methods for antioxidants. *Free Radical Research*, 49(5), 633-49.

10.3109/10715762.2014.996146

Anderson, L., Orme, P., Di Michele, R., Close, G.L., Morgans, R., Drust, B., & Morton, J.P. (2016). Quantification of training load during one-, two- and three-game week schedules in professional soccer players from the English premier league: implications for carbohydrate periodisation. *Journal of Sports Sciences*, 1-10.

10.1080/02640414.2015.1106574

Andersson, H., Bohn, S.K., Raastad, T., Paulsen, G., Blomhoff, R., & Kadi, F. (2010). Differences in the inflammatory plasma cytokine response following two elite female soccer games separated by a 72-h recovery. *Scandinavian Journal of Medicine and Science in Sports*, 20, 740-747.

10.1111/j.1600-0838.2009.00989.x

Andersson, H., Raastad, T., Nilsson, J., Paulsen, G., Garthe, I., & Kadi, F. (2008). Neuromuscular fatigue and recovery in elite female soccer: effects of active recovery. *Medicine & Science in Sports and Exercise*, 40, 372-380.

10.1249/mss.0b013e315b8497

Andreacci, J.L., LeMura, L.M., Cohen, S.L., Urbansky, E.A., Chelland, S.A., & Von Duvillard, S.P. (2002). The effects of frequency of encouragement on performance during maximal exercise testing. *Journal of Sports Science*, 20(4), 345-52.

10.1080/026404102753576125

Andres, S., Pevny, S., Zeigenhagen, R., Bakhiya, N., Schafer, B., Hirsch-Ernst, K., & Lampen, A. (2018). Safety aspects of the use of quercetin as a dietary

supplement. *Molecular Nutrition and Food Research*, 62(1), Special Issue Reviews.

10.1002/mnfr.201700447

Antonioni, A., Fantini, C., Dimauro, I., & Caporossi, D. (2019). Redox homeostasis in sport: Do athletes really need antioxidant support? *Research in Sports Medicine*, 27(2), 147-165.

10.1080/15438627.20.1563899

Aoi, W., Naito, Y., Takanami, Y., Kawai, Y., Sakuma, K., Ichikawa, H, Yoshikawa, T. (2004). Oxidative stress and delayed-onset muscle damage after exercise. *Free Radical Biology & Medicine*, 37(4), 480-487.

10.1016/j.freeradbiomed.2004.05.008

Ascensão, A., Rebelo, A., Oliveira, E., Marques, F., Pereira, L., & Magalhães, J. (2008). Biochemical impact of a soccer match - analysis of oxidative stress and muscle damage markers throughout recovery. *Clinical Biochemistry*, 41, 841-851.

10.1016/j.clinbiochem.2008.04.008

Askari, G., Ghiasvand, R., Feizi, A., Ghanadian, S.M., & Karimian, J. (2012). The effects of quercetin supplementation on selected markers of inflammation and oxidative stress. *Journal of Research in Medical Sciences*, 17(7), 637-641.

doi n/a

Aughey, R.J. (2011). Applications of GPS technologies to field sports. *International Journal of Sports Physiology and Performance*, 6(3), 295-310.

10.1123/ijsp.6.3.295

Bangsbo, J. (1994). Energy demands in competitive soccer. *Journal of Sports Sciences*, 12, Spec No: S5-12.

10.1080/02640414.1994.12059272

Bangsbo, J., Mohr, M., & Krustup, P. (2006). Physical and metabolic demands of training and match play in the elite football player. *International Journal of Sports Science*, 24, 665-674.

10.1080/02640410500482529

Barbero-Alvarez, J.C., Coutts, A., Granda, J., Barbero-Alvarez, V., & Castagna, C. (2010). The validity and reliability of a global positioning satellite system device to assess speed and repeated sprint ability (RSA) in athletes. *Journal of Science and Medicine in Sport*, 13(2), 232-235.

10.1016/j.jsams.2009.02.005

Bartosz, G. (2010). Non-enzymatic antioxidant capacity assays: limitations of use in biomedicine. *Free Radical Research*, 44(7), 711-20.

<https://doi.org/10.3109/10715761003758114>

Bazzucchi, I., Patrizio, F., Ceci, R., Sgro, P., Sabatini, S., Di Luigi, L., Sacchetti, M., & Felici, F. (2019). The effects of quercetin supplementation on eccentric exercise-induced muscle damage. *Nutrients*, 11(1), pii: E205.

10.3390/nu110102-5

Becatti, M., Mannucci, A. & Barygina, V., Mascherini, G., Emmi, G., Silvestri, E., ... Fiorillo, C. (2017). Redox status alterations during the competitive season in elite soccer players: Focus on peripheral leukocyte-derived ROS. *Internal and Emergency Medicine*, 12(6), 777-788.

10.1007/s11739-017-1653-5

Beedie, C., Coleman, D.A., Flowers, E., & Hettinga, F.J. (2018). Consensus statement on placebo effects in sports and exercise: The need for conceptual clarity, methodological rigour, and the elucidation of neurobiological

mechanisms. *European Journal of Sport Science*, 18(10), 1383-1389.

10.1080/17461391.20.1496144

Bell, P.G., Stevenson, E., Davison, G.W., & Howatson, G. (2016). The effects of Montmorency tart cherry concentrate supplementation on recovery following prolonged, intermittent exercise. *Nutrients*, 8(7), 440:1-11.

10.3390/nu8070441

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

<https://doi.org/10.3390/n>

Bengtsson, H., Ekstrand, J., & Hägglund, M. (2013). Muscle injury rates in professional football increase with fixture congestion: An 11-year follow-up of the UEFA Champions League injury study. *British Journal of Sports Medicine*, 47(12), 743-747.

<https://doi.org/10.1136/bjsports-2013-092383>

Bigelman, K.A., Fan, E.H., Chapman, D.P., Freese, E.C., Trik, J.L., & Cureton, K.J. (2010). Effects of six weeks of quercetin supplementation on physical performance in ROTC cadets. *Military Medicine*, 175(10), 791-798.

10.7205/MILMED-D-09-00088

Bishop, P.A., Jones, E., & Woods, A.K. (2008). Recovery from training: a brief review. *Journal of Strength and Conditioning Research*, 22(3), 1015-1024.

10.1519/jSC.0b013e31816eb518

Bland, J.M., & Altman, D.G. (1986). Statistical method for assessing agreement between two methods of clinical measurement. *Lancet*, 327, 307-10.

10.1016/S0140-6736(86)90837-8

- Boots, A.W., Looneke, C.W., Swennen, E.L.R., Kleinjans, J.C.S., Bast, A., & Haenen, G.R.M.M. (2008). In vitro and ex vivo anti-inflammatory activity of quercetin in healthy volunteers. *Nutrition*, 24(7-8), 703-710.
10.1016/j.nut.2008.03.023
- Borg, G. (1970). Perceived exertion as an indicator of somatic stress. *Scandinavian Journal of Rehabilitation Medicine*, 2, 92-98.
doi n/a
- Borg, G. (1998). *Borg's Perceived Exertion and Pain Scales*. Champaign, Illinois: Human Kinetics.
- Borresen, J., & Lambert, I. M. (2009). The quantification of training load, the training response and the effect on performance. *Sports Medicine*, 39(9), 779-795.
<https://doi.org/10.2165/11317780-000000000-00000>
- Bowtell, J. & Kelly, V. (2019). Fruit-derived polyphenol supplementation for athlete recovery and performance. *Sports Medicine*, 49(1), S3-S23.
10.1007/s40279-0-0998-x
- Braakhuis, A. (2010). The role of dietary antioxidants in exercise-induced oxidative stress and athletic performance. A thesis submitted to the Auckland University of Technology in fulfillment of the degree of Doctor of Philosophy, October 2010.
- Braakhuis, A.J., & Hopkins, W.G. (2015). Impact of Dietary Antioxidants on Sport Performance: A Review. *Sports Medicine*, 45(7), 939-955.
10.1007/s40279-015-0323-x
- Braakhuis, A.J., Hopkins, W.G., Lowe, T.E., & Rush, E.C. (2011) Development and validation of a food-frequency questionnaire to assess short-term antioxidant

intake in athletes. *International Journal of Sports Nutrition and Exercise Metabolism*, 21(2), 105-12.

10.1123/ijsnem.21.2.11

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 7(72), 284-254.

10.1016/0003-2697(76)90527-3

Bradley, P.S., Di Mascio, M., Peart, D., & Sheldon, B. (2010). High-Intensity activity profiles of elite soccer players at different performance levels. *Journal of Strength and Conditioning Research*, 24, 2343-2351.

10.1519/JSC.0b013e31aeb1b3

Bradley, P.S., Sheldon, W., Wooster, B., Olsen, P., Boanas, P., & Krstrup, P. (2009). High-intensity running in English FA premier league soccer matches, *Journal of Sports Sciences*, 27(2), 159-168.

10.1080/02640410802512775

Brancaccio, P., Limongelli, F.M. & Maffulli, N. (2007). Creatine kinase monitoring in sport medicine. *British Medical Bulletin*, 81 - 82, 209-230.

10.1093/bmb/ldm014

Brancaccio, P., Lippi, G. & Maffulli, N. (2010). Biochemical markers of muscular damage. *Clinical Chemistry and Laboratory Medicine*, 48(6), 757-767.

10.1515/CCLM.2010.179

Brancaccio, P., Maffulli, N., Politano, L., Lippi, G., & Limongelli, F.M. (2011). Persistent hyperCKaemia in athletes. *Muscles, Ligaments and Tendons Journal*, 1(1), 31-5.

doi n/a

- Brites, F.D., Evelson, P.A., Christiansen, M.G., Nicol, M.F., Basilico, M.J., Wikinski, R.W. & Llesuy, S.F. (1999). Soccer players under regular training show oxidative stress but an improved plasma antioxidant status. *Clinical Science*, 96(4), 381-385.
10.1042/CS19980269
- Bryer, S.C., & Goldfarb, A.H. (2006). Effect of high dose vitamin C supplementation on muscle soreness, damage, function and oxidative stress to eccentric exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, 16(3), 270-280.
doi n/a
- Buford, T.W., Cooke, H.B., Shelmadine, B.P., Hudson, G.M., Redd, L. & Willoughby, D.S. (2009). Effects of eccentric treadmill exercise on inflammatory expression in human skeletal muscle. *Applied Physiology, Nutrition and Medicine*, 34(4), 745-753.
10.1139/H09-067
- Burke, L., & Deakin, V. (2015). *Clinical Sports Nutrition (4th Ed.)*. New South Wales: McGraw-Hill.
10.1519/JSC.0b013e32548af1
- Casamichana, D., Morencos, E., Romero-Moraleda, B., & Gabbett, T.J. (2018). The use of generic and individual speed thresholds for assessing the competitive demands of field hockey. *Journal of Sports Science and Medicine*, 17, 366-371.
doi n/a
- Cazzola, R., Russo-Volpe, S., Cervato, G., & Cestaro, B. (2003). Biochemical assessments of oxidative stress, erythrocyte membrane fluidity and antioxidant

status in professional soccer players and sedentary controls. *European Journal of Clinical Investigations*, 33, 924-930.

10.1046/j.1365-2362.2003.01227.x

Chatzinikolaou, A., Draganidis, D., Avlonti, A., Karipidis, A., Jamurtas, A., Z., Chrysanthi, L., ... Fatouros, I. (2014) The microcycle of inflammation and performance changes after a basketball match. *Journal of Sports Sciences*, 32(9), 870-882.

10.1080/02640414.2013.865251

Chen, J.C., Ho, F.M., Chao, P.D.L., Chen, C.P., Jeng, K.C.G., & Hsu, H.B. (2005). Inhibition of iNOS gene expression by quercetin is mediated by the inhibition of I κ B kinase, nuclear factor kappaB and STAT1, and depends on heme oxygenase-1 induction in mouse BV-2 microglia. *European Journal of Pharmacology*, 521, 9-20.

10.1016/j.ejphar.2005.08.005

Cheuvront, S.N., Ely, B.R., Kenefick, R.W., Michniak-Kohn, B.B., Rood, J.C., & Sawka, M.N. (2009). No effect of nutritional adenosine receptor antagonists on exercise performance in the heat. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 296, R394-401.

10.1152/ajpregu.90812.2008

Clarke, N. D., Campbell, I. T., Drust, B., Evans, L., Reilly, T., & Maclaren, D. P. M. (2012). The ingestion of combined carbohydrates does not alter metabolic responses or performance capacity during soccer-specific exercise in the heat compared to ingestion of a single carbohydrate. *Journal of Sports Sciences*, 30(7), 699–708.

10.1080/02640414.2012.665941

- Clarke, N.D., Drust, B., MacLaren, D.P.M., & Reilly, T. (2008). Fluid provision and metabolic responses to soccer-specific exercise. *European Journal of Applied Physiology*, *104*, 1069-1077.
doi n/a
- Claus, D.R., Osmand, A.P., & Gewurz, H. (1976). Radioimmunoassay of human C-reactive protein and levels in normal sera. *Journal of Laboratory Clinical Methods*, *87*(1), 120-128.
doi n/a
- Cobley, J.N., Close, G.L., Bailey, D.M., & Davison, G.W. (2017). Exercise redox biochemistry: Conceptual, methodological and technical recommendations. *Redox Biology*, *12*, 540-548.
10.1016/j.redox.2017.03.022
- Cohen, J. (1988). *Statistical power analysis for the behavioural sciences (2nd Ed.)*. New York: Lawrence Erlbaum Associates, Inc.
- Cohen, S., Kamarck, T., & Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behaviour*, *24*(4), 385-396.
10.2307/2136404
- Comalada, M., Camuesco, D., Sierra, S., Ballester, I., Xaus, J., Gálvez, J., & Zarzuelo, A. (2005). In vivo quercetin anti-inflammatory effect involves release of quercetin, which inhibits inflammation through down-regulation of the NF- κ B pathway. *European Journal of Immunology*, *35*(2), 584-592.
<https://doi.org/10.1002/eji.200425778>
- Conquer, J. A., Maiani, G., Azzini, E., Raguzzini, A., & Holub, B. J. (1998). Supplementation with quercetin markedly increases plasma quercetin

- concentration without effect on selected risk factors for heart disease in healthy subjects. *The Journal of Nutrition*, 128(3), 593-597.
<https://doi.org/10.1093/jn/128.3.593>
- Coolican, H. (2019). *Research Methods and Statistics in Psychology (7th Ed.)*
New York: Routledge.
- Coutts, A.J., & Duffield, R. (2010). Validity and reliability of GPS units for measuring movement demands of team sports. *Journal of Science & Medicine in Sport*, 13(1), 133-135.
10.1016/j.jsams.2008.09.015
- Cronin, J.B., & Templeton, R.L. (2008). Timing light height affects sprint times. *Journal of Strength and Conditioning Research*, 22(1), 318-320.
10.1519/JSC.0b013e315fa3d3
- Cuevas, M.J., Almar, M., García-Glez, J.C., García-López, D., de Paz, J.A., Alvear-Órdenes, I., & González-Gaslego, J. (2005). Changes in oxidative stress markers and NF κ B activation induced by sprint exercise. *Free Radical Research*, 39(4), 431-439.
10.1080/1071576050007214
- Cummins, C., Orr, R., O'Connor, H., & West, C. (2013). Global Positioning Systems (GPS) and microtechnology sensors in team sports: A systematic review. *Sports Medicine*, 43, 1025-1042.
10.1007/s40279-013-0069-2
- Cureton, K.J., Tomporowski, P.D., Singhal, A., Pasley, J.D., Bigelman, K.A., Lambourne, K., Trilk, J.L., McCully, K.K., ... Zhao, Q. (2009). Dietary quercetin supplementation is not ergogenic in untrained men. *Journal of Applied Physiology*, 107, 1095-1104.

10.1152/jappphysiol.00234.2009.

Dacie, J.V., & Lewis, S.M. (1963). *Practical Haematology (3rd Ed.)*, London: Churchill.

da Silva Barbosa, C.V., Silva, A.S., de Oliveira, C.V.C., Massa, N.M.L., de Sousa, Y.R.F., da Costa, W.K.A., ... Magnani, M. (2017). Effects of sesame (sesamum indicum L.) supplementation on creatine kinase, lactate dehydrogenase, oxidative stress markers, and aerobic capacity in semi- professional soccer players. *Frontiers in Physiology*, 8(196), 1-7.

10.3389/fphys.2017.00196

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

[https://doi.org/10.1016/S0006-291X\(82\)80124-1](https://doi.org/10.1016/S0006-291X(82)80124-1)

Davis, J.M., Carlstedt, C.J., Chen, S., Carmichael, M.D., & Murphy, E.A. (2010). The dietary flavonoid quercetin increases $\dot{V}O_2\text{max}$ and endurance capacity. *International Journal of Sports Nutrition and Exercise Metabolism*, 20(1), 56-62.

10.1123/ijsnem.20.1.56

Davis, J.M., Murphy, E.A., & Carmichael, M.D. (2009a). Effects of the dietary flavonoid quercetin upon performance and health. *Current Sports Medicine Reports*, 8(4), 206-213.

10.1249/JSR.0b013e31ae8959

Davis, J.M., Murphy, E.A., Carmichael, M.D. & Davis, B. (2009b). Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *The American Journal of Physiology—Regulatory, Integrative and Comparative Physiology*, 296: R1071-1077.

