The phytochemical content and bioavailability of beetroot (*Beta Vulgaris* L.) and its application as a recovery intervention following strenuous exercise

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PhD

2016

The phytochemical content and bioavailability of beetroot (*Beta Vulgaris* L.) and its application as a recovery intervention following strenuous exercise

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A thesis submitted in partial fulfilment of the requirements of Northumbria University for the degree of Doctor of Philosophy.
No part of this thesis has been submitted in the past, or is to be submitted for any degree at any other University.

Faculty of Health and Life Sciences

October 2016

Abstract

Athletes and practitioners are continually looking for new strategies that could minimise the negative effects of exercise-induced muscle damage (EIMD) and hasten the recovery process. One strategy that has shown promise in recent years is the use of functional foods rich in phytonutrients. A functional food that has attracted a lot of attention recently, although primarily for its performance enhancing effects, is beetroot (*Beta Vulgaris* L.).In addition to displaying a range of physiological effects that may benefit athletic performance, beetroot also exhibits antioxidant (AOX) and anti-inflammatory effects, both of which could be of benefit for reducing secondary damage and enhancing exercise recovery. Nonetheless, the effect of beetroot on exercise recovery is yet to be systematically investigated in the literature. Thus, the overarching aim of this thesis was to test the efficacy of beetroot supplementation, in the form of beetroot juice (BTJ), as a recovery intervention following strenuous exercise.

The series of investigations that set out to address this aim have led to many novel and interesting findings. To start with, study 1 was the first to show that a commercially available BTJ is a rich source of phytonutrients and therefore possesses a high AOX capacity in comparison to several other fruit and vegetable juices. Secondly, and perhaps the most novel finding in this thesis, was that BTJ showed promise as an efficacious recovery intervention following some bouts of muscle-damaging exercise. Although not a consistent finding throughout this thesis, BTJ was able to improve the recovery of dynamic muscle function and reduce muscle pain after eccentric-heavy exercise. Another important finding was that BTJ, despite being rich in AOXs, did not adversely affect the acute adaptive response to exercise, as measured by the repeated bout effect (RBE). In contrast to the findings in the earlier studies, however, BTJ was not found to be beneficial for recovery after long distance running. Importantly, the final study in this thesis provided the first evidence that BTJ is more beneficial than sodium nitrate (SN) for

enhancing some aspects of recovery. This study highlighted the importance of the phytonuteints in BTJ other than nitrate on recovery after exercise. In summary, the collective findings of this thesis provide new information on the potential application of a phytonutient rich functional food, in BTJ, for recovery from strenuous athletic performance.

Publications

Peer reviewed publications arising from this course of investigation

Clifford, T., Howatson, G., West, D. J., & Stevenson, E. J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients*, *7*(4), 2801-2822.

Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise. *European Journal of Applied Physiology*, *116*(2), 353-362.

Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. (2016). Antioxidant-rich beetroot juice does not adversely affect acute neuromuscular adaptation following eccentric exercise. *Journal of Sports Sciences*, 1-8.

Clifford, T., Constantinou, C. M., Keane, K. M., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The plasma bioavailability of nitrate and betanin from Beta vulgaris rubra in humans. *European Journal of Nutrition*, 1-10.

Clifford, T., Berntzen, B., Davsion, W. G., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The effects of beetroot juice on recovery and performance between bouts of repeated sprint exercise. *Nutrients, 8*(8), 506-523.

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Harper, L. D., **Clifford, T.,** Briggs, M. A., McNamee, G., West, D. J., Stevenson, E., & Russell, M. (2016). The effects of 120 Minutes of simulated match play on indices of acid-base balance in professional academy soccer players. *The Journal of Strength & Conditioning Research*, 30(6), 1517-1524.

Keane, K., George, T., Constantinou, C., Brown, M., **Clifford, T.,** & Howatson, G. (2016). Effects of tart Montmorency cherry (Prunus Cerasus L.) consumption on vascular function in males with early hypertension. *The American Journal of Clinical Nutrition*.

Conference communications and published abstracts during doctoral studies

Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. Beetroot juice as a recovery aid following strenuous eccentric exercise. British association of Sport and Exercise Sciences (BASES) Student Conference, 2015, Liverpool, UK.

***Clifford, T**., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise. European College of Sport Sciences Annual Congress, 2015, Malmö, Sweden.

Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. The effects of beetroot Juice and sodium nitrate on exercise-induced muscle damage. Physiological Society, The Biomedical Basis of Elite Performance, 2016, Nottingham, UK.

Clifford, T., Berntzen, B., Davison, G, W., West, D. J., Howatson, G., Stevenson, E, J. (2016) Effects of beetroot juice on recovery and performance between bouts of repeated sprint exercise. American College of Sports Medicine Annual Congress, 2016, Boston, USA.

Clifford, T. The phytochemical content and bioavailability of beetroot and its application as a recovery aid following muscle-damaging exercise. Beetroot and Nitrate Symposium, Leeds Beckett University, 2016, Leeds, UK.

*Winner of European College of Sport Sciences Young Investigator Award for best mini oral communication.

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List of abbreviations

The following abbreviations have been defined in the text in the first instance.

AA-Fe	H2O2-activated metmyoglobin and free iron
AP-1	Activator protein-1
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AOPP	Advanced oxidation protein products
Antioxidant	AOX
ARE	Antioxidant response element
$A^{\bullet-}$	Ascorbyl radical
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
AU	Arbitrary units
Ca^{2+}	Calcium
CCl_4	Carbon tetrachloride
CCL2	Chemokine (C-C motif) ligand 2
CHO	Carbohydrate
CK	Creatine kinase
CMJ	Counter movement jump
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CV	Coefficient of variation
DMBA	7,12-dimethylbenz[a]anthracene
DMSO	Dimethyl sulfoxide
DOMS	Delayed onset muscle soreness
EC	Excitation-contraction
EDF	Epidermal growth factor
EDTA	Ethylenediaminetetraacetic acid
EIMD	Exercise-induced muscle damage
EPR	Electron paragmagnetic resonance
ES	Effect size
ESR	Electron spin resonance
FGF-2	Fibroblast growth factor
FOX	Ferrous oxidation of xylenol orange
FRAP	Ferric-ion-reducing antioxidant-power assay
GAE	Gallic acid equivalent
GPX	Glutathione peroxidase
GR	Glutathione reductase
Gy	Gray unit
GRO-α,	Regulated oncogene-alpha

GSH	Glutathione (reduced)
GSSH	Glutathione (oxidized)
GST	Glutathione S-transferase
H-BT	High beetroot juice
HPLC	High performance liquid chromatography
HPLC-GS	High pressure liquid gas chromatography/gas spectroscopy
Hs-CRP	High sensitivity C-reactive protein
IGF-1	Insulin like growth factor
ICR	Imprinting control region
IL-1	Interleukin-1
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-10	Interleukin-10
IL-1ra	Interleukin-1 receptor agonist
IL-1-β	Interleukin-1-beta
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-15	Interleukin-15
L-BT	Low beetroot juice
LC	Liquid chromatography
LCMS	Liquid chromatography mass spectroscopy
LDH	Lactate dehydrogenase
LOX	Lipoxygenase
LOX-5	Lipoxygenase-5
LOX-12	Lipoxygenase-12
LIST	Loughborough intermittent shuttle test
LOOH	Lipid hydroperoxides
LSD	Least significant differences
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
mRNA	Messenger RNA
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
MS	Mass spectroscopy
MIVC	Maximum isometric voluntary contraction
MVC	Maximum voluntary contraction
N	Newtons
NAC	N-acetylcysteine
NDEA	N-nitrosodiethylamine
NF-κB	Nuclear factor kappa-B
NGF	Nerve growth factor
NQO1	NAD(P)H:quinone oxidoreductase 1

NOx	Nitric oxide
Nrf2	Nuclear factor (erythroid-derived 2)-like
NSAIDs	Non-steroidal anti-inflammatory drugs
ORAC	Oxygen radical absorbance capacity
PC	Protein carbonyls
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE_2	Prostaglandin E2
PGF_2	Prostaglandin F2
PLA	PLA
PLA_2	Phospholipase A2
PPT	Pressure pain threshold
PRO	Protein
RANTES	Regulated upon activation normal T cell growth
RSI	Reactive strength index
RONS	Reactive oxygen and nitrogen species
RPE	Rate of perceived exertion
RSE	Repeated sprint exercise
RST	Repeated sprint test
RST1	Repeated sprint test 1
RST2	Repeated sprint test 2
RT	Retention time
SERCA	Sarcoplasmic Ca^{2+} ATPase
SD	SD
SN	Sodium nitrate
SSC	Stretch-shortening cycle
SR	Sarcoplasmic reticulum
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive species
TEAC	Trolox equivalent antioxidant capacity
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumour necrosis factor alpha
VAS	Visual analogue scale
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximal volume of oxygen uptake
8-OHdG	8-Hydroxyl-2′-Deoxyguanosine

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisors Prof. Emma Stevenson and Prof. Glyn Howatson for their continued support and guidance throughout this PhD. I could not have asked for a more welcoming, friendly, knowledgeable, and helpful team of supervisors and I will forever be indebted to you both. Despite how busy you both are, you have always bent over backwards to assist me at the drop of a hat, even if it required you to get up before 5am or stay up past 11pm to read over my work or return an email. I have learnt so much from you both, and hope that I continue to throughout the rest of my academic career. I would also like to thank Dr. Dan West for his valuable input on several aspects of my PhD, especially with regards to study design and my published work.

I would also like to thank those who assisted me with data collection or data analysis throughout my PhD; specifically, Prof. Gareth Davison, Dr. Costas Constantinou, Mr. Oliver Bell and Mr. Bram Berntzen. I also wish to thank all of the participants who took part in my studies who, in most cases, endured several days of pain as a result.

I also want to thank everyone in NB431 for their help and friendship in the past few years. I particularly want to thank those in my adopted year group: Dean Allerton, Meghan Brown, Liam Harper, Steven Horsburgh and Karen Keane (Ben Green also) for, firstly, putting up with me and, secondly, their continued assistance and support throughout my PhD.

I am fortunate to have the best friends in the world and would like to thank all of them for their support and love during my PhD, with a special thanks to Ben and Sam. I would also like to thank my family and Jim and Maggie Landry who took me in over 10 years ago and have always treated me as one of their own.

Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Faculty Ethics Committee at Northumbria University.