10.1152/ajpregu.90925.2008

Davison, G.W., Ashton, T., McEneny, J., Young, I.S., Davies, B., & Bailey, D.M. (2012). Critical difference applied to exercise-induced oxidative stress: the dilemma of distinguishing biological from statistical change. *Journal of Physiology and Biochemistry*, 68, 377-384.

10.1007/s13105-012-0149-z

Day, M.L., McGuigan, M.R., Brice, G., & Foster, C. (2004) Monitoring exercise intensity during resistance training using the session RPE scale. *Journal of Strength and Conditioning Research*, 18(2), 353-358.

Dias, A.S., Porawski, M., Alonso, M., Marroni, N., Collado, P.S., & Gonzalez-Gallego J. (2005). Quercetin decreases oxidative stress, NF- κ B activation, and iNOS overexpression in liver of streptozotocin-induced diabetic rats. *Journal of Nutrition*, 135, 2299-2304.

10.1093/jn/135.10.2299

Dill, D.B., & Costill, D.L. (1974). Calculation of percentage changes in volume of blood, plasma and red cells in dehydration. *Journal of Applied Physiology*, 37(2), 247-248.

10.1152/jappl.1974.37.2.24

Dillard, C. J., Litov, R. E., Savin, W. M., Dumelin, E. E., & Tappel, A. L. (1978). Effects of exercise, vitamin E, and ozone on pulmonary functioning and lipid peroxidation. *Journal Applied Physiology*, 45(6), 927-932.

Drury, J.A., Nycyk, J.A., & Cooke, R.W.I. (1997). Comparison of urinary and plasma malondialdehyde in preterm infants. *Clinica Chimica Acta*, 263(2), 177-185.

10.1016/S0009-8981(97)00051-X

- Drust, B., Atkinson, G., & Reilly, T. (2007). Future perspectives in the evaluation of the physiological demands of soccer. *Sports Medicine*, 37(9), 783-805.
10.2165/00007256-200737090-00003
- Drust, B., Reilly, T., & Cable, N.T. (2000). Physiological responses to laboratory-based soccer-specific intermittent and continuous exercise. *Journal Sports Science*, (11), 885-892.
10.1080/026404100750017814
- Dumke, C.L., Nieman, D.C., Utter, A.C., Rigby, M.D., Quindry, J.C., Triplett, N.T., ... McAnulty, L.S. (2009). Quercetin's effect on cycling efficiency and substrate utilization. *Applied Physiology, Nutrition and Metabolism*, 34(6), 993-1000.
10.1139/H09-099
- Dwyer, D.B., & Gabbett, T.J. (2012). Global Positioning system data analysis: Velocity ranges and a new definition of sprinting for field sport athletes. *Journal of Strength and Conditioning Research*, 26, 818-824.
10.1519/JSC.06013e32276555
- Edwardsen, E., Hem, E., & Anderssen, S.A. (2014). End criteria for reaching maximal oxygen uptake must be strict and adjusted to sex and age: A cross-sectional study. *PLoS ONE*, 9(1): e86276.
10.1371/journal.pone.0085276
- Egert, S., Wolfram, S., Bosy-Westphal, A., Boesch-Saadatmandi, C., Wagner, A.E., Frank, J.F., ... Mueller, M.J. (2008). Daily quercetin supplementation dose-dependently increases plasma quercetin concentrations in healthy humans. *The Journal of Nutrition*, 138(9), 1615-1621.
10.1093/jn/138.9.1615
- Erdfelder, E., Faul, F., & Buchner, A. (1996). GPOWER: A general power analysis

program. *Behaviour Research Methods, Instruments, & Computers*, 28(1), 1-11.

doi n/a

Fatouros, I.G., Chatzinikolaou, A., Douroudos, I.I., Nikolaidis, M.G., Kyparos, A.M., Margonis, K., ... Jamurtas, A.Z. (2010). Time-course changes in oxidative stress and antioxidant responses following a soccer game. *Journal of Strength and Conditioning Research*, 24(12), 3278-3286.

10.1519/JSC.0b013e31b60444

Fatouros, I.G., & Jamurtas, A.Z. (2016). Insights into the molecular etiology of exercise-induced inflammation: Opportunities for optimising performance. *Journal of Inflammation Research*, 9, 175-186.

10.2147/JIR.S114635

Ferreira, E.B., Rocha, M.C., & Mequelino, D.B. (2012). Monte Carlo evaluation of the ANOVA's F and Kruskal-Wallis tests under binomial distribution. *Sigmae, Alfenas*, 1(1), 126-139.

doi n/a

Ferrer, M.D., Tauler, P., Sureda, A., Pujol, P., Drobic, F., & Tur, J.A. (2009). A soccer match's ability to enhance lymphocyte capability to produce ROS and induce oxidative damage. *International Journal of Sport, Nutrition and Exercise Metabolism*, 19(3), 243-258.

10.1123/ijsnem.19.3.243

Field, A., Corr, L.D., Haines, M., Lui, S., Naughton, R., Page, M.R., & Harper, L.D. (2020). Biomechanical and physiological responses to 120 min of soccer-specific exercise. *Research Quarterly for Exercise and Sport*, 1-13.

10.1080/02701367.2019.1698698

- Finaud, J., Lac, G., & Filaire, E. (2006a). Oxidative stress relationship with exercise and training. *Sports Medicine* 36(4), 327-358.
10.2165/00007256-200636040-00004
- Finaud, J., Scislowski, V., Lac, G., Durand, D., Vidalin, H., Robert, A., ... Filaire, E. (2006b). Antioxidant status and oxidative stress in professional rugby players: evolution throughout a season. *International Journal of Sports Medicine*, 27, 87-93.
10.1055/s-2005-837489
- Fischer, C.P. (2006). Interleukin-6 in acute exercise and training: what is the biological relevance? *Exercise Immunology Review*, 12, 6-33.
- Food Standards Agency (2002). McCance and Widdowson's The Composition of Foods (Sixth summary edition). Compiled by Food Standards Agency and Institute of Food Research. Royal Society of Chemistry: Cambridge.
- Foster, C., Florhaug, J.A., Franklin, J., Gottschall, L., Hrovatin, L.A., Parker, S., ... Dodge, C. (2001). A new approach to monitoring exercise training. *Journal of Strength and Conditioning Research*, 15(1), 109-115.
10.1519/00124278-200102000-0001
- Fraga, C.G., Actis-Goretta, L., Ottaviana, J.I., Carrasquedo, F.I., Lotito, S.B., Lazarus, S., Schmitz, H.H., & Keen, C.L. (2005). Regular consumption of a flavanol-rich chocolate can improve oxidant stress in young soccer players. *Clinical & Developmental Immunology*, 12(1), 11-17.
10.1080/10446670410001722159
- Fraga, C.G., Oteiza, P.I., & Galleano, M. (2014). In vitro measurements and interpretation of total antioxidant capacity. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1840(2), 931-934.

<https://doi.org.10.1016/j.bbagen.2013.06.030>

Gabbett, T.J. (2016). The training-injury prevention paradox: should athletes be training smarter *and* harder? *British Journal of Sports Medicine*, *50*(5), 273-280.

10.1136/bjsports-2015-095788

Gabbett, T.J., Jenkins, D.G., & Abernethy, B. (2012). Physical demands of professional rugby league training and competition using microtechnology. *Journal of Science and Medicine in Sport*, *15*, 80-86.

10.1016/j.jsams.2011.07.004

Ganio, M.S., Armstrong, L.E., Johnson, E.C., Klau, J.F., Ballard, K.D., Michniak-Kohn, B., ... Maresh, C.M. (2010). Effect of quercetin supplementation on maximal oxygen uptake in men and women. *Journal of Sports Sciences*, *28*(2), 201-208.

10.1080/02640410903428580

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

doi n/a

García-López, D., Cuveas, M., Almar, M., Lima, E., De Paz, González-Gallego J. (2007). Effects of eccentric exercise on NF-kappaB activation in blood mononuclear cells. *Medicine and Science in Sports and Exercise*, *39*(4), 653-664.

10.1249/mss.0b013e31802f04f6

García-Mediavilla, V., Crespo, I., Collado, P.S., Esteller, A., Sánchez-Campos, S., Tuñón, M.J., & González-Gallego, J. (2007). The anti-inflammatory flavones quercetin and kaempferol cause inhibition of inducible nitric oxide synthase,

cyclooxygenase-2 and reactive C-protein and down-regulation of the nuclear factor kappaB pathway in Chang Liver cells. *European Journal of Pharmacology*, 557(2-3), 221-229.

10.1016/j.ejphar.2006.11.014

Gilmore, T.D. (2006). Introduction to NF-κB: Players, pathways, perspectives. *Oncogene*, 25(51), 6680-6684.

<https://doi.org/10.1038/sj.onc.1209954>

Girden, E.R. (1992). *ANOVA: Repeated Measures*. Sage: CA.

10.4135/9781412983419

Giudice, M.D., & Gangestad, S.W. (2018). Rethinking IL-6 and CRP: Why they are more than inflammatory biomarkers, and why it matters. *Brain, Behaviour and Immunity*, 70, 61-75.

10.1016/j.bbi.20.02.013

Gomes, E.C., Silva, A.N., & Rubino de Oliveira, M. (2012). Oxidants, antioxidants, and the beneficial roles of exercise-induced production of reactive species. *Oxidative Medicine and Cellular Longevity*. Article ID 756132. Epub 2012, Jun 3.

10.1155/2012/756132

Gomez-Cabrera, M.C., Borrás, C., Pallardo, F.V., Sastre, J., Ji, L.L., & Viña, J. (2005). Decreasing xanthine oxidase-mediated oxidative stress prevents useful cellular adaptations to exercise in rats. *Journal of Physiology*, 567(Pt1), 113-120.

10.1113/jphysiol.2004.080564

- Gomez-Cabrera, M. C., Domenech, E., & Viña, J. (2008). Moderate exercise is an antioxidant: Upregulation of antioxidant genes by training. *Free Radical Biology and Medicine*, 44(2), 126-131.
<https://doi.org/10.1016/j.freeradbiomed.2007.02.001>
- Gomez-Cabrera, M.C., Martinez, A., Santangelo, G., Pallardó, F.V., Sastre. J., & Viña, J. (2006). Oxidative stress in marathon runners: Interest of antioxidant supplementation. *British Journal of Nutrition*, 96, Suppl. 1, S31-33.
10.1079/BJN20061696
- Gomez-Cabrera, M.C., Ristow, M., & Viña, J. (2012). Antioxidant supplements in exercise: Worse than useless? *American Journal of Physiology – Endocrinology and Metabolism*, 302: E476-477.
10.1152/ajpendo.00567.2011
- Gomez-Cabrera, M.C., Salvador-Pascual A., Cabo, H., Ferrando, B., & Viña, J. (2015). Redox modulation of mitochondriogenesis in exercise. Does antioxidant supplementation blunt the benefits of exercise training? *Free Radical Biology and Medicine*, 86, 37-46.
10.1016/j.freeradbiomed.2015.04.006
- Gomez-Cabrera, M.C., Viña, J., & Ji, LL. (2009). Interplay of oxidants and antioxidants during exercise: Implications for muscle health. *The Physician and Sports Medicine*, 37, 116-123.
10.3810/psm.2009.12.1749
- Gomez-Cabrera, M.C., Viña, J., & Ji, L.L. (2016). Role of redox signalling and inflammation in skeletal muscle adaptations to training. *Antioxidants*, 5(4), pii: E48.
10.3390/antiox5040048

- Hadžović-Džuvo, A., Valjevac, A., Lepara, O., Pjanić, S., Hadžimuratović, A., & Mekić, A. (2014). Oxidative stress status in elite athletes engaged in different sport disciplines. *Bosnian Journal of Basic Medical Sciences*, *14*(2), 56-62.
<https://doi.org/10.17305/bjbms.2014.2262>
- Halliwell, B., & Gutteridge, J.M.C. (1989). *Free radicals in biology and medicine*. Claredon Press: Oxford.
10.1093/acprof:oso/9780198717478.001
- Halliwell, B., & Gutteridge, J.M.C. (2015). *Free radicals in biology and medicine* (5th Ed). University Press: Oxford.
- Halliwell, B., & Lee, C.Y.J. (2010). Using isoprostanes as biomarkers of oxidative stress: Some rarely considered issues. *Antioxidants and Redox Signalling*, *13*(2), 145–156.
<https://doi.org/10.1089/ars.2009.2934>
- Halson, S.L. (2014) Monitoring training load to understand fatigue in athletes. *Sports Medicine*, *44*(Supp2), S192-147.
10.1007/s40279-014-0253-z
- Halvorsen, B.L., Carlsen, M.H., Phillis, K.M., Bohn, S.K., Holte, K., Jacobs Jnr, D.R., & Blomhoff, R. (2006). Content of redox-active compounds (i.e., antioxidants) in foods consumed in the United States. *The American Journal of Clinical Nutrition*, *84*(1), 95-135.
10.1093/ajcn/84.195
- Harper, D.J., Carling, C., & Kiely, J. (2019) High-intensity acceleration and deceleration demands in elite team sports competitive match play: A systematic review and meta-analysis of observational studies. *Sports Medicine*, *49*, 1923-

47.

<https://doi.org/10.0007/s40279-019-01180-1>

Harriss, D.J., & Atkinson, G. (2011). Update - Ethical standards in sport and exercise science research. *International Journal Sports Medicine*, 32, 819-821.

10.1055/s-0035-15656

Harriss, D.J., & Atkinson, G. (2015). Ethical standards in sport and exercise science research: Update. *International Journal Sports Medicine*, 36(14), 1121-1124.

10.1055/s-0035-15656

Harriss, D. J., Macsween, A., & Atkinson, G. (2017). Standards for Ethics in Sport and Exercise Science Research: 20 Update. *International Journal of Sports Medicine*, 38(14), 1126–1131.

10.1055/s-0043-124001

Harty, P.S., Cottet, M.L., Malloy, J.K., & Kerksick, C.M. (2019). Nutritional and supplementation strategies to prevent and attenuate exercise-induced muscle damage: A brief review. *Sports Medicine Open*, 5(1), 1.

10.16/s40798-0-0176-6

Harwood, M., Danielewska-Nikiel, B., Borzelleca, J.F., Flamm, G.W., Williams, G.M. & Lines, T.C. (2007). A critical review of the data related to the safety of quercetin and lack of evidence of *in vivo* toxicity, including lack of genotoxic/carcinogenic properties. *Food and Chemical Toxicology*, 45, 2179-2205.

10.1016/j.fct.2007.05.015

Hazar, F. (2012). Leptin, high-sensitivity C-reactive protein and malondialdehyde concentrations in elite adolescent soccer players and physically active adolescents. *African Journal of Microbiology Research*, 6(12), 3047-3051.

10.5897/AJMR12.204

Heaton, L.E., Davis, J.K., Rawson, E.S., Nuccio, R.P., Witard, O.C., Stin, K.W., ... & Baker, L.B. (2017). Selected in-season nutritional strategies to enhance recovery for team sport athletes: a practical overview. *Sports Medicine*, 47, 2201-2218.

10.1007/s40279-017-0759-2

Heisterberg, M.F., Fahrenkrug, J., Krstrup, P., Storskov, A., Kjær, M., & Andersen, J.L. (2013). Extensive monitoring through multiple blood samples in professional soccer players. *Journal of Strength & Conditioning Research*, 27(5), 1260-1271.

10.1519/JSC.0b013e32653d17

Henríquez-Olguín, C., Meneses-Valdes, R., & Jensen, T., E. (2020).

Compartmentalized muscle redox signals controlling exercise metabolism-current state, future challenges. *Redox Biology*, 35, 1-7.

10.1016/j.redox.2020.101473

Hopkins, W.G. (2000). Measures of reliability in Sports medicine and science. *Sports Medicine*, 30(1), 1-15.

10.2165/00007256-200030010-00001

Hopkins, W.G., Marshall, S.W., Batterham, A.M., & Hanin, J. (2009). Progressive statistics for studies in sports medicine and exercise science. *Medicine and Science in Sports & Exercise*, 41(1), 3-13.

10.1249/MSS.0b013e3cb278

Howatson, G., McHugh, M.P., Hill, J.A., Brouner, J., Jewell, A.P., van Someren, K.A., Shave, R.E., & Howatson, S.A. (2010). Influence of tart cherry juice on

indices of recovery following marathon running. *Scandinavian Journal of Medicine and Science in Sports*, 20(6), 843-852.

10.1111/j.1600-0838.2009.01005x

Howatson, G., & van Someren, K.A. (2008). The prevention and treatment of exercise-induced muscle damage. *Sports Medicine*, 38(6), 483-503.

10.2165/00007256-200838060-00004

Howatson, G., van Someren, K.A., & Hortobágyi (2007). Repeated bout effect after maximal eccentric exercise. *Physiology and Biochemistry*, 28, 557-563.

10.1055/s-2007-964866

Howley, E.T., Bassett, D., & Welch, H.G. (1995). Criteria for maximal oxygen uptake: Review and commentary. *Medicine and Science in Sport and Exercise*, 27(9), 1292-1301.

10.1249/00005768-199509000-00009

Huggins, R.A., Fortunati, A.R., Curtis, R.M., Looney, D.P., West, C.A., Lee, E.C., ... Casa, D.J. (2018). Monitoring blood biomarkers and training load throughout a collegiate soccer season. *Journal of Strength and Conditioning Research*, 33(11), 1-13.

10.1519/JSC.0000000000002622

Hyldahl, R.D., Chen, T.C., & Nosaka, K. (2017). Mechanisms and mediators of the skeletal muscle repeated bout effect. *Exercise and Sport Sciences Reviews*, 45(10), 24-33.

10.1249/JES.0000000000000095

Iaia, M. F., Rampinini, E., & Bangsbo, J. (2009). High-intensity training in football. *International Journal of Sports Physiology and Performance*, 4(3), 291-306.

<https://doi.org/10.1123/ijsp.4.3.291>

- Impellizzeri, F.M., Rampinini, E., & Marcora, S.M. (2005). Psychological assessment of aerobic training in soccer. *Journal of Sports Sciences*, 23(6), 583-92.
10.1080/02640410400021278
- Ispirilidis, I., Fatourous, I.G., Jamurtas, A.Z., Athanasios, Z., Nikolaidis, M.G.,
Douroudos, I., ... Taxildaris, K. (2008). Time-course changes in inflammatory and performance responses following a soccer game. *Clinical Journal of Sport Medicine*, (5), 423-431.
10.1249/01.mss.0000322840.72766.9f
- Jackson, A.S., & Pollock, M.L. (1978). Generalized equations for predicting body density of men. *British Journal of Nutrition*, 40, 497-504.
10.1079/BJN19780152
- Jackson, A.S., Pollock, M.L., & Ward, A. (1980). Generalized equations for predicting body density of women. *Medicine and Science in Sports and Exercise*, 12(3), 175-2.
10.1249/00005768-198023000-00009
- Jaffe, M. Z. (1886) About generates the precipitation which picric acid in normal urine and a new reaction of creatinine. *Physiological Chemistry*, 10, 391-400.
doi n/a
- Jaffe, M.Z. (1886). Ueber den niederschlag welchen pikrinsäure in normalen harn erzeugt und über eine neue reaction des kreatinins. *Physiological Chemistry*, 10, 391-400.
- Jamurtas, A.Z. (2018). Exercise-induced muscle damage and oxidative stress. *Antioxidants*, 7(4), 50.
10.3390/antiox7040050
- Jaspers, A., Brink, M.S., Probst, S.G.M., Frencken, W.G.P. & Helsen, W.F. (2017).

Relationships between training load indicators and training outcomes in professional soccer. *Sports Medicine*, 47, 533-544.

10.1007/s40279-016-0591-0

Jeacocke, N.A., & Burke, L.M. (2010). Methods to standardise dietary intake before performance testing. *International Journal of Sport Nutrition & Exercise Metabolism*, 20(2), 87-103.

10.1123/ijsnem.20.2.87

Jennings, D., Cormack, S., Coutts, A.J., Boyd, L., & Aughey, R.J. (2010). The validity and reliability of GPS units for measuring distance in team sport-specific running patterns. *International Journal Sports Physiology Performance*, 5, 328-341.

10.1123/ijsp.5.3.328

Jeong, T.S., Reilly, T., Morton, J., Bae, S.W., & Drust, B. (2011). Quantification of the physiological loading of one week of “pre-season” and one week of “in-season” training in professional soccer players. *Journal of Sports Sciences*, 29(11), 1161-1166.

10.1080/02640414.2011.583671

Ji, L.L., Gomez-Cabrera, M.C., Steinhafel, N., & Viña, J. (2004). Acute exercise activates nuclear factor (NF)-κB signalling pathway in rat skeletal muscle. *FASEB Journal*, (13), 1499–1506.

<https://doi.org/10.1096/fj.04-46com>

Ji, L.L., Gomez-Cabrera, M. C., & Viña, J. (2006). Exercise and hormesis: Activation of cellular antioxidant signalling pathway. *Annals of the New York Academy of Sciences*, 1067(1), 425–435.

<https://doi.org/10.1196/annals.1354>

- Ji, L.L., Gomez-Cabrera, M. C., & Viña, J. (2007). Role of nuclear factor κ B and mitogen-activated protein kinase signalling in exercise-induced antioxidant enzyme adaptation. *Applied Physiology, Nutrition, and Metabolism*, 32, 930-935.
doi n/a
- Jin, F., Nieman, D.C., Shanely, R.A., Knab, A.M., Austin, M.D., & Sha, W. (2010). The variable plasma Quercetin response to 12-week quercetin supplementation in humans. *European Journal of Clinical Nutrition*, 64, 692-697.
10.1038/ejcn.2010.91
- Johnston, R.D., Gibson, N.V., Twist, C., Gabbett, T.J., MacNay, S.A., & MacFarlane, N.G. (2013). Physiological responses to an intensified period of rugby league competition. *Journal of Strength and Conditioning Research*, 27(3), 643-654.
10.1519/JSC.0b013e325bb469
- Johnstone, J.A., Ford, P.A., Hughes, G., Watson, T., Mitchell, A.C.S., & Garrett, A.T. (2012). Field based reliability and validity of the Bioharness™ multivariable monitoring device. *Journal of Sports Science and Medicine*, 11(4), 643-652.
doi n/a
- Jones, C.M., Griffiths, P.C., & Mellalieu, S.D. (2017). Training load and fatigue marker associations with injury and illness: A systematic review of longitudinal studies. *Sports Medicine*, 47, 943-974.
10.1007/s40279-016-0619-5
- Justino, G.C., Santos, M.R., Canario, S., Borges, C., Florencio, M.H., & Mira, L. (2004). Plasma quercetin metabolites: Structure-antioxidant activity relationships. *Archives of Biochemistry and Biophysics*, 432, 109-121.
10.1016/j.abb.2004.09.007
- Kao, M-F., Lu, H-K., Jang, T-R., Yang, W-C., Chen, C-H., & Hsieh, K-C. (2010).

- Comparison of different measurement equations for body composition estimation in male athletes. *International Journal of Sport and Exercise Science*, 3(1), 11-16. 10.29901/RJS.201106.000
- Kawamura, T., & Muraoka, I. (2018). Exercise-induced oxidative stress and the effects of antioxidant intake from a physiological viewpoint. *Antioxidants*, 7(9), pii E119.
10.3390/antiox709011
- Keane, K. M., Salicki, R., Goodall, S., Thomas, K., & Howatson, G. (2015). Muscle damage response in female collegiate athletes after repeated sprint activity. *Journal of Strength and Conditioning Research*, 29(10), 2802-2807.
10.1519/JSC0000000000000961
- Kelly, D.M., Strudwick, A.J., Atkinson, G., Drust, B., & Gregson, W. (2019). Quantification of training and match-load distribution across a season in elite English premier league soccer players. *Science and Medicine in Football*, 4(1), 59-67.
10.1080/2473398.2019.16151934
- Khan, A., & Rayner, G.D. (2003). Robustness to non-normality of common tests for the many-sample location problem. *Journal of Applied Mathematics & Decision Sciences*, 7(4), 7-206. 10.1155/S1173912603000178
- Koenig, R.T., Dickman, J.R., Kang, C-H., Zhang, T., Chu, Y., & Ji, L.L. (2015). Avenanthramide supplementation attenuates eccentric exercise-inflicted blood inflammatory markers in women. *European Journal Applied Physiology*, 116(1), 67-76.
10.1007/s00421-015-3244-3
- Konrad, M., Neiman, D.C., Henson, D.A., Kennerly, K.M., Jin, F., & Wallner-

Liebmann. (2011). The acute effect of ingesting a quercetin-based supplement on exercise-induced inflammation and immune changes in runners. *International Journal of Sport Nutrition and Exercise Metabolism*, 21(4), 338-346.

10.1123/ijsnem.21.4.338

Kramer, H.F., & Goodyear, L.J. (2007). Exercise, MAPK, and NF- κ B signalling in skeletal muscle. *Journal of Applied Physiology*, 103, 388-395.

10.1152/jappphysiol.00085.2007

Kressler, J., Millard-Stafford, M., & Warren, G.L. (2011). Quercetin and endurance exercise capacity: A systematic review and meta-analysis. *Medicine and Science in Sports and Exercise*, 44(3), 558-559.

10.1249/MSS.0b013e322495a7

Krustrup, P., Mohr, M., Armstrup, T., Rysgaard, T., Johansen, J., Steensberg, A., Pedersen, P.K. & Bangsbo, J. (2003). The yo-yo intermittent recovery test: Physiological response, reliability, and validity. *Medicine and Science in Sports and Exercise*, 35(4), 697-705.

10.1249/01.MSS.0000058441.94520.32

Lamprecht, M., Greilberger, J. & Oettl, K. (2004). Analytical aspects of oxidatively modified substances in sports and exercises. *Nutrition*, 20, 728-730.

10.1016/j.nut.2004.04.016

Larsson, P. (2003). Global Positioning System and Sport-Specific Testing.

Sports Medicine, 33(15), 1093-1101.

<https://doi.org/10.2165/00007256-200333150-00002>

- Lau, W.Y., Muthalib, M., & Nosaka, K. (2013). Visual analogue scale and pressure pain threshold for delayed onset muscle soreness assessment. *Journal of Musculoskeletal Pain, 21*(4), 320-326.
10.3109/10582452.2013.848967
- Lee, E.C., Fragala, M.S., Kavouras, S.A., & Queen, R.M., Pryor, J.L., & Casa, D.J. (2017). Biomarkers in sports and exercise: Tracking health, performance, and recovery in athletes. *Journal of Strength and Conditioning Research, 31*(10), 2920-2937.
10.1519/JSC.0000000000002122
- Le Moal, E., Groussard, C., Paillard, T., Chaory, K., Le Bris, R., Plantet, J., ... Zouhal, H. (2016). Redox status of professional soccer players is influenced by training load throughout a season. *International Journal Sports Medicine, 37*(9), 680-686.
10.1055/s-0035-1565199
- Levers, K., Dalton, R., Galvan, E., O'Connor, A., Goodenough, C., Simbo, S., ... Kreider, R. B. (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), 1-23.
<https://doi.org/10.16/s12970-016-0133-z>
- Lewis, N.A., Newell, J., Burden, R., Howatson, G., & Pedlar, C.R. (2016). Critical difference and biological variation in biomarkers of oxidative stress and nutritional status in athletes. *PLoS ONE, 11*(3), 1-12.
<https://doi.org/10.1371/journal.pone.0149927>

- Lindsay, A., & Costello, J.T. (2017). Realising the potential of urine and saliva as diagnostic tools in sport and exercise medicine. *Sports Medicine*, 47(1), 11-31.
10/1007/s40279-016-0558-1
- Liu, J., & Mori, A. (1999). Stress, aging, and brain oxidative damage. *Neurochemical Research*, 24(11), 1479-1497.
10.1023/A:1022597010078
- Lovell, R., Knapper, B., & Small, K. (2008). Physiological responses to SAFT90: a new soccer-specific match simulation. *Coach Sport Science*, 3, 46-47.
doi n/a
- MacRae, H.S., & Mefferd, K.M. (2006). Dietary antioxidant supplementation combined with quercetin improves cycling time trial performance. *International Journal of Sport Nutrition and Exercise Metabolism*, 16(4), 405-419.
10.1123/ijsnem.16.4.405
- Malm, C. (2001). Exercise-induced muscle damage and inflammation: Fact or fiction? *Acta Physiologica Scandinavica*, 171(3), 233-239.
<https://doi.org/10.1046/j.1365-201X.2001.00825.x>
- Malm, C., Ekblom, O., & Ekblom, B. (2004). Immune system alteration in response to two consecutive soccer games. *Acta Physiologica Scandinavica*, 0, 143-155.
10.1046/j.0001-6722.2003.01232.x
- Malm, C., Sjödin, T.L., Sjöberg, B., Lenkei, R., Renstör, P., Lundberg, I.E., ...Ekblom, E. (2004). Leukocytes, cytokines, growth factors and hormones in human skeletal muscle and blood after uphill or downhill running. *Journal Physiology*, 556 (Pt 3), 983-1000.
10.1113/jphysiol.2003.056598

- Magalhães, J., Rebelo, A., Oliveira, E., Silva, J.R., Marques, F., & Ascensão, A. (2010). Impact of Loughborough Intermittent Shuttle Test versus soccer match on physiological, biochemical and neuromuscular parameters. *European Journal of Applied Physiology*, *108*(1), 39-48.
<https://doi.org/10.1007/s00421-009-1161-z>
- Malone, J.J., Di Michele, R., Morgans, R., Burgess, D., Morton, J.P. & Drust, B. (2015a). Seasonal training-load quantification in elite English premier league soccer players. *International Journal of Sports Physiology and Performance*, *10*(4), 489-497.
10.1123/jspp.2014-0352
- Malone, J.J., Murtagh, C.F., Morgans, R., Burgess, D.J., Morton, J.P. & Drust, B. (2015b). Countermovement jump performance is not affected during an in-season training microcycle in elite youth soccer players. *Journal of Strength and Conditioning Research*, *29*(3), 752-757.
10.1519/JSC.0000000000000701
- Manach, C., Williamson, G., Morand, C., Scalbert, A., & Rémésy, C. (2005). Bioavailability and bioefficacy of polyphenols in humans. Review of 97 bioavailability studies. *American Journal Clinical Nutrition*, *81*(1 Suppl), 230S-242S.
10.1093/ajcn/81.1.230S
- Marrocco, I., Altieri, F., & Peluso, I. (2017). Measurement and clinical significance of biomarkers of oxidative stress in humans. *Oxidative Medicine and Cellular Longevity*, Article ID 6501046, 1-32.
10.1155/2017/6501046

- Martinez-Moral, M-P., & Kannan, K. (2019). How stable is oxidative stress level? An observational study of intra- and inter-individual variability in urinary oxidative stress biomarkers of DNA, proteins and lipids in healthy individuals. *Environment International* 123, 382-389.
10.1016/j.envint.20.12.009
- Massard, T., Eggers, T., & Lovell, R. (2017). Peak speed determination in football: is sprint testing necessary? *Science and Medicine in Football*, 2(2), 123-126.
10.1080/24733938.2017.1398409
- Matomäki, P., Kainulainen, H., & Kyröläinen, H. (2018). Corrected whole blood biomarkers-the equation of Dill and Costill revisited. *Physiological Reports*, 6(12), 1-3.
10.14814/phy2.13749
- McAnulty, L.S., Miller, L.E., Hosick, P.A., Utter, A.C., Quindry, J.C. & McAnulty, S.R. (2013). Effect of resveratrol and quercetin supplementation on redox status and inflammation after exercise. *Applied Physiology, Nutrition and Metabolism*, 38(7), 760-765.
10.1139/apnm-2012-0455
- McAnulty, S.R., McAnulty, L.S., Nieman, D.C., Hosick, P.A., Hudson, M.H., Still, L., & Dibarnardi, A. (2008). Chronic quercetin ingestion and exercise-induced oxidative damage and inflammation. *Applied Physiology, Nutrition and Metabolism*, 33(2), 254-262.
10.1139/H07-177
- McHugh, M.P. (2003). Recent advances in the understanding of the repeated bout effect against muscle damage from a single bout of eccentric exercise. *Scandinavian Journal of Medicine and Science in Sports*, 13(2), 88-97.

<https://doi.org/10.1034/j.1600-0838.2003.02477.x>

McLean, B.D., Coutts, A.J., Kelly, V., McGuigan, M.R., & Cormack, S.J. (2010). Neuromuscular, endocrine, and perceptual fatigue responses during different length between-match microcycles in professional rugby league players. *International Journal of Sports Physiology and Performance*, 5, 367-383.

<https://doi.org/10.1123/ijsp.5.3.367>

Mello, R., Mello, R., Gomes, D., Paz, G.A., Nasser, I., Humberto, M., & Salerno, V. (2017). Oxidative stress and antioxidant biomarker responses after a moderate-intensity soccer training session. *Research in Sports Medicine*, 25(3), 322-332.

10.1080/15438627.2017.1345738

Merry, T.L. & Ristow, M (2016). "Do antioxidant supplements interfere with skeletal muscle adaptation to exercise training?" *Journal of Physiology*, 594(18), 5143-5147.

10.113/JP270654

Metin, G., Gümüştas, M.K., Uslu, E., Belce, A., & Kayserilioglu, A. (2003). Effect of regular training on plasma thiols, malondialdehyde and carnitine concentrations in young soccer players. *Chinese Journal of Physiology*, 46(1), 35-39.

doi n/a

Michaildis, Y., Jamurtus, A.Z., Nikolaidis, M.G., Fatouros, I.G., Koutedakis, Y., Papassotiriou, I., & Kouretas, D. (2007). Sampling time is crucial for measurement of aerobic exercise-induced oxidative stress. *Medicine and Science in Sports and Exercise*, 39(7), 1107-1113.

10.1249/01.mss.0b013e3053e7ba

Miller, N.J., Rice-Evans, C., Davies, M.J., Gopinathan, V. & Milner, A. (1993). A novel method for measuring antioxidant capacity and its application to

monitoring the antioxidant status in premature neonates. *Clinical Science*, 84, 407-412.

10.1042/cs0840407

Mohr, M., Draganidis, D., Chatzinikolaou, A., Barbero-Álvarez, J.C., Castagna, C., Douroudos, I., ... Fatouros, I.G. (2015). Muscle damage, inflammatory, immune and performance responses to three football games in 1 week in competitive male players. *European Journal of Applied Physiology*, 116(1), 179-193.