I declare that the Word Count of this Thesis is 44, 361 words

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Signature:

Date:

1 Introduction

1.1 Introduction

It is now well-established that exercise, particularly if highly strenuous for the individual, can result in ultrastructure damage to skeletal muscle fibres (i.e., myofibrils) and components of the extracellular matrix (ECM) (i.e., desmin and dystrophin) (Beaton, Tarnopolsky, & Phillips, 2002; Friden & Lieber, 2001; Friden, Sjostrom, & Ekblom, 1981, 1983; Hoppeler, 1986; Stupka, Tarnopolsky, Yardley, & Phillips, 2001). The consequences of such damage have also been well described in the literature, and include transient increases in biomarkers of inflammation and oxidative stress (Andersson et al., 2010a; Andersson, Karlsen, Blomhoff, Raastad, & Kadi, 2010b; Close Ashton, McArdle, & Maclaren, 2005; Pizza, Peterson, Baas, & Koh, 2005), muscle soreness (Paulsen et al., 2010; Smith, 1992), impaired range of movement (Howatson, Goodall, & van Someren, 2009; Warren, Lowe, & Armstrong, 1999) and, perhaps of most importance, marked and prolonged declines in muscle function (Ebbeling & Clarkson, 1989; Howatson & van Someren, 2008; Paulsen, Mikkelsen, Raastad, & Peake, 2012).

The aforementioned symptoms of muscle damage are typically more pronounced after exercise that incorporates some form of eccentric contraction, in which a muscle is placed under tension to produce force (Child et al., 1999; Ebbeling & Clarkson, 1989; Paulsen et al., 2012). Because many sports and athletic activities, particularly those that involve the stretch shortening cycle (SSC), such as running, jumping, sprinting, change of direction and decelerating employ high force eccentric muscle contractions, they often result in at least some degree of muscle damage (Byrne, Twist, & Eston, 2004; Duffield, Cannon, & Kind, 2010; Hyldahl & Hubal, 2014; Warhol, Siegel, Evans, & Silverman, 1985).

Despite extensive research efforts, the precise aetiology of muscle damage is not yet completely understood (Hyldahl & Hubal, 2014). However, there is generally an agreement that it probably occurs in two phases (Howatson &

van Someren, 2008; Hubal, Chen, Thompson, & Clarkson, 2008; Toumi & Best, 2003). Indeed, it is widely accepted that physical damage to the muscle ultrastructure and/or impairments in excitation-contraction (EC) coupling are probably the main stimuli responsible for initiating the muscle damage process, i.e., primary damage (Hyldah & Hubal, 2014; Proske & Morgan, 2001; Warren, Ingalls, Lowe, & Armstrong, 2002). Such damage is then thought to trigger a series of biochemical changes that have the capacity to further harm the muscle and connective tissues, a response commonly referred to as secondary muscle damage (Howatson & van Someren, 2008; Hubal et al., 2008; McArdle et al., 1999; Sousa, Teixeira, & Soares, 2013; Toumi & Best 2003). The hallmarks of secondary damage are inflammation and oxidative stress, which typically develop in the hours and days after the damaging event (Nikolaidis et al., 2008; Smith, Kruger, Smith, & Hyburgh, 2008). Although this biological response is innately a beneficial process, triggered to regenerate damaged tissues, somewhat paradoxically, it might also have deleterious effects for muscle function recovery, at least in the short term (Best, Fiebig, Corr, Brickson, & Ji, 1999; Lapointe, Frenette, & Cote, 2002; McArdle et al., 1999; Pizza et al., 2005; Toumi & Best, 2003).

Irrespective of the mechanisms involved, muscle damage remains an obstinate problem for both athletes and recreational exercisers. Not only can the acute force loss affect subsequent performance, but the lingering effects of muscle soreness may heighten injury risk and negatively impact adherence to exercise programs (Howatson & van Someren, 2008; Smith, 1992). Because in many sports athletes are expected to train or compete several times in a weekly cycle (Montgomery et al., 2008; Nédélec et al., 2013), there is an increased need for effective recovery strategies that may help to reduce the magnitude of EIMD in the days after exercise.

In the past two decades, a number of investigations have examined the effects of nutritional or pharmacological supplements on EIMD (see Sousa et al., 2013 for review). Particular interest has focused on the potential benefit

of oral supplements that exhibit AOX or anti-inflammatory effects (Sousa et al., 2013). The potential benefits of these supplements are that they counteract the potentially harmful effects of inflammation and oxidative stress (i.e., the hallmarks of secondary damage) by bolstering endogenous defences when they might be compromised from an exercise or non-exercise stressor (Paulsen et al., 2014b; Nikolaidis, Kerksick, Lamprecht, & McAnulty, 2012a). The majority of studies in this area have focused on the effects of vitamin C and E, which have primarily AOX effects (Nikolaidis et al., 2012a), or non-steroidal anti-inflammatory drugs (NSAIDS), which have primarily anti-inflammatory effects (Schoenfeld, 2012). However, after extensive research, these supplements have been deemed largely ineffective at alleviating symptoms of EIMD, and if taken chronically, or in high doses, might actually have deleterious effects for training adaptations or regeneration (Bjørnsen et al., 2015; Close et al., 2006; Paulsen et al., 2014a, 2014b). In light of these findings, many researchers have shifted their focus onto the effects of other AOX and anti-inflammatory interventions on EIMD, most notably that of functional foods (Sousa et al., 2013). These foods typically refer to fruits and vegetables that are not only naturally rich in anti-inflammatory and AOX phytonutrients, but also possess additional biological effects that may be of benefit in minimising EIMD (Nikolaidis et al., 2012a; Sousa et al., 2013). To date, functional food supplements have shown more promise than vitamin C and E or NSAIDS for enhancing recovery after muscle-damaging exercise (Myburgh, 2014; Sousa et al., 2013). Indeed, cherry (Bell et al., 2014a; Howatson et al., 2010), blueberry (McLeay et al., 2009) and pomegranate juice (Trombold, Barnes, Critchley, & Coyle, 2010; Trombold, Reinfeld, Casler, & Coyle, 2011) have all been shown to help alleviate some of the symptoms of EIMD.

One of most studied functional foods in recent years, in both the clinical and exercise domains, has been beetroot (*Beta Vulgaris L.*). Indeed, there has been an enormous research effort over the past decade to delineate the physiological and biochemical effects that beetroot (and its constituents)

might afford, and how these effects could be exploited to improve health outcomes (Gilchrist et al., 2013; 2014; Joris and Mensink, 2013; Webb et al., 2008) or exercise performance (Bailey et al., 2009; Lansley et al., 2010; Jones, 2014a; 2014b). There is now strong evidence that beetroot juice can positively influence the metabolic and vascular systems, effects that have been largely attributed to its high inorganic nitrate content (Jones, 2014a). Although less well studied, an emerging body of work has also shown that beetroot has AOX and anti-inflammatory properties (El Gamal et al., 2014; Ninfali & Angelino, 2013). These are thought to be mediated by the high number of phenolic and betalainic compounds in beetroot (Esatbeyoglu, Wagner, Schini-Kerth, & Rimbach, 2015; Ninfali & Angelino, 2013). However, as with the aforementioned physiological effects of beetroot, additive and synergistic effects with nitrate, which is found in abundance in beetroot, cannot be ruled out. Furthermore, nitrate, via its conversion to nitric oxide (NOx), has been shown to demonstrate potent anti-inflammatory effects independent of any interactions with phenolic compounds (Jädert et al., 2012; Justice et al., 2015). From an exercise perspective, while beetroot, via a juice beverage, has emerged as an effective and popular ergogenic aid for performance enhancement (Jones, 2014a; 2014b), it is yet to be explored whether BTJ can expedite recovery following strenuous exercise. However, the proposed relationship between inflammation, secondary muscle damage and muscle function loss (Howatson & van Someren, 2008; Pizza et al., 2005; Toumi & Best, 2003), makes the expectation tenable that a phytochemical-rich BTJ has the potential to be a beneficial recovery aid after exercise. Thus, the overarching objective of this thesis was to investigate whether acute supplementation with beetroot, consumed as a juice, can attenuate indices of muscle damage and enhance recovery following different modes of strenuous exercise. Specifically, this idea was addressed in six experimental Chapters, which aimed to:

1) Establish the phytonutrient content of a commercially available BTJ, and the plasma bioavailability of two of its most bioactive components, betanin and nitrate.
2) Examine whether supplementation with BTJ can attenuate indices of muscle damage following an eccentric-heavy exercise task, and whether these effects are dependent on the dose given.
3) Examine the effects of BTJ supplementation on the acute adaptive response, i.e., the RBE to eccentric exercise.
4) Investigate the effects of acute BTJ supplementation on indices of muscle damage and recovery between bouts of repeated sprint exercise in team-sports players.
5) Examine the influence of BTJ on muscle damage and inflammation following a marathon.
6) To compare the effects of a nitrate-matched BTJ and sodium nitrate (SN) drink on indices of EIMD.

2 Literature review

Publication arising from this Chapter: Clifford, T., Howatson, G., West, D. J., & Stevenson, E. J. (2015). The potential benefits of red beetroot supplementation in health and disease. *Nutrients*, *7*(4), 2801-2822.

2.1 Introduction

The objective of this literature review is to highlight the research that has led to formulating the hypotheses to be investigated in this this thesis; whether BTJ is an efficacious recovery aid following strenuous exercise. The literature review begins by describing the aetiological mechanisms associated with EIMD and the deleterious consequences of these effects. The markers typically used to assess the presence and magnitude of EIMD are then discussed. This is followed by a brief discussion on the use of AOX and anti-inflammatory supplements to alleviate EIMD and, where relevant, their potential role in exercise-induced adaptation. The review finishes by highlighting evidence from studies showing the physiological and biological actions of beetroot and its constituents, and aims to provide a rationale for exploring its use as a nutritional intervention for exercise recovery.

2.2 Exercise-induced muscle damage

Typically, EIMD refers to the signs and symptoms that manifest in the days after unaccustomed or especially strenuous exercise; more specifically, either the direct changes at the local level (i.e., sarcomere disruption and EC coupling failure) or, perhaps more commonly, the indirect changes, such as muscle soreness/pain, presence of intramuscular proteins in the blood, and reduced muscle function (Clarkson & Hubal, 2002; Ebbeling & Clarkson, 1989; Hyldahl & Hubal, 2014; Paulsen et al., 2012; Warren et al., 1999).

As alluded to in the introduction, although the precise aetiology of EIMD is not yet fully understood, it has been proposed that it occurs in a biphasic manner (Howatson & van Someren, 2008; Lapointe et al., 2002). The first phase, typically known as the primary event, refers to the mechanical and metabolic damage induced during the exercise task (Howatson & van Someren, 2008). The second phase, typically known as secondary muscle damage, encompasses the ensuing biochemical changes (i.e., inflammatory response) in the hours and days after the primary event (Faulkner, Brooks, &

Opiteck, 1993; Howatson & van Someren, 2008; Lapointe et al., 2002; Smith et al., 2008). Figure 1 provides a general overview of the proposed morphological and biochemical changes associated with EIMD and attempts to differentiate the changes attributed to primary or secondary events. A more detailed discussion of the most relevant processes and the potential mechanisms involved is presented in the following sections.