10.1007/s00421-015-3245-2

Mohr, M., Krstrup, P., & Bangsbo, J. (2003). Match performance of high – standard soccer players with special reference to development of fatigue. *Journal of Sports Sciences*, 21, 519-528.

10.1080/02640410310000712

Mohr, M., Krstrup, P., & Bangsbo, J. (2005). Fatigue in soccer: A brief review. *Journal of Sports Sciences*, 23, 593-599.

10.1080/02640410400021286

Morgan, P.T., Barton, M.J., & Bowtell, J.L. (2019). Montmorency cherry supplementation improves 15-km cycling time-trial performance. *European Journal of Applied Physiology*, 119(3), 675-684.

<https://doi.org/10.1007/s00421-0-04058-6>

Mougios, V. (2007). Reference intervals for serum creatine kinase in athletes. *British Journal of Sports Medicine*, 41(10), 674-678.

<https://doi.org/10.1136/bjism.2006.034041>

Mukherjee, S., & Chia, M. (2009). Urinary total antioxidant capacity in soccer players. *Acta Kinesiologica*, 3(1), 26-33.

doi n/a

- Nair, M.P., Mahajan, S., Reynolds, J.L., Aalikeel, R., Nair, H., & Schwartz, S.A. (2006). The flavonoid quercetin inhibits pro-inflammatory cytokine (tumour necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF- κ B system. *Clinical Vaccine and Immunology*, 13(39), 9-328.
10.1128/CVI.13.3.319-328.2006
- Nédélec, M., McCall, A., Carling, C., Legall, F., Berthoin, S., & Dupont, G. (2012). Recovery in soccer: part I-post-match fatigue and time course of recovery. *Sports Medicine*, 42(12), 997-1015.
10.2165/11635270-000000000-00000
- Nédélec, M., McCall, A., Carling, C., Legall, F., Berthoin, S., & Dupont, G. (2013). Recovery in soccer: part II-recovery strategies. *Sports Medicine*, 43(1), 9-22.
10.1007/s40279-012-0002-0
- Nelson, M., Atkinson, M., & Meyer, J. (1997). *A photographic atlas of food portion sizes*. U.K.: MAFF (Ministry of Agriculture Fisheries and Food) publications.
- Nicholas, C. W., Nuttall, F. E., & Williams, C. (2000). The Loughborough intermittent shuttle test: A field test that simulates the activity pattern of soccer. *Journal of Sports Sciences*, (2), 97-104.
10.1080/026404100365162
- Nieman, D.C. (2008). Immunonutrition support for athletes. *Nutrition Review*, 6(6), 310-320.
10.1111/j.1753-4887.2008.00038x
- Nieman, D.C. & Bishop, N.C. (2006) Nutritional strategies to counter stress to the immune system in athletes, with special reference to football. *Journal of Sports Sciences*, 24, 763-772.

10.1080/02640410500482982

Nieman, D.C., Davis, J.M., Henson, D.A., Gross, D.J., Dumke, C.L., & Utter A.C. (2005). Skeletal muscle cytokine mRNA and plasma cytokine changes after 2.5h cycling: influence of carbohydrate. *Medicine and Science in Sports and Exercise*, 37, 1283-1290.

10.1097/00005768-200505001-00668

Nieman, D.C., Davis, J.M., Henson, D.A., Walberg-Rankin, J., Shute, M., & Dumke, C.L. (2003). Carbohydrate ingestion influences skeletal muscle cytokine and mRNA and plasma cytokine levels after a 3-hr run. *Journal of Applied Physiology*, 94, 1917-1925.

10.1152/jappphysiol.01130.2002

Nieman, D.C., Henson, D.A., Davis, J.M., Murphy, E.A., Jenkins, D.P., & Gross S.J. (2007a). Quercetin's influence on exercise-induced changes in plasma cytokines and muscle and leukocyte cytokine mRNA. *Journal of Applied Physiology*, 103(5), 1728-1735.

10.1152/jappphysiol.00707.2007

Nieman, D.C., Henson, D.A., Gross, S.J., Jenkins, D.P., Davis, J.M., Murphy, E.A., & Mayer, E.P. (2007b). Quercetin reduces illness but not immune perturbations after intensive exercise. *Medicine and Science in Sports and Exercise*, 39(9), 1561-1569.

10.1249/mss.0b013e3076b566

Nieman, D.C., Henson, D.A., Maxwell, K.R., Williams, A.S., McAnulty, S.R., Jin, F., Shanley, R.A., & Lines, T.C. (2009). Effects of Quercetin and EGCG on mitochondrial biogenesis and immunity. *Medicine and Science in Sports and Exercise*, 41(7), 1467-1475.

10.1249/MSS.0b013c3199491f

Nieman, D.C., Luo, B., Dréau, D., Henson, D.A., Shanley, R. A., Dew, D., & Meaney, M.P. (2014). Immune and inflammation responses to a 3-day period of intensified running versus cycling. *Brain, Behaviour, and Immunity*, 39, 0-5.

10.1016/j.bbi.2013.09.004

Nieman, D.C., & Mitmesser, S.H. (2017). Potential impact of nutrition on immune system recovery from heavy exertion: A metabolomics perspective. *Nutrients*, MDPI, 9, 1-23.

<https://doi.org/10.3390/nu905051>

Nieman, D.C., Nehlsen-Cannarella, S.L., Fagoaga, O.R., Henson, D.A., Utter, A., Davis, J.M., ... & Butterworth, D.E. (1998). Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Medicine and Science in Sport and Exercise*, 30(5), 671-678.

10.1097/00005768-199805000-00005

Nieman, D.C., Williams, A.S., Shanely, R.A., Jin, F., McAnulty, S.R. Triplett, N.T.,...Henson, D.A. (2010). Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. *Medicine and Science in Sports and Exercise*, 42(2), 338-345.

10.1249/MSS.0b013e31bfa3

Nikolaidis, M.G., Kyparos, A., Spanou, C., Paschalis, V., Theodorou, A.A., & Vrabas, I.S. (2012). Redox biology of exercise: An integrative and comparative consideration of some overlooked issues. *Journal of Experimental Biology*, 215, 1615-1625.

10.1242/jeb.067470

- O'Fallon, K., S., Kaushik, D., Michniak-Kohn, B., Dunne, C.P., Zambraski, E.J., & Clarkson, P.M. (2012). Effects of quercetin supplementation on markers of muscle damage and inflammation after eccentric exercise. *International Journal of Sport Nutrition and Exercise Metabolism*, 22, 430-437.
doi n/a
- Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, 95(2), 351-358.
10.1016/0003-2697(79)90738-3
- Oliver, I.T. (1955). A spectrophotometric method for the determination of creatine phosphokinase and myokinase. *Biochemical Journal*, 61(1), 116-122.
10.1042/bj0610116
- Oliveira, C., Ferreira, D., Caetano, C., Granja, D., Pinto, R., Mendes, B., & Sousa, M. (2017). Nutrition and Supplementation in Soccer. *Sports*, 5(28), 1-35.
<https://doi.org/10.3390/sports5020028>
- Owens, D.J., Twist, C., Cobley, J.N., Howatson, G. & Close, G.L. (2019). Exercise-induced muscle damage: What is it, what causes it, and what are the nutritional solutions? *European Journal of Sports Science*, 19(1), 71-85.
10.1080/17461391.20.1505957
- Packer, L. (1997). Oxidants, antioxidants, nutrients and the athlete. *Journal of Sports Sciences*, 15, 353-363.
doi n/a
- Pandolf, K.B. (1982). Differentiated ratings of perceived exertion during physical exercise. *Medicine and Science in Sports and Exercise*, 14(5), 397-405.

- Parker, L., Trewin, A., Levinger, I., Shaw, C.S., & Stepto, N.K. (2017). The effect of exercise-intensity on skeletal muscle stress kinase and insulin protein signalling. *PLoS ONE* 12(2), 1-15.
10.1371/journal.pone.0171613
- Peake, J.M., Neubauer, O., Della Gatta, P.A., & Nosaka, K. (2017). Muscle damage and inflammation during recovery from exercise. *Journal of Applied Physiology*, 122(3), 559-570.
<https://doi.org/10.1152/jappphysiol.0090712016>
- Pedersen, B.K. (2000). Exercise and cytokines. *Immunology & Cell Biology*, 78, 532-535.
10.1111/j.1440-1711.2000.t01-11-x
- Pedersen, B.K. & Febbraio, M.A. (2008). Muscle as an endocrine organ: focus on muscle-derived interleukin-6. *Physiological Reviews*, 88, 1379-1406.
10.1152/physrev.90100.2007.
- Pedersen, B.K., Steensberg, A., Fischer, C., Keller, C., Ostrowski, K., & Schjerling, P. (2001). Exercise and cytokines with particular focus on muscle derived IL-6. *Exercise Immunology Review*, 7, 18-31.
doi n/a
- Pelletier, D.M., Lacerte, G., & Goulet, E.D.B. (2013). Effects of quercetin supplementation on endurance performance and maximal oxygen consumption: A meta-analysis. *International Journal of Sport Nutrition and Exercise Metabolism*, 23(1), 73-82.
<https://doi.org/10.1123/ijsnem.23.1.73>
- Peternelj, T.T. & Coombes, J.S. (2011). Antioxidant supplementation during exercise training: Beneficial or detrimental? *Sports Medicine*, 1, 41(12), 1043-1069.

10.2165/11594400-000000000-00000

Petersen, A.M., & Pedersen, B.K. (2005). The anti-inflammatory effect of exercise. *Journal Applied Physiology*, 98(4), 1154-1162.

10.1152/jappphysiol.00164.2004

Pizza, F., Koh, T.J., McGregor, S.J., & Brooks, S.V. (2002). Muscle inflammatory cells after passive stretches, isometric contractions, and lengthening contractions. *Journal of Applied Physiology*, 92, 1873-8.

10.1152/jappphysiol.01055.2001

Plunkett, B. A., Callister, R., Watson, T. A., & Garg, M. L. (2010). Dietary antioxidant restriction affects the inflammatory response in athletes. *British Journal of Nutrition*, 103(8), 1179-1814.

<https://doi.org/10.1017/S0007114509993011>

Poole, D. & Jones, A.M. (2017). CORP: Measurement of the maximum oxygen uptake ($\dot{V}O_{2max}$): VO_{2peak} is no longer acceptable. *Journal of Applied Physiology*, 122(4), 997-1002.

10.1152/jappphysiol.01063.2016

Powers, S.K. (2017). Teaching myocytes new tricks. *Journal of Applied Physiology*, 123(2), 460-472.

10.1152/jappphysiol.004.2017

Powers, S.K., Criswell, D., Lawler, J., Li, L.J., Martin, R.A.H., & Dudley, G (1994). Influence of exercise and fiber type on antioxidant enzyme activity in rat skeletal muscle. *American Journal Physiology*, 266, R375-R380.

10.1152/ajpregu.1994.266.2.R375

Powers, S.K., DeRuisseau, K.C., Quindry, J., & Hamilton, K.L. (2004). Dietary antioxidants and exercise. *Journal Sports Science*, 22(1), 81-94.

10.1080/0264041031000140563

Powers, S.K., Duarte, J., Kavazis, A.N., & Talbert, E.E. (2010a). Reactive oxygen species are cell signalling molecules for skeletal muscle adaptation. *Experimental Physiology*, 95(1), 1-9.

10.1113/expphysiol.2009.050526

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

10.1152/physrev.00031.2007

Powers, S.K., Nelson, W.B., & Hudson, M.B. (2011). Exercise-induced oxidative stress in humans: Cause and consequences. *Free Radical Biology and Medicine*, 51(5), 942-950.

10.1016/j.freeradbiomed.2010.12.009

Powers, S.K., Radak, Z., & Ji, L. (2016). Exercise-induced oxidative stress: Past, present and future. *Journal of Physiology*, 594(18), 5081-5092.

10.1113/JP270646

Powers, S.K., Smuder, A.J., Kavazis, A.N., & Hudson, M.B. (2010b) Experimental guidelines for studies designed to investigate the impact of antioxidant supplementation on exercise performance. *International Journal of Sports Nutrition and Exercise Metabolism*, 20(1), 2-14.

doi n/a

Proske, U., & Allen, T.J. (2005). Damage to skeletal muscle from eccentric exercise. *Exercise and Sport Sciences Reviews*, 33(2), 98-104.

0091-6331/3302/98-104

- Proske, U., & Morgan, D.L. (2001). Muscle damage from eccentric exercise: mechanism, mechanical signs, adaptation, and clinical applications. *Journal of Physiology*, 537(2), 333-345.
10.1111/j.1469-7793.2001.00333.x
- Pyne, D.B. (1994a). Regulation of Neutrophil Function During Exercise. *Sports Medicine: An International Journal of Applied Medicine and Science in Sport and Exercise*, 17(4), 245-258.
<https://doi.org/10.2165/00007256-199417040-00005>
- Pyne, D.B. (1994b). Exercise-induced muscle damage and inflammation: a review. *Australian Journal of Science and Medicine in Sport*, 26(3-4), 49-58.
doi n/a
- Quindry, J.C., McAnulty, S.R., Hudson, M.B., Hosick, P.A., Dumke, C.L., McAnulty, L., ... Nieman, D.C. (2008). Oral quercetin supplementation and blood oxidative capacity in response to ultramarathon competition. *International Journal of Sport Nutrition and Exercise Metabolism*, (6), 601-616.
10.1123/ijsnem..6.601
- Radak, Z., Chung, H.Y., & Goto, S. (2008). Systematic adaptation to oxidative challenge induced by regular exercise. *Free Radical Biology and Medicine*, 44(2), 153-159.
10.1016/j.freeradbiomed.2007.01.029
- Radak, Z., Chung, H.Y., Koltai, E., Taylor, A.W, & Goto, S. (2007). Exercise, oxidative stress and hormesis. *Ageing Research Reviews*, 7(1), 34-42.
10.1016/j.arr.2007.04.004
- Radak, Z., Ishihara, K., Tekus, E., Varga, C., Posa, A., Balogh, L., Boldogh,, ...

- Koltai, E. (2017). Exercise, oxidants, and antioxidants change the shape of the bell-shaped hormesis curve. *Redox Biology*, *12*, 285-290.
10.1016/j.redox.2017.02.015
- Rahnama, N., Reilly, T, Lees, A., & Graham-Smith, P. (2006) Muscle fatigue induced by exercise simulating the work rate of competitive soccer. *Journal of Sports Science*, *21*(11), 933-42.
<https://doi.org/10.1080/0264041031000140428>
- Ramezani, A.R., & Moonikh, K. (2017). Effect of quercetin supplementation on oxidative stress and exhaustion in male soccer players. *Journal of Medicinal Plants*, *16*(62), 136-144.
10.1136/bjism.22.4.141
- Ramsbottom, R., Brewer, J., & Williams, S. (1988). A progressive shuttle run test to estimate maximal oxygen uptake. *British Journal of Sports Medicine*, *22*(4),141-4.
10.1136/bsjm.22.4.141
- Ranchordas, M.K., Dawson, J.T., & Russell, M. (2017a). Practical nutritional recovery strategies for elite soccer players when limited time separates repeated matches. *Journal of the International Society of Sports Nutrition*, *14*(1), 1-14.
<https://doi.org/10.16/s12970-017-0193-8>
- Ranchordas, M.K., Rogerson, D., Soltani, H., & Costello, J.T. (2017b). Antioxidants for preventing and reducing muscle soreness after exercise. *Cochrane Database of Systematic Review*, 1-190.
10.1002/146558.CD009789.pub2
- Reid, M.B. (2001). Invited Review: Redox modulation of skeletal muscle contraction:

what we know and what we don't. *Journal of Applied Physiology*, 90(2), 724-731.

10.1152/jappl.2001.90.2.724

Reid, M.B., Khawli, A., & Moody, M.R. (1993). Reactive oxygen in skeletal muscle III. Contractility of unfatigued muscle, *Journal of Applied Physiology*, Sept, 1081-1087.

10.1152/jappl.1993.75.3.1081

Reid, M.B., Stokić, D.S., Koch, S.M., Khawli, F.A., & Leis, A.A. (1994). N-acetylcysteine inhibits muscle fatigue in humans. *Journal of Clinical Investigation*, 94(6), 2468-2474

10.1172/JCI117615

Reilly, T. & Thomas, V. (1976) A motion analysis of work-rate in different contact roles in professional football match-play. *Journal of Human movement Studies*, 2, 87-97.

doi n/a

Ristow, M., Zarse, K., Oberbach, A., Klötting, N., Birringer, M., Kiehntopf, M., ... Blüher, M. (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.

10.1073/pnas.0903485106

Roberts, J.D., Roberts, M.G., Tarpey, M.D., Weekes, J.C., & Thomas, C.H. (2015). The effect of a decaffeinated green tea extract formula on fat oxidation, body composition and exercise performance. *Journal of the International Society of Sports Nutrition*, 12(1), 1-9.

<https://doi.org/10.16/s12970-014-0062-7>

- Robson-Ansley, P.J., Blannin, A., & Gleeson, M. (2007). Elevated plasma interleukin-6 levels in trained male triathletes following an acute period of intense interval training. *European Journal Applied Physiology*, 99, 353-360.
10.1007/s00421-006-0354-y
- Romagnoli, M., Sanchis-Gomar, F., Alis, R., Risso-Ballester, J., Bosio, A., Graziani, R.L., ... Rampinini, E. (2016). Changes in muscle damage, inflammation, and fatigue-related parameters in young elite soccer players after a match. *The Journal of Sports Medicine and Physical Fitness*, 56(10), 1198-1205.
doi n/a
- Rushall, B.S. (1990). A tool for measuring stress tolerance in elite athletes. *Journal of Applied Sport Psychology*, 2(1), 51-66.
10.1080/10413209008406420
- Russell, M., Rees, G., Benton, D., & Kingsley, M. (2011). An exercise protocol that replicates soccer match play. *International Journal Sports Medicine*, 32(7), 511-518.
10.1055/s-0031-1273742
- Sari-Saraf, V., Reilly, T., Doran, D.A., & Atkinson, G. (2007). The effects of single and repeated bouts of soccer-specific exercise on salivary IgA. *Archives of Oral Biology*, (52)6, 526-532.
doi.10.1016/j.archoalbio.2006.11.016
- Scholten, S.D. & Sergeev, I.N. (2013). Long-term quercetin supplementation reduces lipid peroxidation but does not improve performance in endurance runners. *Open Access Journal of Sports Medicine*, 4, 53-61.
doi: 10.2147/OAJSM.S39632

- Scott, B.R., Lockie, R.G., Knight, T.J., Clark, A.C. & Janse de Jonge, X.A.K. (2013). A comparison of methods to quantify the in-season training load of professional soccer players. *International Journal of Sports Physiology and Performance*, 8, 195-202.
10.1123/ijsp.8.2.195
- Scott, A., Khan, K.M., Roberts, C.R., Cook, J.L., and Duronio, V. (2004). What do we mean by the term “inflammation”? A contemporary basic science update for sports medicine. *British Journal of Sports Medicine*, 38, 372-380.
10.1136/bj.2004.011312
- Scott, M.T.U., Scott, T.J., Kelly, V. (2016). The validity and reliability of global positioning systems in team sport a brief review. *The Journal of Strength and Conditioning*, 30(5), 1470-90.
10.1519/JSC.0000000000001221
- Sen, C.K., & Packer, L. (1996). Antioxidant and redox regulation of gene transcription. *FASEB Journal*, 10(7), 709-720.
10.1096/fasebj.10.7.8635688
- Shing, C.M., Peake, J.M., Ahert, S.M., Strobel, N.A., Wilson, G., Jenkins, D.G., & Coombes, J.S. (2007). The effect of consecutive days of exercise on markers of oxidative stress. *Applied Physiology, Nutrition, and Metabolism*, 32(4), 677-685.
10.1139/H07-051
- Shirvani, H., Nikbakht, H., Ebrahim, K., & Gaeini, A.A. (2012). The effects of coenzyme Q10 supplementation with high intensity intermittent exercise on serum IL-6 and TNF- α in well-trained soccer players. *European Journal of Experimental Biology*, 2(5), 1664-167.

- Sies, H., & Jones, D. (2007) *Oxidative Stress*. In Fink, G. (Ed.) *Encyclopaedia of Stress* (2nd Ed.) (pp.45-48), Amsterdam: Elsevier.
- Silva, J.R., Ascensão, A., Marques, F., Seabra, A., Rebelo, A., & Magalhães, J. (2013). Neuromuscular function, hormonal and redox status and muscle damage of professional soccer players after a high-level competitive match. *European Journal Applied Physiology*, 113(9), 2193-2201.
10.1007/s00421-013-2633-8
- Silva, J.R., Rumpf, M.C., Hertzog, M., Castagna, C., Farooq, A., Girard, O., ... Hader, K. (2018). Acute and residual soccer match-related fatigue: A systematic review and meta-analysis. *Sports Medicine*, 48(10), 539-583.
10.1007/s40279-017-0798-8
- Siri, W.E. (1961) Body composition from fluid space and density: Analysis of methods. *Nutrition*, 9(5), 480-491.
doi n/a
- Somerville, V., Bringans, C., & Braakhuis, A. (2017). Polyphenols and performance: A systematic review and meta-analysis. *Sports Medicine*, 47(8), 1589-1599.
10.1007/s40279-017-0675-5
- Souglis, A., Bogdanis, G.C., Giannopoulou, I., Papadopoulos, C.H., & Apostolidis, N. (2015). Comparison of inflammatory responses and muscle damage following a soccer, basketball, volleyball and handball game at an elite competitive level. *Research in Sports Medicine*, 23(1), 59-72.
10.1080/15438627.2014.975814

- Sparks, M., Coetzee, B., & Gabbett, T. (2016). Internal and external match loads of university-level soccer players: A comparison between methods. *The Journal of Strength and Conditioning Research*, 31(4), 1072-1077.
10.15.19/JSC.0000000000001560
- Steensberg, A. (2003). The role of IL-6 in exercise-induced immune changes and metabolism. *Exercise Immunology Review*, 9, 40-47.
doi n/a
- Steensberg, A., Van Hall, G., Osada, T., Sacchetti, M., Saltin, B., & Pedersen, B. K. (2000). Production of interleukin-6 in contracting human skeletal muscles can account for the exercise-induced increase in plasma interleukin-6. *Journal of Physiology*, 529(1), 237-242.
<https://doi.org/10.1111/j.1469-7793.2000.00237.x>
- Stevens, T., Twisk, J.W.R., Savelsbergh, G.J., & Beek, P.J. (2017). Quantification of in-season training load relative to match load in professional Dutch Eredivisie football players. *Science and Medicine in Football*, 1(2), 117-25.
10.180/24733938.2017.1282163
- Strimbu, K., & Tavel, J.A. (2010). What are Biomarkers? Author manuscript. *Current Opinion HIV AIDS*. 5(6), 463-466.
10.1097/COH.0b013e32833ed177
- Subudhi, A.W., Davis, S.L., Askew, E.W., Walker, J.A. & Johnson, S.C. (2002). Time course of urinary oxidative stress markers following sub-maximal and maximal exercise. *Journal of Human Movement Studies*, 43, 35-47.
- Sureda, A., Ferrer, M.D., Tauler, P., Romaguera, D., Drobic, F., Pujol, P., ... Pons A. (2009). Effects of exercise intensity on lymphocyte H₂O₂ production and

antioxidant defences in soccer players. *British Journal of Sports Medicine*, 43, 186-190.

10.1136/bjism.2007.043943

Suzuki, K. (2018). Cytokine response to exercise and its modulation. *Antioxidants*, 7(1).

<https://doi.org/10.3390/antiox7010017>

Suzuki, K., Nakaji, S., Yamada, M., Totsuka, M., Sato, K., & Sugawara, K. (2002).

Systemic inflammatory response to exhaustive exercise. Cytokine kinetics. *Exercise Immunology Review*, 8, 6-48.

doi n/a

Suzuki, K., Totsuka, M., Ige, S. H., Nakaj, Y., Yamada, M., Kudoh, S., ... Sato, K.

(1999). Endurance exercise causes interaction amongst stress hormones, cytokines, neutrophil dynamics, and muscle damage. *Journal of Applied Psychology*, 4, 1360-1367.

10.1152/jappl.1999.87.4.1360

Tanita Corporation, UK Ltd.

Body Composition Analyser BC-4MA. Instruction Manual.

Tanita: Middlesex.

Taylor, L., Fitch, N., Castle, P., Watkins, S., Aldous, J., Sculthorpe, N., ... Mauger, A.

(2014). Exposure to hot and cold environmental conditions does not affect the decision-making ability of soccer referees following an intermittent sprint protocol. *Frontiers in Physiology*, 5, 1-9.

<https://doi.org/10.3389/fphys.2014.005>

Taylor, H., Buskirk, E., & Henschel, A. (1955). Maximal Oxygen intake as an

objective measure of cardio-respiratory performance. *Journal of Applied*

Physiology, 73-80.

doi n/a

Tejada, S., Nabavi, S.M., Copo, X., Martorell, M., del Mar Bibiloni, M., Josep, A.T., Pons, A., & Sureda, A. (2017). Quercetin effects on exercise induced oxidative stress and inflammation. *Current Organic Chemistry*, 21(4), 348-356.

10.2174/1385272820666161017122202

Tiidus, P.M. (2000). Oestrogen and gender effects on muscle damage, inflammation and oxidative stress. *Canadian Journal of Applied Physiology*, 25(4), 274-287.

10.1139/h00-022

Totsuka, M., Nakaji, S., Suzuki, K., Sugawara, K., & Sato, K. (2002). Break point of serum creatine kinase release after endurance exercise. *Journal of Applied Physiology*, 93(4), 1280-1286.

<https://doi.org/10.1152/jappphysiol.01270.2001>

Traka, M.H., & Mithen, R.F. (2011). Plant science and human nutrition: Challenges in assessing health-promoting properties of phytochemicals.

The Plant Cell, 23, 2483-2497.

10.1105/tpc.111.087916

Twist, C., & Eston, R. (2005). The effects of exercise-induced muscle damage on maximal intensity intermittent exercise performance. *European Journal of Applied Physiology*, 94(5-6), 652-658.

10.1007/s00421-005-1357-9

USDA Database for the Flavonoid Content of Selected Foods. Release 3.2 (November 2015). Department of Agriculture. Pub: USDA NAL.

<https://data.nal.usda.gov/system/files/Flav3.2.pdf>

Utter, A.C., Nieman, D.C., Kang, J., Dumke, C.L., Quindry, J.C., McAnulty, S.R., &

- McAnulty, L.S. (2009). Quercetin does not affect rating of perceived exertion in athletes during the western states endurance run. *Research in Sports Medicine, 17*, 71-83.
- 10.1080/15438620902901474
- Vella, L., Caldow, M.K., Larsen, A.E., Tassoni, D., Gatta, P.A.D., Gran, P., Cameron-Smith, D. (2012). Resistance exercise increases NF- κ B activity in human skeletal muscle. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology, 302*, R667-R673.
- 10.1152/ajpregu.00336.2011
- Vella, L., Markworth, J. F., Peake, J. M., Snow, R. J., Cameron-Smith, D., & Russell, A.P. (2014). Ibuprofen supplementation and its effects on NF- κ B activation in skeletal muscle following resistance exercise. *Physiological Reports, 2*(10), 1-11.
- <https://doi.org/10.14814/phy2.12172>
- Vider, J., Laasonen, D.E., Kilk, A., Atalay, M., Lethmaa, J., Zilmer, M., & Seni, C.K. (2001). Physical exercise induces activation of NF- κ B in human peripheral blood lymphocytes. *Antioxidants & Redox Signalling, 3*(6), 111-1137.
- doi n/a
- Viitala, P., & I.J. Newhouse. (2004). Vitamin E supplementation, exercise and lipid peroxidation in human participants. *European Journal Applied Physiology, 93*, 108-115.
- 10.1007/s00421-004-1178-293
- Vollard, N.B., Shearman, J.P., & Copper, C.E. (2005). Exercise-induced oxidative stress: myths, realities and physiological relevance. *Sports Medicine, 35*(12), 1045-1062.

10.2165/00007256-200535120-00004

Wacker, W.E.C., Ulmer, D.D., & Vallee, B.L. (1956). Metalloenzymes and myocardial infarction-malic and lactic dehydrogenase activities and zinc concentrations in serum. *The New England Journal of Medicine*, 255, 449-456.

10.1056/NEJM195609062551001

Waldron, M., Worsfold, P., Twist, C., & Lamb, K. (2011). Concurrent validity and test-retest reliability of a global positioning system (GPS) and timing gates to assess sprint performance variables. *Journal of Sports Sciences*, 29(15), 1613-1619.

10.1080/02640414.2016.608703

Warren, G.L., Lowe, D.A., & Armstrong, R.B. (1999) Measurement tools used in the study of eccentric contraction-induced injury. *Sports Medicine*, 27(1), 43-59.

10.2165/00007256-199927010-00004

Watson, T. A., MacDonald-Wicks, L. K., Garg, M. L. (2005). Oxidative stress and antioxidants in athletes undertaking regular exercise training. *International Journal of Sport Nutrition and Exercise Metabolism*, 15, 131-146.

doi n/a

Weitner, T., Iničč, S., Jablan, J., Gabričević, M., & Domijan, A-M. (2016). Spectrophotometric determination of malondialdehyde in urine suitable for epidemiological studies. *Croatica Chemica Acta*, 89(1), 133-139.

10.5562/cca2902

Williamson, J., Hughes, C.M., & Davison, G.W. (20). Exogenous plant-based nutraceutical supplementation and peripheral cell mononuclear DNA damage following high intensity exercise. *Antioxidants*, 7(5), pii: E70, 1-14.

10.3390/antiox7050070

- Winter, E.M., Jones, A.M., Davison, R.C.R., Bromley, P.D., & Mercer, T.H. (Eds). (2007). *Sport and Exercise Physiology Testing Guidelines: Volume II - Exercise and Clinical Testing. The British Association of Sport and Exercise Sciences Guide*. London: Routledge.
- Woodward, K. A., Draijer, R., Thijssen, D.H.J., & Low, D.A. (2018). Polyphenols and microvascular function in humans: A systematic review. *Current Pharmaceutical Design*, 24, 2.
10.2174/1381612823666171109103939
- Yagi, K. (1976). A simple fluorometric assay for lipoperoxide in blood plasma. *Biochemical Medicine*, 15, 212-216.
10.1016/0006-2944(76)90049-1
- Young, W., Cormack, S., & Crichton, M. (2011). Which jump variables should be used to assess explosive leg muscle function? *International Journal of Sports Physiology and Performance*, 6(1), 51–57.
<https://doi.org/10.1123/ijsp.6.1.51>
- Zhang, J., Sun, Z., Sun, P., Chen, T., & Chen, F. (2014) Microalgal carotenoids: Beneficial effects and potential in human health. *Food and Function*, 5, 413-424.
10.1039/c3fo60607d
- Zhou, X., Fragala, M.S., McElhaney, J.E. & Kuchela, G.A. (2010). Conceptual and methodological issues relevant to cytokine and inflammatory marker measurements in clinical research. *Current Opinion in Clinical Nutrition & Metabolic Care*, 13(5), 541-547.
10.1097/MCO.0b013e32833cf3bc

Appendices

APPENDIX A: Training Log

Training Log

Instructions: Please record all training sessions, being as specific as possible regarding the type, amount and intensity of the exercise. For resistance exercise, please include the weight lifted, number of sets, reps and include length of rest periods. For training intensity please use the training intensity scale or average heart rate (if known):

Please refer to the example below. If you have any questions regarding the training log please email the researcher: c.holland@herts.ac.uk.

Training Intensity Scale:

- 1 = Rest
- 2 = Very Light
- 3 = Light
- 4 = Fairly Light
- 5 = Somewhat hard
- 6 =
- 7 = Heavy (hard)
- 8 = Extremely hard
- 9 = Very hard
- 10 = Maximum effort

Example:

Date commenced: 9.01.2014

Day Time	Type of training (please describe in detail)	Amount/Length (e.g., time, distance, weight lifted, no. of sets & reps)	Training Intensity (& average heart rate if known)	Additional comments
Monday 7pm	Spinning class Abs exercises-sit ups, plank	45 minutes 2 x 20 2 x 1 minute	8	
Wednesday 7.30pm	Football training- Agility drills, shuttle runs, match play	1 1/2 hours	5	

Day Time	Type of training (please describe in detail)	Amount/Length (e.g., time, distance, weight lifted, no. of sets & reps)	Training Intensity (or average heart rate if known)	Additional comments

APPENDIX B: Estimated Food Record

Estimated Food Record

Instructions: Please record all food and drinks eaten, please be as specific as possible regarding the type and amounts of food and drink. Please refer to the example below. If you have any questions regarding the food record please email the researcher: c.holland@herts.ac.uk.

Example:

Day: Monday

Date: 9.1.2012

Supplements: 1 x multivitamin tablet

Time Meal/snack	Food & drink consumed (please describe items in detail)	Amount (household measure e.g., tbsp., tsp.)	Preparation/cooking method	Additional comments
10am breakfast	Orange juice, Tesco value	1 small glass		
	Banana	1 small	Raw, peeled	
	Muesli, Alpen Swiss	1 bowl		
	Milk, skimmed, Tesco organic	Approximately 250ml		

Please start a new page when you start recording for a new day.