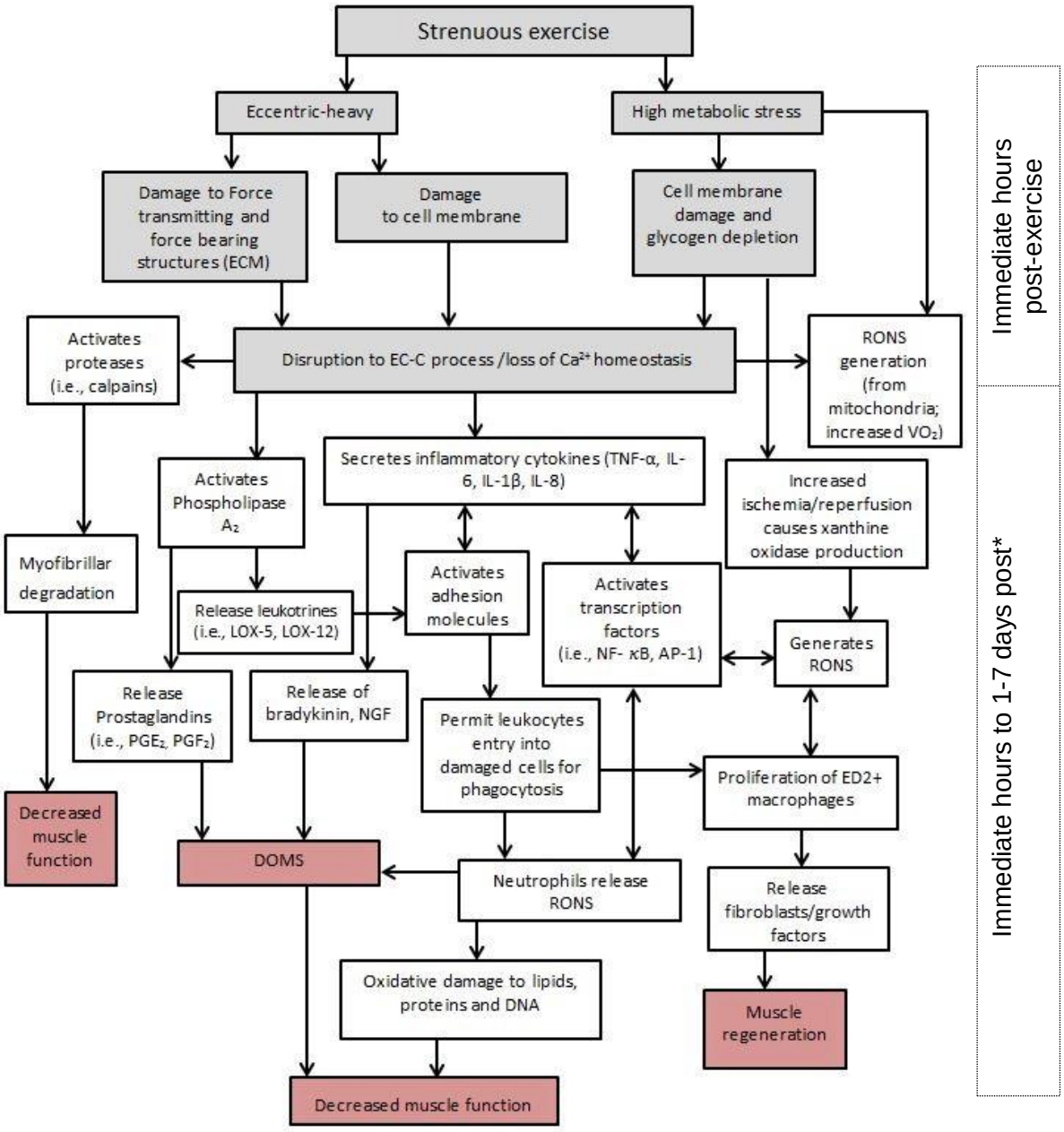

Figure 1 - Overview of the alleged processes that perpetuate muscle damage during and after exercise. Shaded grey boxes represent primary events; where as white boxes represent secondary events. Pink shaded boxes denote the outcome of these processes. *The time course of events and number of days these biochemical processes and symptoms such as DOMS manifest for after exercise is highly dependent on the level of muscle damage sustained (see Paulsen et al., 2012 for a more in-depth discussion).

2.2.1 Primary muscle damage

It is beyond the scope of this thesis to review all the potential mechanisms of how eccentric contractions might cause muscle damage; for this the reader is referred to previous reviews of the topic (Hyldahl & Hubal, 2014; Proske & Morgan, 2001; Warren et al., 2002). Rather, the aim of this section is to give a brief overview of the two main theories that have prevailed to help explain the primary event believed to initiate muscle damage.

The first theory, known as the 'popping sarcomere hypothesis' was originally put forth by Morgan, (1990), and postulates that EIMD is caused by direct mechanical stretch of sarcomeres (Morgan & Proske, 2004; Proske & Morgan, 2001). This theory proposes that when the muscle is actively lengthened, the longer, weaker half-sarcomeres in myofibrils will be recruited first and that repetitive overstretching of these weaker half-sarcomeres (i.e., through repeated eccentric contractions) causes them to weaken to a point where actin and myosin can no longer overlap (Allen, 2001). The overstretched sarcomeres that fail to re-attach will be unable to contribute to further contractions, causing sarcomere non-uniformity and instability at random sections throughout the myofibre (Morgan & Allen, 1999). Because sarcomere lengths are non-uniformly distributed across the fibre, greater mechanical tension is placed on the sarcomeres adjacent to the weaker half sarcomeres and the passive, non-contractile structures, leading to focal disruption within regions of the myofibril (Morgan & Allen, 1999). The increasing number of disrupted sarcomeres then initiates a cascade of events that leads to further physical damage in the ECM and eventually a loss of intracellular Ca^{2+} homeostasis; the cellular event proposed to be largely responsible for the initiation and propagation of secondary muscle damage (Beaton et al., 2002; Gissel & Clausen, 2001; Howatson & van Someren, 2008).

An alternative theory was put forth by Warren and colleagues (2002) who propose that a failure of the EC coupling process is the primary event that initiates EIMD. The first evidence to support this theory was presented by Warren et al. (1993), who found that by exposing muscle fibres (from mice) to caffeine, thereby initiating muscle fibre contraction (by stimulating the release of Ca^{2+} from sarcoplasmic reticulum; SR), they could overcome the decrement in isometric muscle force induced by relatively few (<20) eccentric muscle contractions. This led the authors to propose that the force loss observed after eccentric contractions was due, in large part, to a failure in the EC coupling process up stream of SR Ca^{2+} release (Warren et al., 2002), as opposed to damage to contractile elements (i.e., loss of sarcomere homogeneity) proposed by the popping sarcomere hypothesis. However, Warren and colleagues (2002) do point out that the data to support the EC uncoupling theory has been obtained from investigations using animal models and, therefore, these findings may not directly translate to damage in human skeletal muscle.

As proponents of the popping sarcomere hypothesis, Proske and Allen (2001) countered the view of Warren and colleagues, (1993) and proposed that failure of EC coupling is more likely to be a consequence of the disrupted sarcomeres induced by eccentric contractions, as opposed to the initiating event. Irrespective of the precise mechanisms, and their relative contributions, both sarcomere inhomogeneity and failure of the EC coupling process are observed after muscle damaging exercise and, therefore, the most up to date expert consensus suggests that both are likely to have a prominent role in the manifestation of EIMD (Hyldahl & Hubal, 2014).

2.2.2 Metabolic damage

It has also been suggested that EIMD can arise from metabolic (as opposed to mechanical) stress during exercise (Tee et al., 2007). This is based on studies that observed symptoms of muscle damage (increased serum CK

and force loss) after exercise tasks that do not require significant eccentric muscle contractions, i.e., cycling (Bell et al., 2014; Bell et al., 2015; Saunders et al., 2004). Although they were quite modest rises, Bell et al. (2015), observed increases in plasma CK and markers of inflammation (high sensitivity c-reactive protein; hs-CRP and interleukin-6; IL-6) following a bout of high intensity cycling exercise (109 min). The cycling protocol also precipitated an immediate and significant reduction in isometric strength that had not returned to pre-exercise levels 3 days after. The presence of muscle damage, despite the absence of any significant eccentric muscle contractions, supports the concept that metabolic factors seem to have at least a partial role in precipitating muscle damage following exercise.

The primary mechanisms by which exercise-induced metabolic stress causes muscle damage (in the absence of significant mechanical stress) are not well understood and it is beyond the scope of this thesis to review them in detail. Instead the reader is referred to Tee et al. (2007) who provided a theoretical model to help explain the potential mechanisms involved. Briefly, in their model, Tee and colleagues (2007) postulated that during high intensity exercise, adenosine-triphosphate (ATP) concentrations in the working muscles could be reduced to a point where sarcoplasmic Ca^{2+} ATPase (SERCA) activity is inhibited, preventing the efficient re-uptake of Ca^{2+}. The model goes on to propose that in such instances, a rise in free Ca^{2+} might, in turn, activate cytotoxic molecules with the potential to compromise the integrity of the cell and degrade structural proteins, such as protease enzymes (i.e., calpain) and reactive oxygen and nitrogen species (RONS). There are several studies that report increased RONS production after high intensity cycling exercise that lend some support to this hypothesis (Bell et al., 2014; Bell et al., 2015; Morillas-Ruiz et al., 2005; Nieman et al., 2013). In one such study with 26 trained male cyclists, oxidative damage to proteins, as measured by protein carbonyls (PC), and to DNA, as measured by 8-Hydroxyl-2'-Deoxyguanosine (8-OHdG), was evident following 90 min of cycling at 70% VO_{2max}, suggesting an excess production of potentially

damaging RONS (Morillas-Ruiz et al., 2005). These data also suggest that in exercise with both a high metabolic and mechanical stress, such as field based team-sports and distance running, although the principal cause of EIMD is still likely to be related to mechanical factors (due to the repeated number of eccentric muscle contractions), there is likely to be a contribution from or interactions with metabolic factors.

2.3 Secondary muscle damage

The mechanical and/or metabolic damage outlined in the previous section appears to initiate a series of biochemical alterations that have the capacity to cause further damage to the cell; a phenomenon that has been termed secondary muscle damage (Howatson & van Someren, 2008; Toumi & Best, 2003). The principal initiating event in secondary damage is believed to be the loss of Ca^{2+} homeostasis resulting from sarcolemma damage and opening of stretch activated channels (Armstrong, Warren, & Warren, 1991; Butterfield, Best, & Merrick, 2006; Hyldahl & Hubal, 2014). The disturbance in Ca^{2+} homeostasis then triggers an acute phase inflammatory response to orchestrate the degradation and subsequent regeneration of muscle cells (Armstrong et al., 1991; Toumi, F'Guyer, & Best, 2006) (see overview presented in Figure 1). The inflammatory response is believed to be the main instigator of secondary damage following exercise, and as such will be discussed in further detail below.