Day:

Date:

Supplements:

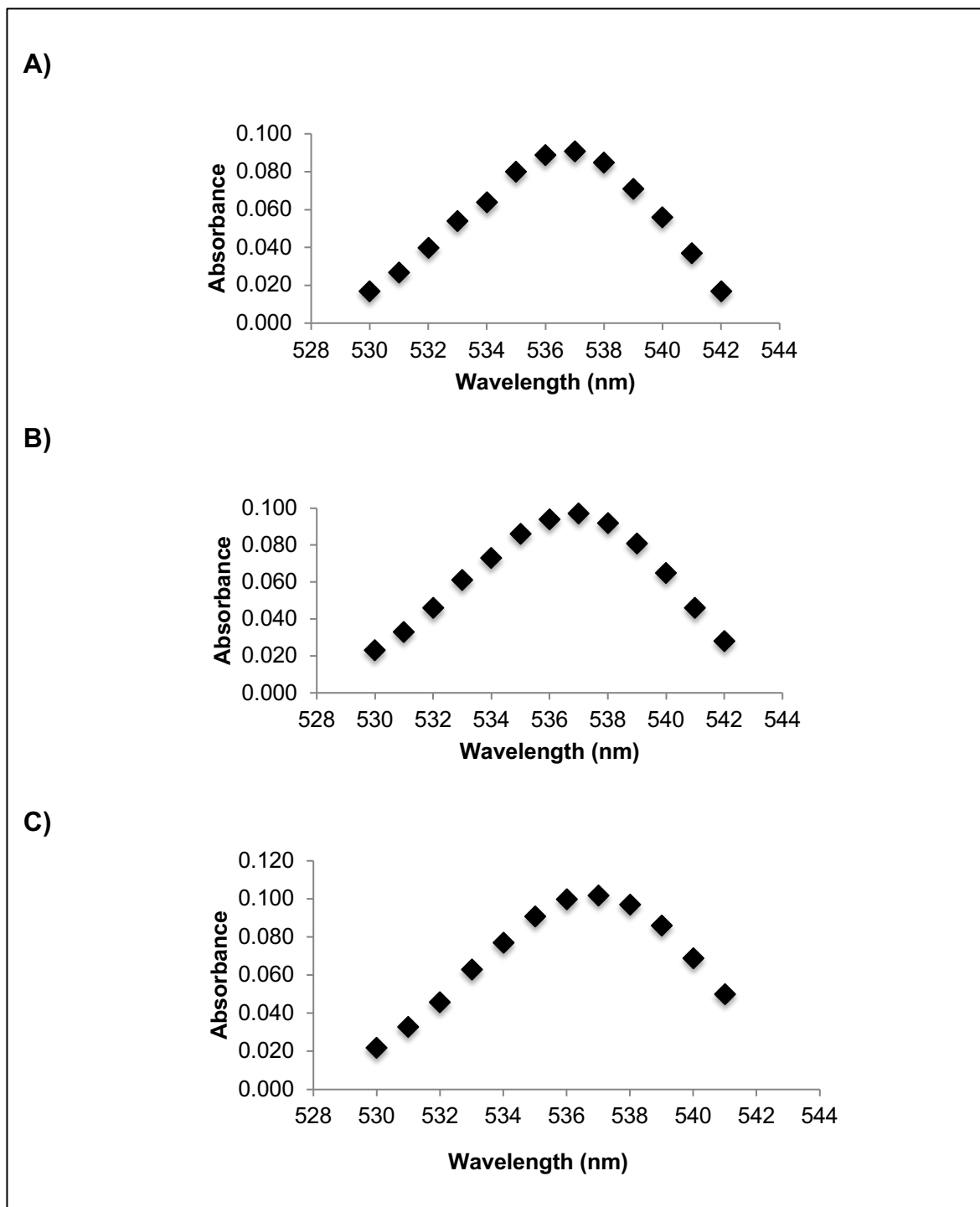
Time Meal/snack	Food & drink consumed (please describe items in detail)	Amount (household measure e.g., tbsp., tsp.)	Preparation/cooking method	Additional comments

Remember to include everything you eat and drink today!

Please start a new page when you start recording for a new day.

APPENDIX C: Spectrophotometer Wavelength Accuracy

Wavelength accuracy was checked throughout chapter 4 (T1: T3) using a certified Holmium oxide glass filter (H5BB, Cecil Instruments, Cambridge: UK) with a peak absorbance at 536.58 (± 0.3) nm; chosen as the filter most closely matching the optimum wavelength for MDA and creatinine (532 nm & 510 nm, respectively).



APPENDIX D: Dietary Antioxidant Questionnaire

Dietary Antioxidant Questionnaire

**INSTRUCTIONS:**

- Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.
- Put a ✓ in the box next to your answer.
- If you make any changes, cross out the incorrect answer and put a ✓ in the box next to the correct answer.
- This questionnaire should take approximately 10 minutes to complete.

Before turning the page please fill in the following:

Today's date:

Date of birth:

How many years have you been involved in the sport?

1. Over the past 1 month, how often did you drink **tomato juice** or **vegetable juice**?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 2) | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

1a. Each time you drank **tomato juice** or **vegetable juice**, how much did you usually drink?

- Less than $\frac{3}{4}$ glass (200mL)
 $\frac{3}{4}$ to $1\frac{1}{4}$ glasses (200-300mL)
 More than $1\frac{1}{4}$ glasses (300mL)

2. Over the past 1 month, how often did you drink **orange juice, apple, pineapple, cranberry** or **grape juice**?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 3) | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

2a. Each time you drank **orange juice, pineapple, apple, cranberry** or **grape juice**, how much did you usually drink?

- Less than $\frac{3}{4}$ glass (200mL)
 $\frac{3}{4}$ to $1\frac{1}{4}$ glasses (200-300mL)
 More than $1\frac{1}{4}$ glasses (300mL)

3. Over the past 1 month, how often did you drink **fruit drinks** containing **blackberry, strawberry, cranberry, raspberry, blackcurrant** or **blueberry**?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 4) | g <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | h <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | i <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | j <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

3a. Each time you drank **fruit drinks** containing **blackberry, strawberry, cranberry, raspberry, blackcurrant** or **blueberry**, how much did you usually drink?

- Less than $\frac{3}{4}$ glass (200mL)
 $\frac{3}{4}$ to $1\frac{1}{2}$ glasses (200-300mL)
 More than $1\frac{1}{2}$ glasses (300mL)

Over the past 1 month....

4. How often did you drink other **fruit drinks** (such as sports drinks or soft drinks, diet or regular)?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 5) | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

4a. Each time you drank other **fruit drinks**, how much did you usually drink?

- Less than $\frac{1}{2}$ bottle (250mL)
 $\frac{1}{2}$ to 1 bottle (250-500mL)
 1 bottle or more (500mL)

4b. How often were your fruit drinks **enriched (added) with vitamin-C**?

- Almost never or never
 About $\frac{1}{4}$ of the time
 About $\frac{1}{2}$ of the time
 About $\frac{3}{4}$ of the time
 Almost always or always
 Don't know

5. How often did you drink hot drinks such as **coffee, black, green** or **oolong tea**?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 6) | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

5a. Each time you drank **coffee, black, green** or **oolong tea**, how much did you usually drink?

- Less than 1 cup (150mL)
 1 to 2 cups (150-300mL)
 3 to 4 cups (450-600-mL)
 More than 4 cups (600mL)

6. How often did you drink **red wine**?

- | | |
|---|--|
| <input type="checkbox"/> Never (go to question 7) | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 1 time per month or less | <input type="checkbox"/> 2-3 times per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 4-5 times per day |
| <input type="checkbox"/> 1-2 times per week | <input type="checkbox"/> 6 or more times per day |
| <input type="checkbox"/> 3-4 times per week | |
| <input type="checkbox"/> 5-6 times per week | |

Questions appear on the next page

Question 6 continues on the next page

6a. Each time you drank **red wine**, how much did you usually drink?

- 1-2 glasses (110-220mL)
 3-4 glasses (330-440mL)
 5-6 glasses (550-660mL)
 More than 7 glasses (770mL)

Over the **past 1 month**.....

7. How often did you drink **beer**?

- Never (go to question 8)
 1 time per month or less
 2-3 times per month
 1-2 times per week
 3-4 times per week
 5-6 times per week
- 1 time per day
 2-3 times per day
 4-5 times per day
 6 or more times per day

7a. Each time you drank **beer**, how much did you usually drink?

- 1/2 pint (110-375mL)
 1 pint (400ml-750mL)
 2 pints (800-1125mL)
 More than 2 pints (1125mL)

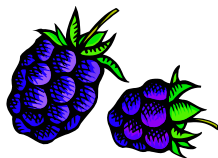
Fruits:

8. How often did you eat **blackberries** or **blackcurrants** (fresh, tinned or frozen)?

- Never (go to question 9)
 1-6 times per year
 7-11 times per year
 1 time per month
 2-3 times per month
 1 time per week
- 2 times per week
 3-4 times per week
 5-6 times per week
 1 time per day
 2 or more times per day

8a. Each time you ate **blackberries** or **blackcurrants**, how many did you usually eat?

- Less than 10 berries
 Between 10 and 20 berries
 More than 20 berries



9. How often did you eat **dried fruit**, such as prunes, raisins or dates?

- Never (go to question 10)
 1-6 times per year
 7-11 times per year
 1 time per month
 2-3 times per month
 1 time per week
- 2 times per week
 3-4 times per week
 5-6 times per week
 1 time per day
 2 or more times per day

9a. Each time you ate **dried fruit**, how much did you usually eat?

- Less than 2 tablespoons
 2 to 5 tablespoons
 More than 5 tablespoons

10. How often did you eat **strawberries**, **blueberries** or **raspberries**?

- Never (go to question 11)
 1-6 times per year
 7-11 times per year
 1 time per month
 2-3 times per month
 1 time per week
- 2 times per week
 3-4 times per week
 5-6 times per week
 1 time per day
 2 or more times per day

10a. Each time you ate **strawberries**, **blueberries** or **raspberries**, how many did you usually eat?

- 1 fruit or less
 1 to 2 fruits
 More than 2 fruits

11. How often did you eat **cranberries** or **cherries**?

- Never (go to question 12)
 1-6 times per year
 7-11 times per year
 1 time per month
 2-3 times per month
 1 time per week
- 2 times per week
 3-4 times per week
 5-6 times per week
 1 time per day
 2 or more times per day

11a. Each time you ate **cranberries** or **cherries**, how much did you usually eat?

- Less than 10 fruit
 10 to 30 fruit
 More than 30 fruit

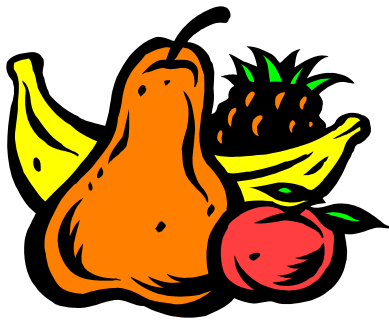
Over the past 1 month.....

12. How often did you eat **plums** or **pineapple** (tinned or fresh)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 13) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1-6 times per year | <input type="checkbox"/> 3-4 times per week |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

12a. Each time you ate either **plums** or **pineapple**, how much did you usually eat?

- Less than 1 plum or less than 1 slice
- Between 2 - 4 plums or 2-4 slices
- More than 4 plums or more than 4 slices



13. How often did you eat **pears**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 14) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1-6 times per year | <input type="checkbox"/> 3-4 times per week |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

13a. Each time you ate **pears**, how much did you usually eat?

- Less than 1
- 1 to 2 pears
- More than 2 pears

14. How often did you eat **oranges** or **kiwifruit**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 15) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1-6 times per year | <input type="checkbox"/> 3-4 times per week |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

14a. Each time you ate **oranges** or **kiwifruit**, how much did you usually eat?

- Less than 1 orange or 2 kiwifruit
- 1-3 oranges or 2-4 kiwifruit
- More than 3 oranges or 4 kiwifruit

Cereals:

Over the past 1 month...

15. How often did you eat **bran (e.g., Bran Flakes)** or **whole grain (e.g., Shredded Wheat)** breakfast cereal?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 16) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1-6 times per year | <input type="checkbox"/> 3-4 times per week |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

15a. Each time you ate **bran** or **whole grain** breakfast cereal, how many did you usually eat?

- 1 small bowl or less
- 1 medium bowl
- 1 large bowl or more

16. How often did you eat **Coco Pops (or other chocolate cereal)**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 17) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1-6 times per year | <input type="checkbox"/> 3-4 times per week |
| <input type="checkbox"/> 7-11 times per year | <input type="checkbox"/> 5-6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2-3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

16a. Each time you ate **Coco Pops (or other chocolate cereal)**, how much did you usually eat?

- 1 small bowl or less
- 1 medium bowl
- 1 large bowl or more



17. How often did you eat **Corn Flakes** or **Rice Krispies** breakfast cereal?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

17a. Each time you ate **Cornflakes** or **Rice Krispies**, how much did you usually eat?

- 1 small bowl or less
 1 medium bowl
 1 large bowl or more

Vegetables:

Over the past 1 month...

How often did you eat **artichokes** or **artichoke hearts**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 19) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

a. Each time you ate **artichokes**, how much did you usually eat?

- 1
 1 to 2
 More than 3

19. How often did you eat **cabbage** (red or white)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 20) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

19a. Each time you ate **cabbage** (red or white), how much did you usually eat?

- Less than a handful
 ½ to 1 handful
 More than 1 handful

Over the past 1 month...

20. How often did you eat **potatoes** (white, red or sweet), regardless of cooking method?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 21) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

20a. Each time you ate **potatoes**, how much did you usually eat?

- Less than 1 medium potato
 1-2 medium potatoes
 More than 2 potatoes

21. How often did you eat **spinach** ?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 22) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

21a. Each time you ate **spinach**, how much did you usually eat?

- Less than 1 tablespoon
 1 to 2 tablespoons
 More than 2 tablespoons

22. How often did you eat **peppers** (red, green or yellow)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 23) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

22a. Each time you ate **peppers**, how much did you usually eat?

- Less than ½ pepper
 ½ pepper to 1 pepper
 More than 1 pepper

Over the past 1 month....

23. How often did you eat **broccoli** (fresh or frozen)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 24) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

23a. Each time you ate **broccoli**, how much did you usually eat?

- 1 spear
 1-2 spears
 More than 2 spears

Other foods:

24. How often did you eat **pecans** or **walnuts**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 25) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

24a. Each time you ate **pecans** or **walnuts**, how much did you usually eat?

- Less than 1 handful
 1 handful
 More than 1 handful

25. How often did you eat **tinned spaghetti** or **baked beans**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 26) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

25a. Each time you ate **tinned spaghetti** or **baked beans**, how much did you usually eat?

- Less than ½ tin
 ½ to 1 tin
 More than 1 tin

26. How often did you eat **milk chocolate** or **dark chocolate**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 27) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

26a. Each time you ate **milk** or **dark chocolate**, how much did you usually eat?

- 1 fun size bar or less
 1 standard size bar
 More than 1/2 block of chocolate

27. How often did you eat **chocolate cake** or **chocolate chip cookies**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 28) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

27a. Each time you ate **chocolate cake** or **chocolate chip cookies**, how much did you usually eat?

- Less than 1 slice or 3 cookies
 1 slice or 3 cookies
 More than 1 slice or 3 cookies

28. How often did you eat **tomato based pasta dishes (e.g., lasagna)** or **pizza** (fresh or frozen)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 29) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

28a. Each time you ate **tomato based pasta dishes (e.g., lasagna)** or **pizza**, how much did you usually eat?

- 1 small serving or small pizza
 1 medium sized serving or medium pizza
 1 large serving or large pizza

Over the past 1 month...

29. How often did you eat **tomato soup (tinned or fresh)**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 30) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

29a. Each time you ate **tomato soup**, how much did you usually eat?

- Less than ½ tin
 ½ to 1 tin
 More than 1 tin

30. How often did you eat **chocolate ice cream**?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 31) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

30a. Each time you ate **chocolate ice cream**, how much did you usually eat?

- Less than 2 scoops
 2 to 4 scoops
 More than 4 scoops

31. How often did you consume **milk** (whole, semi-skimmed or skimmed)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 32) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

31a. Each time you consumed **milk**, how much did you usually eat?

- Less than ¾ glass (200mL)
 ¾ to 1¼ glasses (200–300mL)
 More than 1¼ glasses (300mL)

32. How often did you drink **flavoured milk** or eat **yoghurt** (full or reduced fat)?

- | | |
|--|--|
| <input type="checkbox"/> Never (go to question 33) | <input type="checkbox"/> 2 times per week |
| <input type="checkbox"/> 1–6 times per year | <input type="checkbox"/> 3–4 times per week |
| <input type="checkbox"/> 7–11 times per year | <input type="checkbox"/> 5–6 times per week |
| <input type="checkbox"/> 1 time per month | <input type="checkbox"/> 1 time per day |
| <input type="checkbox"/> 2–3 times per month | <input type="checkbox"/> 2 or more times per day |
| <input type="checkbox"/> 1 time per week | |

32a. Each time you drank **flavoured milk** or ate **yoghurt**, how much did you usually consume?

- Less than ¾ glass (200mL)
 ¾ to 1¼ glasses (200–300mL)
 More than 1¼ glasses (300mL)

33. Please mark any of the following **single herbs** and **spices** you consumed more than once per week either fresh or dried:

- | | |
|-----------------------------------|--|
| <input type="checkbox"/> cinnamon | <input type="checkbox"/> ginger |
| <input type="checkbox"/> basil | <input type="checkbox"/> mustard seeds |
| <input type="checkbox"/> oregano | <input type="checkbox"/> turmeric |

Over the past 1 month...

34. How many servings of **fruit** (not including juices) did you eat per week or per day?

- | | |
|---|--|
| <input type="checkbox"/> Less than 1 per week | <input type="checkbox"/> 2 per day |
| <input type="checkbox"/> 1–2 per week | <input type="checkbox"/> 3 per day |
| <input type="checkbox"/> 3–4 per week | <input type="checkbox"/> 4 per day |
| <input type="checkbox"/> 5–6 per week | <input type="checkbox"/> 5 or more per day |
| <input type="checkbox"/> 1 per day | |

**Supplements:**

The next questions are about your use of antioxidant or vitamin supplements.