1.1.3.2 Inflammation

Exercise-induced inflammation is primarily co-ordinated by a series of intracellular signalling molecules known as cytokines (Cannon & St Pierre, 1998). Cytokines operate by binding to specific receptors on the surface membrane of a target cell and communicating a function (Cannon & St Pierre, 1998). Damaged muscle cells and other cells (i.e., leuckocytes, endothelial cells) synthesize a large number of inflammatory cytokines that are either classed as pro-inflammatory (tumour-necrosis-factor-alpha; TNF–

α, interleukin 1-beta; IL-1β, IL-6, Interleukin-8; IL-8 and interferon-gamma; IFN-γ) or anti-inflammatory (interlukin-1alpha; IL-1α interlukin-4; IL-4 and interleukin-10; IL-10) depending on their primary biological functions in skeletal muscle and other cells (Calle & Fernandez, 2010; Cannon & St Pierre, 1998; Smith, Kruger, Smith, & Myburgh, 2008). The pro-inflammatory cytokines TNF-α and IL-1β play a key role in attracting immune cells to damaged muscle (Butterfield et al., 2006; Smith et al., 2008). More specifically, these cytokines stimulate adhesion molecules, initially those in the selectin family (E-selectin-1 and P-selectin) and then vascular cell adhesion protein 1 (VCAM-1) which, together, help to recruit leukocytes from the circulation into the affected muscle tissue in a process called diapedesis (Butterfield et al., 2006).

The main leukocytes recruited by damaged muscle tissues are neutrophils, which infiltrate muscle tissue immediately following the incitement of muscle damage, and may remain elevated for up to 5 days' post, depending on the intensity and duration of the exercise task (Cannon & St Pierre, 1998; Tidball, 2005; Toumi et al., 2006). Neutrophils are thought to be primarily phagocytic, serving to clear cellular debris and remove necrotic fibres, thus paving the way for the remodelling and regenerative process (Butterfield et al., 2006; Chazaud, 2016). Neutrophils fulfil their phagocytic functions by deploying a range of proteolytic (i.e., proteases) and cytotoxic molecules (i.e., RONS), especially superoxide through means of an oxidative burst (Butterfield et al., 2006; Kharraz, Guerra, Mann, Serrano, & Munoz-Canoves, 2013, Musarò, 2014; Tidball 2005). Furthermore, the invading neutrophils secrete a host of pro-inflammatory cytokines, which attract more neutrophils to the affected area and thus more cytotoxic molecules, forming a positive feedback mechanism that magnifies the local inflammatory response (Butterfield et al., 2006).

It has been proposed, albeit somewhat controversially, that while neutrophil mediated phagocytosis is clearly an important step in the repair process, it

may also exacerbate the existing damage and harm healthy cells; thus, at least in the early stages, phagocytosis might hamper the rate of recovery (Lapointe et al., 2002; Pizza, Baylies, & Mitchell, 2001; Pizza et al., 2005; Tidball, 2005; Toumi et al., 2006). Indeed, in the model of secondary muscle damage proposed by Pizza (2008), neutrophils are suggested to be the main molecules involved, and primarily cause further damage by inhibiting the growth of new myofibrils or degrading undamaged ones, due to the collective activities of RONS (discussed in Chapter 2.1.3.2), proteases and pro-inflammatory cytokines. In some instances, probably depending on the intensity and magnitude of muscle damage sustained, these activities might result in a further or secondary loss in muscle function in the subsequent days (Howatson & van Someren, 2008; Toumi & Best, 2003), although this appears to be absent in many studies (Warren, Call, Farthing, & Baadom-Piaro, 2016). Nonetheless, it has been proposed that regardless of whether or not a further loss in muscle function is observed, the aforementioned biochemical changes might still inflict local muscle damage, secondary to the primary physical damage, and affect the rate or extent of muscle function recovery in the ensuing days (Toumi & Best, 2003; Warren et al., 2016).

There are a number of studies showing a relationship between the early neutrophil response and the magnitude of muscle damage after strenuous exercise or lengthening muscle contractions (Brickson et al., 2003; Pizza et al., 2001; Pizza et al., 2005). In one of these studies, Pizza et al. (2005) found that after a series of muscle damaging eccentric contractions, mice deficient in the integrin CD18 (and therefore who had a blunted capacity to generate neutrophils) experienced less muscle tissue damage and lower force deficits 3 and 7 days' post-contractions compared to non CD18 deficient mice. It is studies such as this, and others reporting attenuations in muscle damage when phagocytosis is blunted (Beaton et al., 2002; Brickson et al., 2003; Lapointe et al., 2002), which has led to the proposal that temporarily suppressing the early inflammatory response after muscle damaging exercise could be useful for enhancing the rate of muscle recovery

(Toumi & Best, 2003). On the other hand, it has also been proposed that this stage of inflammation is a critical step for normal muscle regeneration and, thus, dampening this response might disrupt muscle healing in the long-term (Chauzad, 2015) and/or interfere with training-induced adaptations (Bjørnsen et al., 2015; Paulsen et al., 2014b). The potential for these effects are discussed in section 2.5.

After the invasion of neutrophils in damaged muscle tissue, there is an influx of macrophages, another population of phagocytic immune cells that seem to accumulate in response to the release of chemokines (Chazaud, 2016; Pizza, 2008). The role of macrophages in damaged muscle is far more complex than that of neutrophils though, as they appear to exhibit multiple functions and switch phenotypes according to their surroundings and presence of other cells (Musarò, 2014; Smith et al., 2008; Tidball, 2005). A key distinction between neutrophils and macrophages is that in addition to releasing cytokines and RONS that promote cell lysis, macrophages also produce a series of growth factors that support muscle remodelling (Tidball, 2005).

Whether macrophages have a degradative or beneficial role in muscle damage appears to depend on the functional phenotype activated (Smith et al., 2008). Macrophages have been classified as three distinct populations (ED1+, ED2+, ED3+) that are activated sequentially and play separate roles in the resolution of muscle damage (Butterfield et al., 2006; Musarò, 2014; Smith et al., 2008). The ED1+ subtypes are the first macrophages to enter damaged fibres (within 24 h) and appear to have mainly phagocytic functions (Butterfield et al., 2006). They also act as signalling molecules, attracting additional neutrophils and pro-inflammatory cytokines to the damaged site, which serves to magnify the inflammatory response (Kharraz et al., 2013). Thus, the ED1+ population of macrophages are believed to interact with neutrophils to remove cellular debris and therefore may promote muscle degradation (Tidball, 2005). By contrast, the ED2+ and ED3+ subtypes are

not considered to contribute to phagocytosis (Smith et al., 2008). Instead, ED2+ and ED3+ macrophages may play a beneficial role in muscle repair by secreting cytokines such as IL-6 and IL-10, chemokines such as monocyte chemoattractant protein-1 (MCP-1), and growth promoting factors such as insulin like growth factor 1 (IGF-1), transforming growth factor beta 1 (TGF-β1), interlukin-15 (IL-15), fibroblast growth factor (FGF-2), which are essential for healing and myogenesis (Smith et al., 2008; Urso, 2013).

2.1.3.2 Oxidative stress

Under normal metabolic conditions, the biological environment of a cell is considered to be in a state of redox (reduction: oxidation) balance, or, in other words, equilibrium exists between reducing and oxidising molecules (antioxidants and pro-oxidants, respectively) (Kannan & Jain, 2000; Kohen & Nyska, 2002). Molecules capable of oxidation are known as RONS and are continuously generated in cellular metabolism (Kohen & Nyska, 2002). At these low concentrations, RONS play an important role in a diverse multitude of cellular and biochemical processes, including gene expression, cell proliferation, apoptosis and muscular contraction (Baker, Hayden, & Ghosh, 2011; Powers, Nelson, & Hudson, 2011). However, an excess production of RONS, as seen with strenuous exercise (Nikolaidis et al., 2008), can cause an imbalance in redox homeostasis, which gives rise to the condition typically referred to as oxidative stress (Kohen & Nyska, 2002). This imbalance may overwhelm the endogenous AOX defence network leaving DNA, carbohydrate, protein and lipid structures susceptible to oxidation and functional impairments (Madamanchi, Vendrov, & Runge, 2005; Powers et al., 2011; Reuter, Gupta, Chaturvedi, & Aggarwal, 2010). With regards to exercise, these functional disruptions could have important implications for muscle fatigue and muscle damage and, subsequently, a significant impact on recovery and performance (Powers & Jackson, 2008).

Several investigations support the contention that exercise can shift cellular redox balance towards a pro-oxidant state (for the sake of clarity, this shift will be referred to as oxidative stress throughout this thesis). This has been evidenced by directly observing increased production of RONS in the circulation (Close et al., 2004) and muscle (McArdle et al., 1999; Zerba et al., 1990), or through detecting oxidative damage to target molecules such as nucleic acids, protein and lipid structures during and following acute bouts of exercise (Nikolaidis et al., 2008; Nikolaidis et al., 2012b). Interestingly, oxidative stress is not just confined to the blood and/or skeletal muscle after exercise but has also been observed in several other tissues, including the brain, kidney, and liver, suggesting that exercise may perturb redox homeostasis in several cells (Nikolaidis et al., 2012b).

The degree of oxidative stress is dependent on several factors; the duration (short vs. prolonged) and intensity (high vs. low) of the exercise and predominant mode of muscle contraction (eccentric vs. concentric) (Nikolaidis et al., 2008; Nikolaidis et al., 2012b). It is clear that exercise encompassing eccentric muscle contractions produces significantly more RONS than other types of muscle contractions (i.e., concentric and isometric), which is presumably a consequence of the greater muscle damage and, subsequently, greater inflammatory response imposed by eccentric exercise (Bloomer, 2007; Nikolaidis et al., 2008; Nikolaidis et al., 2012b). Interestingly, oxidative stress arising from eccentric muscle contractions appears to be more pronounced 24–96 h following the initial insult (Nikolaidis et al., 2008). In contrast, after exercise that does not generally induce significant muscle damage, in other words, does not contain a significant eccentric component, the acute increase in oxidative stress has often returned to baseline levels ≤24 h post-exercise (Nikolaidis et al., 2008; Nikolaidis et al., 2012b).

It has been suggested that following lengthening muscle contractions, an excess production of RONS might contribute to secondary muscle damage,

leading to a further loss of muscle force (Zerba et al., 1990; McArdle et al., 1999) and an increase in muscle soreness (Lee et al., 2002). In support of such effects, in a study by Zerba et al. (1990), mice treated with an AOX that directly targets superoxide generation (polyethylene glyocl-superoxide dismutase) had less myofibril damage and a smaller loss of muscle force 3 days' post *in situ* lengthening contractions compared to untreated mice. These data suggested that excessive superoxide production resulting from mechanical stress is associated with secondary muscle damage and the magnitude of force loss. Similar findings were found in the previously mentioned study by Pizza et al. (2005), in which they investigated muscle damage, inflammation and oxidative stress in healthy mice and mice deficient in CD18 after *in situ* lengthening muscle contractions. This study found that inhibiting neutrophil accumulation attenuated cellular protein carbonyl content, a marker of oxidative stress, and that this was associated with reduced muscle force deficits 3 days' post exercise. Thus, akin to the findings from Zerba et al. (1990), this study appears to support the concept that oxidant production, which is derived, in large part, from infiltrating neutrophils (Jackson, Vasilaki, & McArdle, 2016; Nikolaidis et al., 2008) contributes, at least to some extent, to the prolonged loss in muscle function after eccentric-heavy exercise.

It is important to note however, that at present, most of the evidence that suggests a more direct relationship between oxidative stress and muscle function loss after exercise has been derived from animal models and, therefore, might not directly translate to humans. In fact, as Nikolaidis et al. (2008) point out, a number of studies suggest that systemic oxidative stress might not be directly related to EIMD in humans, as assessed by changes in muscle function, because the two do not appear to follow a similar time-course in recovery from exercise. Clearly, more work is required to clarify the role of oxidative stress in EIMD.

Other degradative pathways such as the caplain or ubiquitin-proteasome pathway are also significantly upregulated after muscle-damaging exercise (Raastad et al., 2010; Zhang et al., 2008) and thus, are likely to operate parallel to or in conjunction with the above described inflammatory and oxidative stress responses in secondary muscle damage. However, it was not within the scope of this thesis to investigate these pathways and therefore a detailed overview of their potential role in secondary damage has not been discussed. For this, the reader is referred to previous reviews on the topic (Belcastro, Shewchuk, & Raj, 1998; Goll, Thompson, Li, Wei, & Cong, 2003).

2.4 Markers of exercise-induced muscle damage

A number of markers have been used to quantify the presence and magnitude of EIMD (Duffield et al., 2008). These markers are typically classified as either direct: collecting and analysing sections of muscle tissue, or indirect: examining changes in muscle function, muscle soreness, range of movement, limb girth, efflux of intramuscular proteins in the blood, inflammation and oxidative stress (Duffield et al., 2008; Paulsen et al., 2012). It is not within the scope of this thesis to describe all of these markers; this information is available elsewhere (Duffield et al., 2008; Paulsen et al., 2012). Rather, the aim of this section is to provide a general overview of the markers that were used to quantify muscle damage throughout the course of this thesis.

2.4.1 Muscle function

Tests of muscle function that delineate the power or force generating capacity of skeletal muscle are widely considered the best measures for evaluating muscle damage following strenuous exercise (Clarkson & Hubal, 2002; Duffield et al., 2008; Ebbeling & Clarkson, 1989; Paulsen et al., 2012; Warren et al., 1999). Perhaps the greatest advantage of muscle function tests over other markers of EIMD is that muscle function is the most relevant

to athletes, whose principal goal during recovery is to restore optimal muscular performance as quickly as possible (Byrne et al., 2004).

Muscle function is most commonly assessed by monitoring changes in maximal voluntary contractile force (MVC) before and after exercise (Duffield et al., 2008). There are several methods by which MVC has been evaluated but the most common include isometric muscle force (at a fixed range of motion) (Wilson & Murphy, 1996) or concentric and/or eccentric muscle torque (between a given range of motion or at a fixed velocity) (Byrne & Eston, 2002; Byrne et al., 2004; Warren et al., 1999).

Reductions in MVC are thought to give a reliable estimation of the extent of muscle damage induced by an exercise bout (Duffield et al., 2008; Warren et al., 1999). However, the magnitude of change in MVC can vary widely depending on the nature of the exercise task and training status of the individual (Sayers & Clarkson, 2001). On reviewing the literature, Paulsen et al. (2012) found that the largest and most prolonged reductions in MVC occurs after activities that encompass repetitive, high force, eccentric muscle contractions, particularly of isolated muscle groups. It is not uncommon for MVC to be reduced by 25-60% after exercise involving repetitive max eccentric contractions of the elbow flexors or knee extensors (King & Duffield, 2009; Paulsen et al., 2010), and in severe cases, force may not be recovered ≥7 days after the damaging insult (Paulsen et al., 2012). In contrast, Paulsen et al. (2012) reported that other eccentrically-biased exercise tasks, such as downhill running or resistance exercise tend to induce more modest reductions in MVC (10-40%) and are recovered to pre-exercise values by 24-96 h post.

Nevertheless, the use of MVC as a marker of muscle function does have its limitations. Because MVCs are typically performed with isolated muscle groups, and at low velocities, they are unlikely to give a valid reflection of the loss of function associated with dynamic activities (i.e., jumping, sprinting)

that require several muscle groups to work in concert (Byrne et al., 2004; Gathercole, Sporer, Stellingwerff, & Sleivert, 2015; Twist & Highton, 2013; Wilson & Murphy, 1996). To overcome this limitation, several studies have also assessed movements that can be more easily extrapolated to a sporting context, such as jump tests (i.e., countermovement jumps; CMJ or squat jumps) and max effort sprints, which can estimate power output and speed, respectively (Byrne et al., 2004; Gathercole et al., 2015; Twist & Highton, 2013). It therefore seems that in order to get a more complete picture of the magnitude and time course of EIMD, changes in both isometric and dynamic movements should be evaluated.

2.4.2 Muscle soreness

Muscle soreness or muscle pain (used interchangeably throughout this thesis) is another marker frequently used to assess EIMD. In fact, a review by Warren et al. (1999) found that muscle soreness is more commonly used as a marker of EIMD than muscle function. At the time of writing, muscle soreness was measured in 73% of studies investigating EIMD versus 55% for muscle function. With regards to the time course for the development of muscle soreness, it is known to vary considerably between individuals. However, in most cases, it appears ≤24 h post exercise, not immediately post (hence it is often referred to as delayed onset muscle soreness; DOMS), and peaks between 48-72 h post-exercise (Clarkson & Hubal, 2002; Smith, 1992).

Muscle soreness is frequently measured with the use of a visual analogue scale (VAS) (MacIntyre, Reid, & McKenzie, 1995). Typically, a VAS for measuring muscle soreness requires individuals to rate their perceived level of soreness on a scale that has some iteration of ‘no soreness’ and ‘unbearably sore’ at opposite ends of a fixed length spectrum (Howatson et al., 2009; Howatson et al., 2012; Paulsen et al., 2010). Participants can rate their soreness either passively (sitting or standing in a relaxed position) or

actively (while holding a squat position, for example). The simplicity and ease of measurement makes the VAS method attractive for quantifying muscle soreness; however, its usefulness for repeated measurements across days has been questioned (MacIntyre et al., 1995), as participants may recall their previous scores and erroneously gauge their present score according to this response.

Another measure commonly used to quantify muscle soreness is pressure pain threshold (PPT). Typically, PPT represents the first point at which the force or pressure applied to a muscle evokes a sensation of discomfort or pain (Areces et al., 2015). PPT is often measured with a flat headed cylindrical probe attached to a strain gauge, such as an algometer (Borsa, Kaiser, & Martin, 2013; Connolly, McHugh, Padilla-Zakour, Carlson, & Sayers, 2006; Sands, McNeal, Murray, & Stone, 2015). The probe is used to apply force perpendicular to the muscle belly and at a constant rate; the point at which discomfort is first felt is recorded as newtons or kg/force and represents PPT (Areces et al., 2015). Importantly, it has been shown that an algometer can generate valid and reliable measures of PPT when used across consecutive days (Areces et al., 2015; Nussbaum, & Downes, 1998). Furthermore, PPT seems to be sensitive to exercise-induced changes in muscle pain. Indeed, a number of studies have reported marked and prolonged reductions in PPT that can last up 72 h after muscle damaging exercise (Bowtell, Sumners, Dyer, Fox, & Mileva, 2011; Ceci et al., 2015; Close et al., 2006; Connolly et al., 2006). Perhaps its biggest advantage over the VAS method is that it is less subjective for the participant. Thus, PPT seems a useful method for monitoring muscle soreness in the days after muscle damaging exercise.

It is important to note, that irrespective of how muscle soreness is measured, its intensity and time course does not necessarily correspond with changes in histological muscle damage or other indirect markers of EIMD such as muscle function, CK efflux (Duffield et al., 2008; Hyldahl & Hubal, 2014;

Nosaka, Newton, & Sacco, 2002) or inflammation (Crameri et al., 2007; Malm et al., 2004). Consequently, it has been suggested that quantifying muscle soreness is not sufficient to describe EIMD, but should be measured in conjunction with other aforementioned indices (Paulsen et al., 2012). Notwithstanding, it has been demonstrated that muscle soreness or pain might heighten an individual's risk of injury due to pain-related alterations in movement patterns during activity (Smith, 1992), and influence the degree of effort (Fletcher et al., 2016). Thus, evaluating muscle soreness is still considered a useful tool for monitoring recovery and athlete preparedness in the days after exercise.

2.4.3 Intramuscular proteins as markers of membrane damage

Systemic concentrations of the intramuscular proteins, CK, myoglobin, lactate dehydrogenase (LDH) and troponin are often measured as surrogate markers of damage to the muscle membrane (Clarkson & Hubal, 2002). Of these markers, CK is the most frequently measured (Baird et al., 2012; Ebbeling & Clarkson, 1989). As CK was the only marker of membrane permeability measured as part of this thesis the other aforementioned markers will not be discussed. However, it should be noted that the same limitations associated with CK as a marker of EIMD can also be applied to LDH and myoglobin.

Normal plasma CK levels in healthy individuals are low (<175 $IU \cdot ^{-1}$) but in response to muscle damaging exercise can rise substantially (1000-5000 $IU \cdot ^{-1}$), which is presumably part of the reason it is so commonly used to estimate the extent of muscle damage associated with a particular task (Clarkson & Hubal, 2002). The magnitude and time course of exercise induced CK efflux is highly variable (Ebbeling & Clarkson, 1989; Nosaka, Newton, & Sacco, 2002). This is probably because it can be influenced by so many different variables, including an individual's physical characteristics (training status, gender, genetics, fibre type distribution) and the nature of the exercise task

undertaken (intensity, volume, type, muscle group) (Baird et al., 2012). As one would probably expect, exercise that is high intensity and requires repetitive eccentric loading of the muscle appears to evoke the largest and most prolonged increases in plasma CK (Baird et al., 2012). This becomes even more evident when smaller muscle groups such as the elbow flexors are subjected to high force eccentric actions (Paulsen et al., 2010; Paulsen et al., 2012). For example, plasma CK activity following eccentric contractions with the elbow flexors has been shown to reach peak values 96 h post, increasing to 4000–6000 $IU \cdot^{-1}$ (Hirose et al., 2004; Paulsen et al., 2010a). By contrast, plasma CK values following two popular experimental models of muscle damage: downhill running and high volume plyometric activity (i.e., drop jumps, CMJ) is reported to peak 24-48 h post, and evoke more modest increases between 300-850 $IU \cdot^{-1}$ (Dousset et al., 2007; Duffield et al., 2010; Etheridge, Philp, & Watt, 2008; Howatson, Goodall & van Someren, 2009; Howatson et al., 2012).