35. Over the past 1 month, how often did you take **multivitamins**?

- Never (go to question 36)
 Less than 1 day per month
 1–3 days per month
 1–3 days per week
 4–6 days per week
 Every day

35a. Does your **multivitamin** usually contain **antioxidants** (such as vitamin C, vitamin E or selenium)?

- NO
 YES
 Don't know

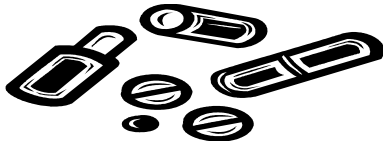
35b. For how many years have you taken **multivitamins**?

- Less than 1 year
 1–4 years
 5–9 years
 10 or more years



35c. Did you take any **vitamins, minerals, or other herbal supplements** other than multivitamins?

- No (go to end of questionnaire)
 Yes (go to question 36)



Over the past 1 month...

36. How often did you take **Beta-carotene** (NOT as part of a multivitamin in Question 35)?

- Never (go to question 37)
 Less than 1 day per month
 1–3 days per month
 1–3 days per week
 4–6 days per week
 Every day

36a. When you took **Beta-carotene**, about how much did you take in one day?

- Less than 10,000 IU
 10,000–14,999 IU
 15,000–19,999 IU
 20,000–24,999 IU
 25,000 IU or more
 Don't know

36b. For how many years have you taken **Beta-carotene**?

- Less than 1 year
 1–4 years
 5–9 years
 10 or more years



37. How often did you take **Vitamin A** (NOT as part of a multivitamin in Question 35)?

- Never (go to question 38)
 Less than 1 day per month
 1–3 days per month
 1–3 days per week
 4–6 days per week
 Every day

37a. When you took **Vitamin A**, about how much did you take in one day?

- Less than 8,000 IU
 8,000–9,999 IU
 10,000–14,999 IU
 15,000–24,999 IU
 25,000 IU or more
 Don't know

37b. For how many years have you taken **Vitamin A**?

- Less than 1 year
 1–4 years
 5–9 years
 10 or more years

38. How often did you take **Vitamin C** (NOT as part of a multivitamin in Question 35)?

- Never (go to question 39)
 Less than 1 day per month
 1–3 days per month
 1–3 days per week
 4–6 days per week
 Every day

38a. When you took **Vitamin C**, about how much did you take in one day?

- Less than 500 mg
 500–999 mg
 1,000–1,499 mg
 1,500–1,999 mg
 2,000 mg or more
 Don't know

38b. For how many years have you taken **Vitamin C**?

- Less than 1 year
- 1–4 years
- 5–9 years
- 10 or more years

Over the past 1 month...

39. How often did you take **Vitamin E** (NOT as part of a multivitamin in Question 35)?

- Never (go to question 40)
- Less than 1 day per month
- 1–3 days per month
- 1–3 days per week
- 4–6 days per week
- Every day

39a. When you took **Vitamin E**, about how much did you take in one day?

- Less than 400 IU
- 400–799 IU
- 800–999 IU
- 1,000 IU or more
- Don't know

39b. For how many years have you taken **Vitamin E**?

- Less than 1 year
- 1–4 years
- 5–9 years
- 10 or more years

40. How often did you take **selenium** (NOT as part of a multivitamin in Question 35)?

- Never (go to end of questionnaire)
- Less than 1 day per month
- 1–3 days per month
- 1–3 days per week
- 4–6 days per week
- Every day

40a. When you took **selenium**, about how much did you take in one day?

- Less than 20g
- 21–100g
- 101–200g
- 201g or more
- Don't know

40b. For how many years have you taken selenium?

- Less than 1 year
- 1–4 years
- 5–9 years

10 or more years



END

Thank you **very much** for completing this questionnaire!

Please take a moment to review each page.

APPENDIX E: DAILY ANALYSIS OF LIFE DEMANDS IN ATHLETES

QUESTIONNAIRE (DALDA)

This questionnaire is designed to monitor your signs and symptoms of psychological and physiological stress.

Each day:

- a) Please respond to the questions of Part A (page 2) and Part B (page 3) by writing the response (a, b or c) on the score sheet for each day (pages 4-10).
- b) For example:

Question 1. *Diet:* Consider whether you are eating regularly and in adequate amounts. Are you missing meals? Do you like your meals?

a = worse than normal b = normal c = better than normal

The athlete has not been eating regularly and has little appetite. Therefore, the athlete would write 'a' (worse than normal) under the relevant day. However, if the athlete had been eating normally, they would write response 'b' (normal) etc.

- c) Please answer as honestly as possible but do not spend too long thinking about each question - give your initial reaction.

This questionnaire should take approximately 2 minutes to complete.

Part A

1. **Diet:** Consider whether you are eating regularly and in adequate amounts. Are you missing meals? Do you like your meals?
2. **Home-life:** Have you had any arguments with your family members? Are you being asked to do too much around the house? How are your relationships with your family members? Have there been any unusual happenings at home concerning your family?
3. **Work:** Consider the amount of work that you are doing there. Are you required to do more or less at home or in your own time? How are your evaluations? Think of how you are interacting with managers.
4. **Friends:** Have you lost or gained any friends? Have there been any arguments or problems with your friends? Are they complimenting you more or less? Do you spend more or less time with them?
5. **Training and exercise:** How much and how often are you training? Are the levels of effort that are required easy or hard? Are you able to recovery adequately between efforts? Are you enjoying your sport?
6. **Climate:** Is it too hot, cold, wet or dry?
7. **Sleep:** Are you getting enough sleep? Are you getting too much? Can you sleep when you want to?
8. **Recreation:** Consider the activities that you do outside of your sport. Are they taking up too much time? Do they compete with your application to your sport?
9. **Health:** Do you have any infections, a cold, or other temporary health problems?

Part B

1. **Muscle pains:** Do you have sore joints and/or pains in your muscles?
2. **Techniques:** How do your techniques feel?
3. **Tiredness:** What is your general state of tiredness?
4. **Need for a rest:** Do you feel that you need a rest between training sessions?
5. **Supplementary work:** How strong do you feel when you do supplementary training (e.g., weights, resistance work, stretching)?
6. **Boredom:** How boring is your training?
7. **Recovery time:** Do the recovery times between each training effort need to be longer?
8. **Irritability:** Are you irritable? Do things get on your nerves?
9. **Weight:** How is your weight?
10. **Throat:** Have you noticed your throat being sore or irritated?
11. **Internal:** How do you feel internally? Have you had constipation, upset stomachs, etc?
12. **Unexplained aches:** Do you have any unexplained aches or pains?
13. **Technique strength:** How strong do your techniques feel?
14. **Enough sleep:** Are you getting enough sleep?
15. **Between sessions recovery:** Are you tired before you start your second training session of the day?
16. **General weakness:** Do you feel weak all over?
17. **Interest:** Do you feel that you are maintaining your interest in your sport?
18. **Arguments:** Are you having squabbles and arguments with people?
19. **Skin rashes:** Do you have any unexplained skin rashes or irritations?
20. **Congestion:** Are you experiencing congestion in the nose and/or sinuses?
21. **Training effort:** Do you feel you can give your best effort at training?
22. **Temper:** Do you lose your temper?
23. **Swellings:** Do you have any lymph gland swellings under your arms, below your ears, in your groin etc?

24. **Likability:** Do people seem to like you?

25. **Runny nose:** Do you have a runny nose?

DALDA SCORE SHEET: Monday

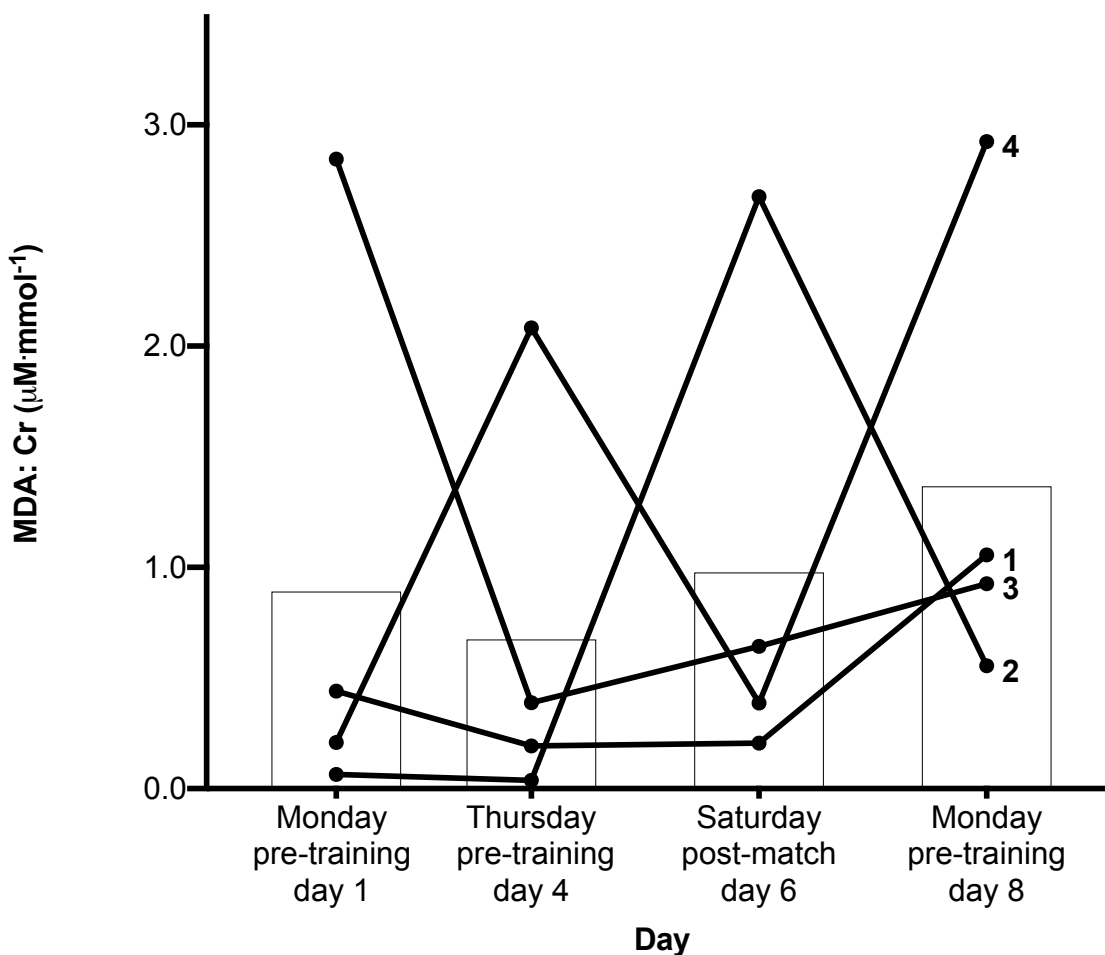
Please **fill in** the appropriate response alongside each item.

a = worse than normal b = normal c = better than normal

<i>Question</i>	Score
<i>PART A</i>	
1. <i>Diet</i>	
2. Home-life	
3. School/college, work	
4. Friends	
5. Sport training	
5. Climate	
6. <i>Sleep</i>	
7. Recreation	
8. Health	
<i>Number of "a" scores</i>	
<i>PART B</i>	
1. <i>Muscle pains</i>	
2. Techniques	
3. Tiredness	
4. Need for a rest	
5. Supplementary work	
6. <i>Boredom</i>	
7. Recovery time	
8. Irritability	
9. Weight	
10. Throat	
11. Internal	
12. Unexplained aches	
13. Techniques/strength	
14. Enough sleep	
15. Recovery	
16. General weakness	
17. Interest	
18. Arguments	
19. Skin rashes	
20. Congestion	
21. Training effort	
22. Temper	
23. Swellings	
24. Likeability	
25. Running nose	
<i>Number of "a" scores</i>	

APPENDIX F: Intra- & Inter-Individual Differences in Lipid Peroxidation

Subgroup analysis of Malondialdehyde (MDA: Cr) concentrations throughout T1 ($n = 4$; experimental group). Lines 1, 2, 3 & 4 represent individual participants.



Urine samples were collected at the following time points:

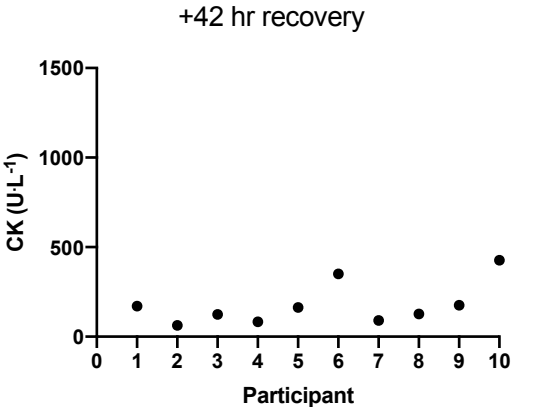
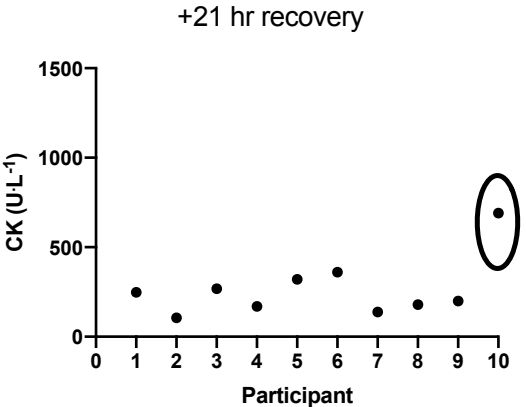
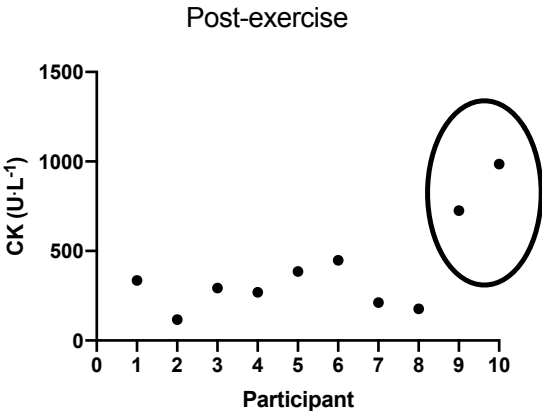
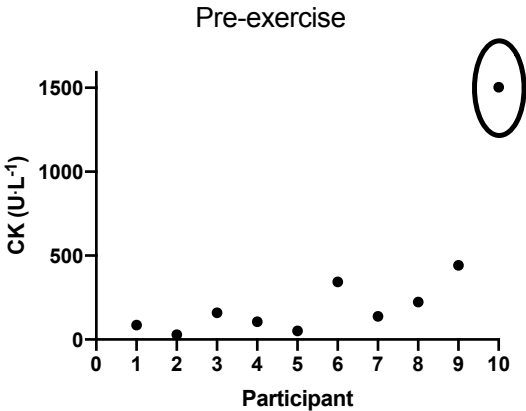
- (i) Baseline: Monday, pre-training, day 1;
- (ii) Mid-week: Thursday, pre-training, day 4;
- (iii) Immediately post-match: Saturday, day 6; and
- (iv) Post-match + 42 hours recovery: Monday, pre-training, day 8.

Inter-individual differences in MDA throughout T1 are presented above. Participant 1 had relatively unchanged MDA concentrations between days 1, 4 and 6 ($0.44 \mu\text{M}\cdot\text{mmol}^{-1}$ vs. $0.19 \mu\text{M}\cdot\text{mmol}^{-1}$ vs. $0.21 \mu\text{M}\cdot\text{mmol}^{-1}$), however, MDA concentration increased approximately five-fold at day 8 from day 6 ($1.06 \mu\text{M}\cdot\text{mmol}^{-1}$), following a match and rest day. Participant 2 had a lower baseline MDA value on day 1 compared to participant 1 ($0.07 \mu\text{M}\cdot\text{mmol}^{-1}$ vs. $0.44 \mu\text{M}\cdot\text{mmol}^{-1}$), this was relatively unchanged at day 4 ($0.04 \mu\text{M}\cdot\text{mmol}^{-1}$), however, increased post-match on day 6 ($2.68 \mu\text{M}\cdot\text{mmol}^{-1}$), decreasing on day 8, but remaining elevated above baseline ($0.56 \mu\text{M}\cdot\text{mmol}^{-1}$). Participant 3 had an increased baseline of MDA on day 1 in comparison to the other participants ($2.85 \mu\text{M}\cdot\text{mmol}^{-1}$ vs. $0.07 - 0.44 \mu\text{M}\cdot\text{mmol}^{-1}$), which decreased at day 4 ($0.39 \mu\text{M}\cdot\text{mmol}^{-1}$), but increased post-match ($0.64 \mu\text{M}\cdot\text{mmol}^{-1}$), increasing further on day 8 ($0.93 \mu\text{M}\cdot\text{mmol}^{-1}$). Participant 4's baseline value was observed as $0.21 \mu\text{M}\cdot\text{mmol}^{-1}$, this increased on day 4 ($2.08 \mu\text{M}\cdot\text{mmol}^{-1}$), decreasing post-match ($0.39 \mu\text{M}\cdot\text{mmol}^{-1}$), however, was elevated on day 8, post-match day plus recovery ($2.93 \mu\text{M}\cdot\text{mmol}^{-1}$) above baseline concentration.