Despite its widespread measurement, CK has several limitations as a marker of EIMD (Clarkson & Hubal, 2002; Paulsen et al., 2012). Firstly, as with muscle soreness, the level of CK activity does not appear to correlate well with decrements in muscle function following EIMD (Warren et al., 1999). Additionally, the high inter-subject variability associated with CK, even amongst relatively heterogeneous cohorts, makes accurate interpretations difficult (Paulsen et al., 2012). Given these limitations, it is important that CK analysis is accompanied by additional measures that are felt to more accurately reflect the time course and magnitude of EIMD, such as muscle function (Paulsen et al., 2012; Warren et al., 1999).

2.4.4 Measuring exercise-induced oxidative stress

As described in section 2.1.3.2, exercise can induce a transient state of cellular oxidative stress in the days after exercise. Because oxidative stress may be harmful to the cell and contribute to secondary muscle damage, it is

become more common to employ markers of oxidative stress in EIMD studies. In general, oxidative stress is more often measured in blood samples than other biological tissues due to the comparative ease of both collecting the sample and performing the measurement (Margaritelis et al., 2015). However, irrespective of the biological sample, a 'gold standard' method or single biomarker that accurately reflects the oxidative state of an organism or RONS production, particularly *in vivo*, has not been identified (Close et al., 2004; Powers, Smuder, Kavazis, & Hudson, 2010). In an attempt to overcome this, the general consensus is that oxidative stress should be estimated by means of presenting results from a variety of different methods that include both direct and indirect techniques, and different cellular targets such as lipids and proteins (Close et al., 2005; Finaud, Lac, & Filaire, 2006; Jackson, 1999; Powers et al., 2010). In view of this, a wide range of biomarkers have been developed and are presented in Table 1. The most common biomarkers measure oxidative damage to lipids, proteins and DNA, or levels of endogenous enzymatic AOX activity (Powers et al., 2010; Vassalle, Pingitore, De Giuseppe, Vigna, & Bamonti, 2014). Less commonly, the direct assessment of radical production has been employed (Powers et al., 2010). It is beyond the scope of this thesis to weigh up the merits of each individual biomarker, for this the reader is referred to Powers et al. (2010), Jackson (1999) and Finaud et al. (2006). Instead, this thesis will provide a brief discussion of the methods and biomarkers most relevant to this thesis.

Table 1 - Overview of common biomarkers used to assess oxidative stress (Grielberger, Greilberger, & Djukic, 2015; Nikolaidis et al., 2015; Powers et al., 2010; Vassalle et al., 2015).

Target	Biomarker
Protein	Protein carbonyls
	Advanced oxidation products
Lipids	F_2-isoprostanes
	Malondialdehyde (MDA)
	Thiobarbituric acid reactive substances (TBARS)
	Lipid hydroperoxides (LOOH)
	Conjugated dienes
DNA	Comet assay
	8-Hydroxyl-2′-Deoxyguanosine (8-OHdG)
AOX capacity	Trolox equivalent antioxidant capacity (TEAC)
	Ferric-ion-reducing antioxidant-power
	Assay (FRAP)
	Oxygen-radical absorbance-capacity
	Assay (ORAC)
Endogenous AOX	Superoxide dismutase
Enzymes	Glutathione peroxidase
	Glutathione reductase
	Glutathione S-transferase
	Catalase
Free radical	Electro paramagnetic resonance spectroscopy
	(EPR)
	Electron spin resonance (ESR)

It is possible to estimate oxidative stress directly by employing electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy

techniques. These methods can be used to detect the presence of unpaired electrons, that is free radicals (FR) or RONS in a biological system, by measuring the energy they absorb in a magnetic field, also known as a resonance absorption signal (Jackson, 1999). Because of the high level of skill and expense associated with this measurement and difficulty applying *in vivo*, it is rarely assessed in studies investigating the relationship between oxidative stress and EIMD (Close et al., 2005). One of the few studies that was conducted by Close et al. (2004), who studied RONS production in the 72 h after 30 min of downhill running. They found significant elevations in the EPR signal (+122% of baseline) 72 h after the exercise bout, and thus provided important evidence of direct RONS production following muscle damaging exercise in humans.

It is far more common for studies assessing oxidative stress and its relationship to EIMD to employ indirect measures, in which oxidative stress is identified by measuring breakdown products of oxidatively modified biomolecules; normally proteins and lipids (Close et al., 2005; Nikolaids et al., 2015; Powers et al., 2010). In most investigations, the rate of lipid peroxidation in a biological sample, typically muscle or blood, will be measured to indicate oxidative stress (Jackson, 1999). This is due to the fact that lipids, more so the unsaturated than saturated lipid molecules, are particularly susceptible to oxidation and, as a result, generate a variety of oxidation products that are detectable in biological samples (Finaud et al., 2006; Nikolaidis et al., 2008; Powers et al., 2010). Those considered primary breakdown products of lipid oxidation, such as hydroperoxides, have been measured in several EIMD studies (Bell et al., 2014; Bell et al., 2015; Fogarty, Hughes, Burke, Brown, & Davison, 2013) including Chapter 7, and with the exception of isoprostanes, are favoured over the use of most secondary breakdown products, such as TBARS and MDA, which are thought to lack specificity to lipid peroxidation (Jackson, 1999; Finaud et al., 2006). With that said, the measurement of LOOH is not without disadvantages (Powers et al., 2010) and, therefore, it is presently suggested

that the most valid and reliable measure of lipid peroxidation is that of isoprostanes, specifically the F_2-isoprostanes, which are breakdown products of polyunsaturated fat oxidation (Nikolaidis et al., 2015). While F_2-isoprostanes can be measured with immunoassay kits, more specialist techniques such as that of high pressure liquid gas chromatography/gas spectroscopy (HPLC-GS) are required to obtain the most accurate results (Powers et al., 2010; Nikolaidis et al., 2015). These techniques require specialist laboratory equipment, are expensive to run and require a great deal of expertise and therefore their measurement is not always possible (Nikolaidis et al., 2015; Powers et al., 2010).

Another popular approach to assess oxidative stress is by estimating cellular modification to proteins (Powers et al., 2010). In most studies, this is determined by analysing systemic concentrations of protein carbonyls (PC), which are formed via reactions between RONS and amino acids (Powers et al., 2010). Although other methods are available, PC formation is considered to give a reliable indication of protein oxidation (Powers et al., 2010) and has therefore been used extensively as an indicator of exercise-induced oxidative stress (Bowtell et al., 2011; Fogarty et al., 2013; Goldfarb, Garten, Cho, Chee, & Chambers, 2011; Goldfarb, Garten, Waller, & Labban, 2014).

It is important to highlight that although these biomarkers are widely measured, they have limitations in their ability to accurately quantify oxidative stress (Close et al., 2005; Powers et al., 2010). This largely stems from the fact that redox biology is very complex *in vivo,* especially when the influence of diet, age and tissue type are factored in and, therefore, irrespective of the biomarker measured, valid and reliable results are difficult to obtain (Fisher-Wellman et al., 2009; Powers et al., 2010). Indeed, the extremely short half-lives of RONS (~10^{-5} s) and their weak concentrations, means measuring oxidative stress at a specific time point in any tissue type will always be prone to some degree of error (Close et al., 2005; Finaud et al., 2006; Fisher-Wellman & Bloomer, 2009). It is also important to stress that an

increase in RONS after exercise is not necessarily indicative of direct oxidant mediated damage to a specific tissue but could merely be a consequence of the initial damaging stimulus, which in terms of EIMD, could be any number of morphological alterations (Close et al., 2005; Nikolaidis et al., 2008). Given the above discussion, it is clear that to truly quantify the extent of oxidative stress and thus, its relationship to EIMD, is extremely difficult in humans and, as such, results should be interpreted with a degree of caution.

2.4.5 Measuring exercise-induced Inflammation

Examining exercise-induced inflammation is generally performed indirectly, by measuring changes in limb girth due to local oedema (Warren et al., 1999) or, directly, by measuring specific biomarkers from blood or tissue samples. In the last two decades, the majority of studies have favoured the measurement of specific biomarkers, particularly cytokines, leukocytes, and acute phase proteins in the blood; consequently, this section will present a brief discussion of the markers that fall under these categories.

A common marker used to assess exercise-induced inflammation is C-reactive protein (CRP); an acute phase protein secreted by hepatocytes that plays a central role in regulating the systemic inflammatory response (Black, Kushner, & Samols, 2004; Kasapis & Thompson, 2005). The main inflammatory functions of CRP are to stimulate the intra and extracellular cytokine network and activate adhesion molecules, which, in turn, help to facilitate phagocytosis (Black et al., 2004; Kasapis &Thompson, 2005). In addition to being an inducer of cytokines, the initial release of CRP is believed to be modulated by the cytokine network, particularly the pleiotropic IL-6, which is significantly elevated in response to exercise (Black et al., 2004; Kasapis & Thompson, 2005). In addition to IL-6, CRP may also be regulated by IL-1β and leukocytes, both of which are key mediators of the acute pro-inflammatory response to exercise (Black et al., 2004). This would suggest that CRP release during and after exercise is likely related to the

activation of the hepatocyte cytokine network. Because CRP release appears to be sensitive to changes in exercise intensity, duration and muscle damage (Kasapis & Thompson, 2005), and is involved in a range of acute inflammatory processes, it could be considered a useful marker for characterising the general inflammatory response to exercise.

Cytokines are probably the most often measured biomarkers of exercise-induced inflammation. Of these, plasma IL-6 has been measured the most, which could be due to the fact that it is secreted in greater volumes than other cytokines, principally during, and in the immediate hours after exercise (Peake, Della Gatta, Suzuki, & Nieman, 2015a). Indeed, studies report marked but transient increases in IL-6 concentrations after resistance training (Calle & Fernandez, 2010; Izquierdo et al., 2009) and plyometric exercise (Chatzinikolaou et al., 2010) with the largest increases observed after high intensity, long duration endurance exercise (Bell et al., 2014; Brenner et al., 1999; Howatson et al., 2010).

Other cytokines typically measured in EIMD include IL-8, IL-1β and TNF-α which are believed to exert mostly pro-inflammatory functions (Calle & Fernandez, 2010; Smith et al., 2008). Both TNF-α and IL-1β have been implicated in the acute inflammatory response after exercise and are thought to play a role in secondary muscle fibre damage (Liao, Zhou, Ji, & Zhang, 2010; Pizza et al., 2005; Reid and Li, 2001; Smith et al., 2008) and the development of DOMS (Schafer, Sorkin, & Sommer 2003) providing a rationale for their measurement. With that said, the greatest increases in these cytokines are normally seen at the local level (Peake et al., 2015a) or after very intense exercise such as marathon running (Scherr et al., 2011; Shanely et al., 2013), with few studies observing increases systemically in the hours and days after less severe exercise (Paulsen et al., 2012; Peake et al., 2015a). An added layer of complexity with measuring these cytokines is that they also appear to be important for macrophage and fibroblast activation and, therefore, muscle regeneration after EIMD (Paulsen et al.,

2012; Tidball & Villalta, 2010). Thus, while measuring cytokines may be useful for identifying the presence of an acute inflammatory response, their seemingly dual roles in the biological processes associated with EIMD makes it difficult to discern whether they are a cause or merely a consequence of muscle damage (Paulsen et al., 2012). A more in-depth discussion of these cytokines and others used as markers of exercise-induced inflammation can be found in Peake et al. (2015a) and Paulsen et al. (2012).

In some studies, systemic leukocytosis has been measured to assess the inflammatory response associated with EIMD (Malm, Lenkei, & Sjödin, 1999; Paulsen et al., 2005; Paulsen et al., 2005; Risoy et al., 2003; Suzuki et al., 1999; Suzuki et al., 2003). Leukocytosis is typically measured as the total number of leukocytes present in a blood sample, and the proportion of which are neutrophils, lymphocytes or monocytes. Significant increases in leukocyte counts are observed very early after exercise, but are believed to peak 5-6 h post, largely due to the delayed neutrophilia (Malm et al., 2004; Paulsen et al., 2005). In contrast to the systemic response, leukocyte accumulation in the muscle may persist for several days' post-exercise and has even been observed in the muscle up to 7 days after severe eccentric exercise (Paulsen et al., 2010b; Paulsen et al., 2012). Intriguingly, an increase in leukocyte activity, predominately neutrophils, has shown a strong positive correlation with post exercise losses in muscle function, and this is evident in both animal (Pizza et al., 2005) and human models (Paulsen et al., 2005). This suggests that leukocytes are important mediators of secondary muscle damage; consequently, they might present as a useful marker of exercise-induced inflammation and its relationship to EIMD.

2.5 Nutritional supplements as recovery interventions

From the above sections, it is clear that muscle-damaging exercise can result in a host of deleterious consequences for both a recreational exerciser

and an athlete. Consequently, a plethora of interventions have been developed and tested for their ability to minimise the negative effects of muscle damage and enhance recovery. Some of the more popular interventions purported to aid recovery are cold water immersion, heat application, massage therapy, compression garments and nutritional or pharmacological therapies (Howatson & van Someren, 2008; Sousa et al., 2013). With regards to the latter category, branch chain amino acids (Howatson et al., 2012; Kirby et al., 2012; Ra et al., 2013), protein and carbohydrates (Etheridge et al., 2008; Saunders et al., 2004), milk (Cockburn, Hayes, French, Stevenson, & St Clair Gibson, 2008; Cockburn, Robson-Ansley, Hayes, & Stevenson, 2012), NSAIDS (Paulsen et al., 2010a; Vella et al., 2016) and AOX supplementation (Beaton et al., 2002; Bloomer, 2007; Thompson et al., 2001) have all been tested for their efficacy as recovery aids following EIMD, with varying degrees of success. It is not in the scope of this thesis to review all of the nutritional interventions purported to enhance recovery; rather, the aim of following sections is to briefly discuss the main findings from studies that are most relevant to this thesis: AOX and anti-inflammatory supplements. For an all-encompassing review of the recovery strategies used to manage EIMD, or just nutrition based strategies, the reader is referred to Howaston and van Someren, (2008) and Sousa et al. (2013), respectively.

2.5.1 AOX and anti-inflammatory supplementation

The potential benefits of consuming AOX and/or anti-inflammatory supplements is cellular protection against exercise-induced increases in inflammation and RONS, which, as described in the above sections, has the potential to cause secondary muscle damage (Paulsen et al., 2014b; Peake & Suzuki, 2004). To test whether this holds true, the vast majority of studies have administered either high doses of nutritional AOXs, such as vitamin C and/or E (Close et al., 2006; Jakeman & Maxwell, 1993; Paulsen et al., 2014b) and N-acetylcysteine (NAC) (Cobley, McGlory, Morton, & Close,

2011) or anti-inflammatory medications, such as NSAIDS (Lanier, 2003; Paulsen et al., 2010a). Despite the high interest in these supplements, and their widespread use amongst athletic populations (Tscholl, Vaso, Weber, & Dvorak, 2015; Warden, 2010), evidence to support their effectiveness in protecting against muscle damage is equivocal. While some studies have reported beneficial effects with these supplements, including lowered muscle soreness with NSAIDS (Paulsen et al., 2010a) and a faster recovery of muscle function with NAC (Cobley et al., 2011) and vitamin C (Jakeman & Maxwell, 1993), the majority report no benefits (Bailey, Williams, Betts, Thompson, & Hurst, 2011; Beaton et al., 2002; Paulsen et al., 2014b; Theodorou et al., 2011).

Interestingly, some studies have actually reported negative effects with these supplements, particularly with regard to exercise-induced adaptations (Bjørnsen et al., 2015; Child, Jacobs, Kaminski, Halliwell, & Leeuwenburgh, 2001; Close et al., 2006). Indeed, there is a growing concern that dampening exercise-induced oxidative stress and/or inflammation could actually mitigate or at least lessen some of the physiological adaptations evoked by training (Gross & Baum, 2015). This is due to the evidence that RONS function as cell-signalling molecules, and their production is required for stimulating molecular pathways necessary for improved aerobic capacity (i.e., peroxisome proliferator-activated receptor-γ coactivator; PCG1-α) and hypertrophy and strength (i.e., mitogen-activated protein kinases; MAPK) (Gross & Baum, 2015; Paulsen et al., 2014a). This has led many to posit that instead of positively influencing exercise performance, as originally proposed, AOXs and anti-inflammatory supplements negatively affect performance, a consequence of suppressing RONS-induced adaptive cellular pathways (Gomez-Caberra et al., 2015; Paulsen et al., 2014a). Nonetheless, this is not a view supported by other research groups (Higashida, Kim, Higuchi, Holloszy, & Han, 2011).

As this topic continues to be hotly debated in the literature and formed the basis of the rationale behind Chapter 6, it was felt that a discussion of the main issues should be presented. However, because of the plethora of studies addressing this phenomenon, both in animals and humans, and with a variety of different supplements and doses, a full review of this topic was beyond the scope of this thesis. For a more in depth discussion on this topic the reader is referred to the reviews by Peake et al. (2015b), Gomez et al. (2015) and Hyldahl, Chen, & Nosaka, 2017). Instead, this section will only focus on the main findings and current knowledge regarding the effects of vitamin C and E and NSAIDS in humans only, with reference to their effects on the RBE, endurance performance, strength and hypertrophy.

2.5.2 Effects of AOX and anti-inflammatory supplements on the repeated bout effect

The acute adaptive response to muscle-damaging exercise is classically illustrated by the RBE, in which the magnitude of damage (i.e., strength loss, muscle soreness) evoked by the same or a similar exercise stimulus is lessened in a subsequent bout performed up to 6 months later (Hubal et al., 2008; McHugh, 2003; Nosaka & Clarkson, 1995; Pizza et al. 2001). The precise mechanisms to explain the RBE have still not been fully elucidated, but it is generally assumed that they are related to adjustments in neural, mechanical and/or cellular pathways (McHugh, 2003; Nosaka & Aoki, 2011; Hyldahl et al., 2017).

In terms of cellular pathways, exercise-induced inflammation, including RONS production, has been proposed as one of the mechanisms driving the adaptations reflected by the RBE (Nosaka & Aoki, 2011). The premise is that the transient increase in inflammation and RONS act as cell signals for transcription factors such as NF-kB, which directly transcribes most gene targets associated with an inflammatory response, including cytokines, chemokines, apoptotic and phagocytic cells (Hubal et al., 2008; Pizza et al.,

2002; Xin, Hyldahl, Chipkin, & Clarkson, 2013). The subsequent up-regulation of these specific genes might reinforce host defence so that in future exercise bouts muscle cells are less susceptible to inflammatory-mediated damage, in other words, secondary muscle damage, that might depress muscle force (Hubal et al., 2008; Pizza, Koh, McGregor, & Brooks, 2002; Xin et al., 2013). In support of this, studies have shown that neutrophil invasion (Pizza et al., 2001; Pizza et al., 2002), cytokine secretion (i.e., IL-6, IL-8) (Smith et al., 2008), NF-kB activation (Xin et al., 2013) and oxidative damage to biomolecules (Nikolaidis et al., 2007) are all increased after an initial bout of muscle damaging exercise, but attenuated when the bout is repeated shortly after. This would suggest that the immune and oxidative stress response to EIMD adapts after just a single bout of exercise. Ostensibly, such adaptations might help to make skeletal muscles and the ECM more resistant to EIMD in future bouts (Hyldahl & Hubal, 2014).

Based on the above evidence, it seems logical to assume that AOX and or anti-inflammatory supplements could supress the production of RONS and inflammatory mediators that help to promote a RBE. In this scenario, the neuromuscular adaptations typically associated with the RBE, such as lowered muscle soreness and faster recovery of muscle function, would be attenuated when consuming these supplements. At present, this possibility has only been tested by a few studies. In one of these, healthy but untrained male and female volunteers, given 400 $mg \cdot day^{-1}$ of NSAIDs (celecoxib) for 9 days after eccentric elbow flexor exercise, showed no signs of maladaptation compared to a PLA group when the same bout was performed 3 weeks later (Paulsen et al., 2010a). Indeed, muscle function, muscle soreness, CK efflux and leukocyte accumulation were similarly attenuated in bout 2 compared to bout 1 for both groups, indicating that an RBE had occurred, irrespective of supplementation. Another study investigated the effects of supplementation with vitamin C (1000 $mg \cdot d \cdot^{-1}$) and E (400 $I \cdot U^{-1}$) versus a PLA on the RBE to downhill running (40 min at −10% grade) (He, Hockemeyer, & Sedlock, 2015). After bout 1, supplementation showed beneficial effects, reducing

soreness in the quadriceps and tibialis anterior by ~45 and ~60%, respectively; however, the authors did not detect any adverse effects of supplementation on the RBE. This was indicated by the similar attenuations in muscle soreness and plasma CK in bout 2 compared to bout 1 for both the supplementation and PLA group. Based on the available data, there is insufficient evidence that vitamin C and E or NSAIDs interfere with the RBE in humans. However, further work with different exercise paradigms and supplements are needed to gain a more in-depth understanding of the potential role of AOX or anti-inflammatory containing supplements in acute exercise adaptation.