These results demonstrate the cumulative effects of within microcycle training and match play on lipid peroxidation. Following a training session, a training tapering session, and a competitive match (completed on three consecutive days, Thursday - Saturday; session durations: 90; 129; 90 min, respectively), all four participants had increased concentrations of lipid peroxidation when returning to train on the following Monday morning in comparison to mid-week values, with three out of the four participants demonstrating an increase in lipid peroxidation in comparison to the Monday before. These results provided rationale for the 3-day exercise protocol used in chapters 5 and 6.

APPENDIX G: Intra- & Inter-Individual Differences in CK

Oval denotes potential high responders > 500 U·L⁻¹.

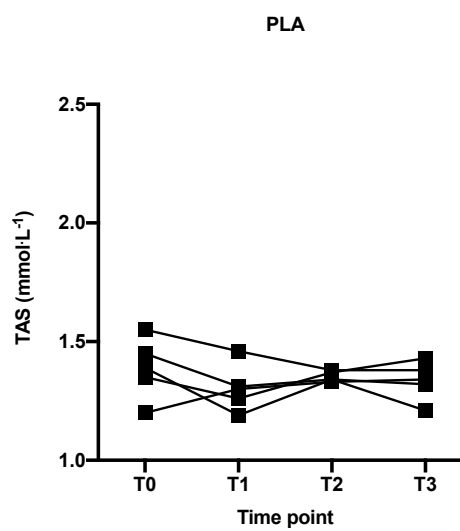
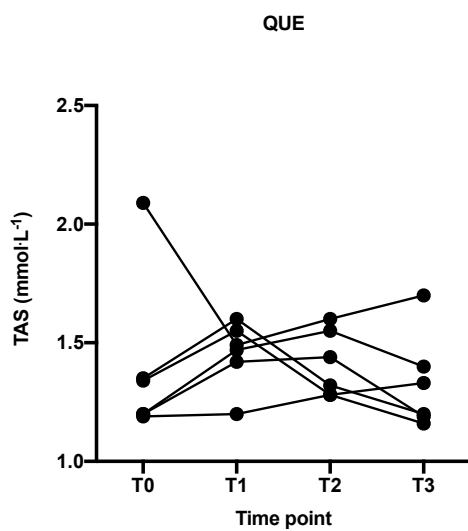
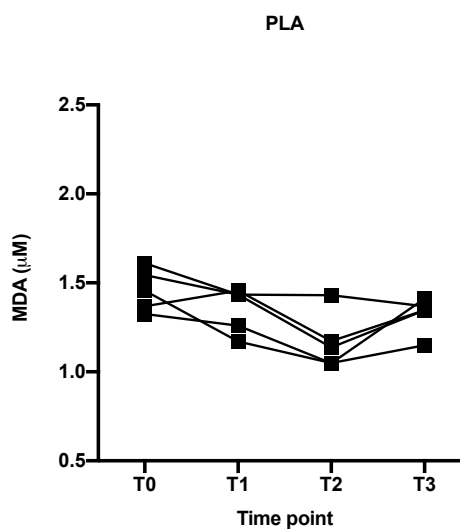
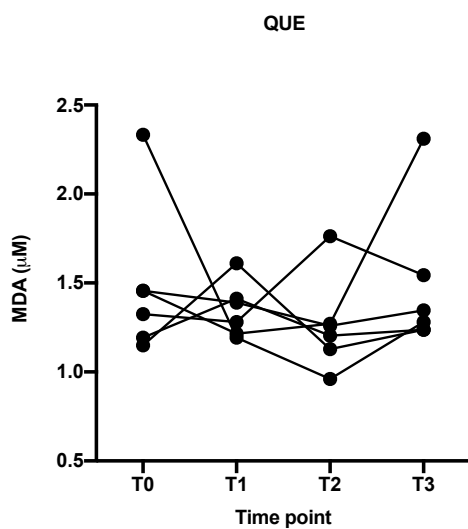


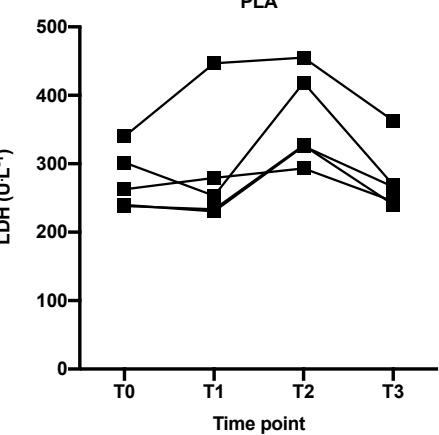
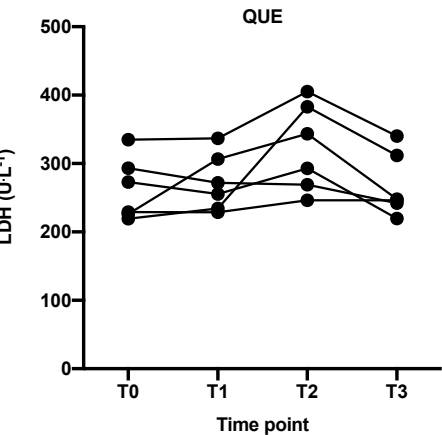
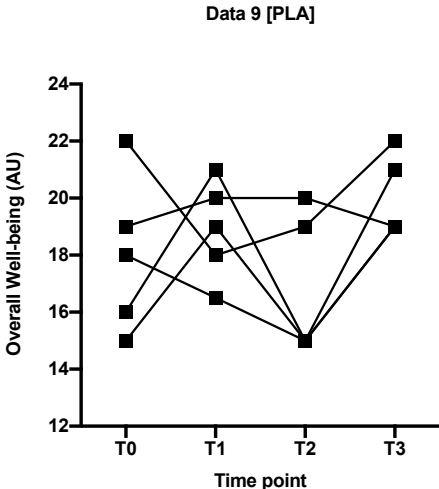
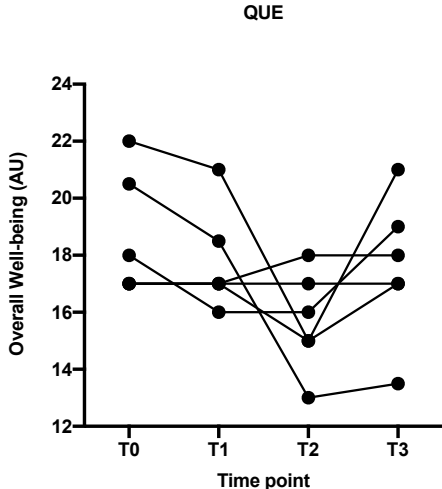
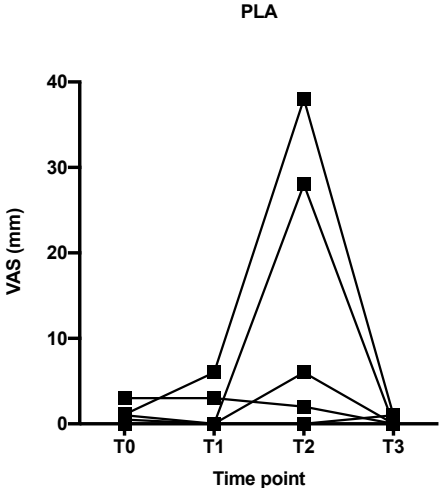
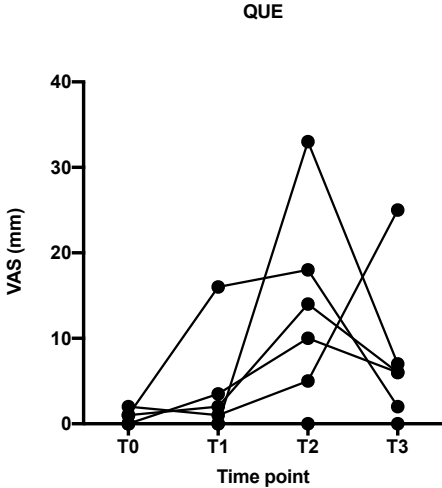
APPENDIX H: Exemplar Perceptual Fatigue Questionnaire (McLean et al., 2010)

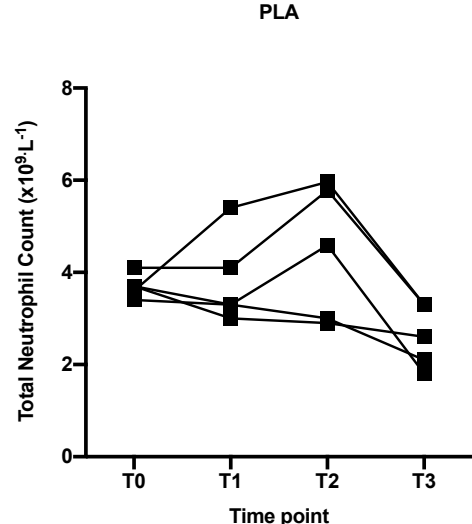
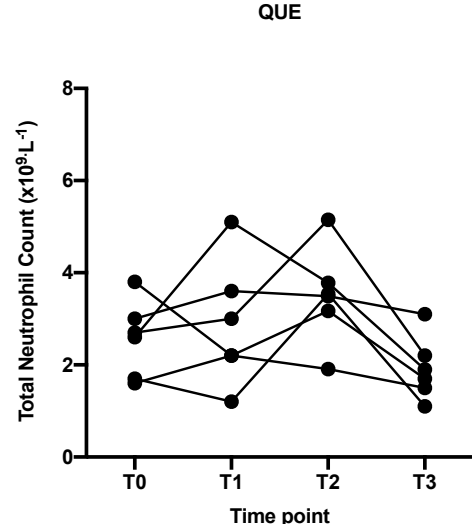
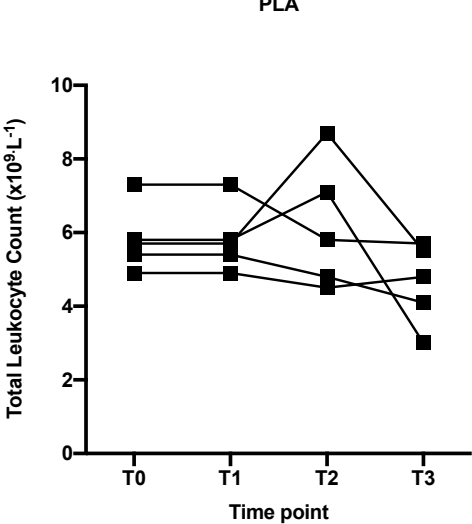
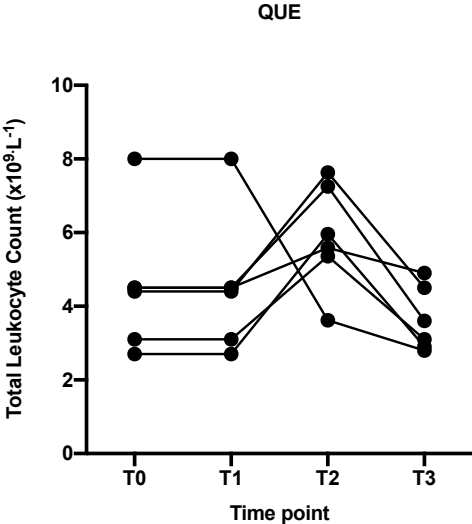
Recovery Questionnaire

	5	4	3	2	1
Fatigue	VERY FRESH	FRESH	NORMAL	MORE TIRED THAN NORMAL	ALWAYS TIRED
Sleep Quality	VERY RESTFUL	GOOD	DIFFICULTY FALLING ASLEEP	RESTFUL SLEEP	INSOMNIA
General Muscle Soreness	FEELING GREAT	FEELING GOOD	NORMAL	INCREASE IN SORENESS/TIGHTNESS	VERY SORE
Sleep Quality	VERY RELAXED	RELAXED	NORMAL	FEELING STRESSED	HIGHLY STRESSED
Mood	VERY POSITIVE MOOD	A GENERALLY GOOD MOOD	LESS INTERESTED IN OTHERS/OR ACTIVITIES THAN USUAL	SNAPINESS AT TEAMMATES/FAMILY/CO-WORKERS	HIGHLY ANNOYED/IRRITABLE/DOWN

APPENDIX I: Intra- & Inter-Individual Differences in Dependent Variables







APPENDIX J: Null Hypotheses

The following null hypotheses were tested in each study in the corresponding experimental chapters 4, 5 and 6. Acceptance or rejection of each null hypothesis is detailed below.

Chapter 4: Assessment of High-Intensity Training Load and Exercise-Induced Lipid Peroxidation in Professional Soccer Players (Study one)

1. H_0 : There will be no change in urinary MDA throughout a competitive in-season in a cohort of professional soccer players. REJECTED.
2. H_0 : MDA concentration will not differ significantly between professional and recreational soccer players. REJECTED.
3. H_0 : Urinary MDA will not be associated with indices of high-intensity training load in a cohort of professional soccer players. ACCEPTED.

Chapter 5: Effects of a Three-Day Period of Prolonged High-Intensity Intermittent Exercise on Biomarkers of Oxidative Stress, Muscle Damage, and Inflammation (Study two)

1. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on TAS or MDA. ACCEPTED.
2. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on CK and LDH. PARTIALLY ACCEPTED (CK).
3. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on NF- κ B p65 activation. ACCEPTED.
4. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on CRP. REJECTED.

5. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on IL-6, IL-8, or MCP-1. REJECTED (IL-6; MCP-1). PARTIALLY ACCEPTED (IL-8).
6. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on IL-10. ACCEPTED.
7. H_0 : The 3-day period of prolonged high-intensity intermittent exercise will have no significant effect on total leukocyte and neutrophil counts. REJECTED.

Chapter 6: Effect of Prolonged Quercetin Supplementation on Blood Biomarkers of Exercise-Induced Cellular Damage and Inflammation Following a Three-Day Period of Prolonged High-Intensity Intermittent Exercise (Study three)

1. H_0 : There will be no effect of prolonged quercetin supplementation, in comparison to a placebo, in attenuating serum LDH concentration following three days of prolonged high-intensity intermittent exercise in trained athletes. ACCEPTED.
2. H_0 : There will be no effect of prolonged quercetin supplementation, in comparison to a placebo, in attenuating inflammatory indices (CRP, NF- κ B p65 activation, IL-6, total leukocyte & neutrophil counts) following three days of prolonged high-intensity intermittent exercise in trained athletes. ACCEPTED.
3. H_0 : There will be no effect of prolonged quercetin supplementation, in comparison to a placebo, on indices of oxidative stress (TAS; MDA) following three days of prolonged high-intensity intermittent exercise in trained athletes. ACCEPTED.

4. H_0 : There will be no effect of prolonged quercetin supplementation, in comparison to a placebo, on subjective measures of perceived fatigue and muscle soreness following three days of prolonged high-intensity intermittent exercise in trained athletes. ACCEPTED.

APPENDIX K: Cytokine & Growth Factor Responses to the 3-Day Exercise Protocol ($M \pm SD$)

Variable	EXP ($n = 10$)				CON ($n = 10$)				p
	T1	T2	T3	T4	T1	T2	T3	T4	
IL-2 (pg·mL ⁻¹)	2.56 ± 2.28	2.36 ± 2.38	2.69 ± 2.54	2.54 ± 2.66	0.79 ± 1.89	0.81 ± 1.95	0.57 ± 1.19	0.54 ± 1.33	.441
IL-4 (pg·mL ⁻¹)	2.13 ± 1.20	1.99 ± 0.57	2.04 ± 0.73	1.78 ± 0.57	1.20 ± 2.42	0.60 ± 1.05	0.57 ± 1.04	0.73 ± 1.05	.758
IL-1α (pg·mL ⁻¹)	0.29 ± 0.44	0.15 ± 0.15	0.15 ± 0.21	0.24 ± 0.26	0.07 ± 0.10	0.07 ± 0.10	0.08 ± 0.08	0.06 ± 0.06	.504
IL-1β (pg·mL ⁻¹)	1.75 ± 1.88	1.55 ± 1.40	1.55 ± 1.61	1.73 ± 1.95	0.65 ± 1.03	0.42 ± 0.63	0.40 ± 0.44	0.27 ± 0.44	.659
VEGF (pg·mL ⁻¹)	96.01 ± 62.60	86.91 ± 73.80	85.93 ± 74.04	94.22 ± 71.00	90.32 ± 64.92	91.91 ± 73.55	88.92 ± 52.87	77.14 ± 52.91	.413
IFNγ (pg·mL ⁻¹)	0.84 ± 1.52	0.58 ± 1.39	0.94 ± 1.60	0.77 ± 1.38	1.66 ± 3.45	0.41 ± 0.87	0.38 ± 0.57	0.33 ± 0.52	.277
TNFα (pg·mL ⁻¹)	2.37 ± 0.48	2.25 ± 0.68	2.33 ± 0.84	2.39 ± 0.65	2.34 ± 1.24	2.48 ± 1.31	2.25 ± 1.07	2.21 ± 1.28	.550

EGF	60.53 ±	37.86 ±	30.09 ±	42.42 ±	51.32 ±	58.75 ±	44.41 ±	46.64 ±	.441
(pg·mL ⁻¹)	34.80	37.16	17.19	34.04	37.55	40.95	29.74	29.52	

Note. CON = Control group; EXP = Experimental group; IL = Interleukin; EGF = Epidermal Growth Factor; IFN γ = Interferon gamma; n = number of participants; T = time point; TNF α = Tumor Necrosis Factor alpha; VEGF = Vascular Endothelial Growth Factor.