2.5.3 Effects of AOX and anti-inflammatory supplements on endurance adaptations

The observation that AOXs might interfere with mitochondrial biogenesis (Gross & Baum, 2015; Paulsen et al., 2014a) raises the possibility that these supplements could blunt training-induced adaptations important for endurance performance, like an enhanced VO_{2max}, for example. This possibility has been the subject of several recent investigations (Paulsen et al., 2014a; Peake et al., 2015b; Yfanti at al., 2010). The vast majority of studies in this category have been conducted with vitamin C and E as opposed to NSAIDS, which are more commonly used for their analgesic properties after muscle-damaging exercise. Although there is evidence that vitamin C and E might blunt training-induced changes in aerobic capacity of rats (Gomez-Cabrera et al., 2008; Peake et al., 2015b), there is currently a lack of evidence supporting similar impairments with AOX supplements in humans (Yfanti et al., 2010; Paulsen et al., 2014a; Peake et al., 2015b). For instance, improvements in VO_{2max} and maximal power output in response to 12 weeks of cycling training were similar between groups administered either a PLA or vitamin C (500 mg·day^{-1}) and E (400 IU·day^{-1}) (Yfanti at al., 2010). In another study, although vitamin C and E supplementation (1000 mg·day^{-1}) and E (400 IU·day^{-1}) suppressed the training induced increase in PGC-1α, a

key marker of mitochondrial biogenesis, no changes in VO_{2max} or endurance capacity were detected between the PLA and vitamin groups (Paulsen et al., 2014a). A recent review by Peake et al. (2015b) concluded that while vitamin C and E appear to blunt some molecular changes induced by endurance type exercise, evidence that this translates to changes at the performance level, at least in humans, is lacking.

2.5.4 Effects of AOX and anti-inflammatory supplements on strength adaptations

The supposition that AOXs or NSAIDS might interfere with endurance adaptations, sparked an interest in their effects on long-term adaptations associated with resistance training or eccentric-heavy exercise, such as muscle hypertrophy and strength (Paulsen et al., 2014b). With regards to NSAIDS, the interest in their potential to blunt such adaptations stems from the fact that when taken acutely alongside eccentric-heavy exercise, as is commonly done in an attempt to reduce EIMD (Tscholl et al., 2015), they can dampen anabolic signalling (Trappe et al., 2002; Markworth, Vella, Figueiredo, & Cameron-Smith, 2014) and reduce satellite cell activity (Mikkelsen et al., 2009). Such effects are believed to be due to NSAIDS inhibitory effect on the pro-inflammatory cyclooxygenase (COX) pathway, especially the blunting of the cyclooxygenase-1 (COX-1) isoform, which is a key precursor enzyme for prostaglandin F_2 formation (PGF_2) (Mikkelsen, Helmark, Kjær, & Langberg, 2008; Trappe et al., 2006). Nonetheless, not all studies have found negative effects with NSAIDS (Paulsen et al., 2010a; Peake et al., 2015b; Vella et al., 2016) and, in fact, a recent study suggested that they might actually enhance satellite cell activity and muscle regeneration following a severe bout of muscle-damaging exercise (Mackey et al., 2016).

Furthermore, although in some instances NSAIDS appear to blunt molecular signals that are believed to promote gains in hypertrophy and strength,

evidence demonstrating that these adaptations are inhibited in humans, is currently lacking (Peake et al., 2015b; Schoenfield et al., 2012; Trappe & Liu, 2013). In fact, more recent studies suggest that NSAIDS may actually induce positive adaptive effects, at least in elderly populations. For instance, Petersen et al. (2011) administered NSAIDS or a PLA to older, osteoarthritic adults and measured changes in hypertrophy and strength after 12 weeks of strength training. No group differences in cross sectional area in the quadriceps were detected, but gains in isometric leg strength were greater for the NSAID group. These data are supported by another study, in which acetaminophen or ibuprofen (NSAIDS) supplementation evoked greater improvements in 1 repetition maximum leg strength and increases in quadriceps hypotrophy than a PLA following 12 weeks of leg extensor exercise in healthy, older adults (>60 y old) (Trappe et al., 2011). These effects have not been shown in younger adults however, who, in an earlier study (Krentz, Quest, Farthing, Quest, & Chilibeck, 2008), were shown to experience similar gains in hypertrophy and strength after 6 weeks of resistance training while taking either NSAIDS (ibuprofen) or a PLA after training sessions. Given the above discussion, it seems that while NSAIDS might blunt some acute molecular responses to eccentric-exercise, they do not impair long-term adaptations (or there is insufficient evidence for this at present) and, at least in elderly individuals, they might augment some of these adaptations. For a more in depth discussion on the effects of NSAIDS on training induced adaptations the reader is referred to the reviews by Peake et al. (2015b), Schoenfeld (2012) or Trappe & Liu, (2013).

The influence of AOXs on strength training adaptations was first investigated by Theodorou et al. (2011), in which healthy young males were administered either vitamin C or E a PLA in conjunction with a 4 week resistance training program. Training-induced improvements in muscle torque were similar between the PLA and vitamin supplemented groups, as were changes in redox biomarkers, leading the authors to conclude that AOXs had no effects on muscle performance following resistance exercise. In a more recent

study, Paulsen et al. (2014b) tested whether vitamin C and E supplementation in conjunction with a 10 week resistance training program would prevent gains in hypertrophy and strength in healthy males. Vitamin supplementation had no effect on hypertrophy, or lower body muscle strength; however, upper body strength was lower in the AOX vs. PLA group, suggesting that it might have interfered with normal adaptations in these muscle groups. Another study from the same group provided further evidence that AOXs might blunt strength-training adaptations. Bjørnsen et al. (2015) administered daily doses of vitamin C and E to older adults (≥60 yrs) during a 12 week strength training program and measured changes in body composition and strength in comparison to a PLA group. Although there were no group differences in strength parameters, gains in lean mass were significantly lower in the vitamin C and E group vs. the PLA group (1.4 vs. 3.9%). The authors speculated that these results could have been due to the effects of the AOXs on RONS-induced signalling pathways associated with muscle hypertrophy (i.e., MAPK) given they were shown to be down-regulated after 11 weeks of vitamin C and E supplementation in their previous study (Paulsen et al., 2014b). Thus, the available evidence suggests that vitamin C and E may impair some of the favourable adaptations that accompany strength training and, therefore, individuals, especially athletes, should be cautious about using them chronically. Nevertheless, it is important to take into consideration that the doses of vitamin C and E used in these studies are very high (~5-10 × the recommended daily allowance; RDA) and provided over 10-12 week periods; as such, these findings cannot be interpreted as evidence that lower doses of these supplements, taken acutely, will have the same effects. In support of this notion, Paulsen et al. (2014b) found that acute recovery of muscle function was unaffected by vitamin C and E supplementation in the 72 h following 4 sets of 10 leg press and knee extension exercise. The potentially differing effect of acute vs. chronic AOX supplementation on exercise adaptations requires further research.

2.5.5 Hormesis

From the above discussion, it is clear that research into the effects of AOXs and NSAIDS on exercise-induced adaptations is equivocal. Perhaps this is because in some instances, a modest increase in RONS and inflammation may be beneficial and, therefore, countermeasures detrimental (i.e., adaptation), while in other scenarios, the opposite may be true (i.e., muscle damage or disease). This apparent dual function of RONS and inflammation may be partly explained by the hormesis theory (Peake et al., 2015b; Radak, Chung, Koltai, Taylor, & Goto, 2008). The hormesis theory refers to Hans Seyle's posit almost 70 years ago, in which he suggested that exposing a biological system to a low/moderate stress will elicit a beneficial effect, but over or excessive exposure to the same stress would have a deleterious effect (i.e., an inverted U-shape pattern) (Nikolaidis et al., 2012b; Peake et al., 2015b). Applying the hormesis theory to EIMD, it could be suggested that an increase in immune cells and RONS could be an important driver of acute and chronic molecular adaptations (see Chapter 2.5.2) but, excessive or chronic production could lead to cell dysfunction, additional muscle damage, and prolonged losses in function (Peake et al., 2015b; Slattery, Bentley, & Coutts, 2015).

In view of hormesis, perhaps the usefulness of AOXs or NSAIDS for athletes, particularly with regards to exercise recovery, is highly context dependent. For instance, their chronic use throughout a competitive season could interfere with molecular signalling processes and possibly hinder long-term training adaptations, therefore being detrimental. However, the acute use of these supplements, when the need to minimise the adverse effects of EIMD (i.e., soreness and losses in muscle function) far outweigh the need for adaptation (i.e., tournaments, fixture congestion), these supplements could form part of a recovery strategy to restore optimal performance.

Furthermore, the hormesis theory could also be extended to explain the effects of the supplements themselves; at low to moderate doses AOX or anti-inflammatory supplements could be ergogenic, but at high doses, deleterious. This could explain why inhibitory effects on exercise-induced adaptations are more commonly reported with very high doses of vitamin C and E (i.e., vitamin C ≥1000 mg·day^{-1}) (Bjørnsen et al., 2015; Close et al, 2006; Paulsen et al, 2014b) than moderate doses (500 mg mg·day^{-1}) (Yfanti et al., 2010; Yfanti et al., 2011). The potential for dose-response effects of these supplements on exercise-induced adaptations is clearly an avenue that needs further exploration.

2.6 Functional foods as recovery interventions

In recent years, perhaps because of the risk of deleterious effects with vitamin C and E and NSAIDS, there has been a growing interest in the potential benefits of other AOX or anti-inflammatory containing supplements to attenuate muscle damage. Of particular interest are the effects of natural or functional food supplements on exercise recovery (Myburgh, 2014; Sousa et al., 2013). This interest stems from the observation that functional foods contain a variety of naturally occurring phytonutrients, such as polyphenols, (i.e., flavonoids, anthocyanins) (Myburgh, 2014). These phytonutrients appear to exhibit a broad range of beneficial physiological effects that include, but are not limited to, AOX and anti-inflammatory (Nikolaidis et al., 2012a; Sousa et al., 2013; Urso, 2013). Unlike studies with NSAIDS and vitamin C and E, more favourable effects on exercise recovery have been demonstrated after supplementing with functional foods. For instance, there are now several reports that cherry juice supplementation can attenuate indices of muscle damage following cycling (Bell et al., 2014; Bell et al., 2015) running (Howatson et al., 2010) and eccentric-heavy resistance exercise (Bowtell et al., 2010; Connolly et al., 2006). Furthermore, a series of studies has also shown that pomegranate juice taken before and after eccentric exercise can enhance the recovery of muscle function in the

ensuing days (Trombold et al., 2010; Trombold et al., 2011; Machin et al., 2014). Numerous other functional foods, including curcumin (Drobnic et al., 2014; Tanabe et al., 2015), blueberry (McLeay et al., 2009) and green tea (Jówko et al., 2011) have also shown favourable effects on EIMD. Why these functional food sources appear to be more beneficial than NSAIDS or vitamin C/E for alleviating EIMD is not completely understood but some potential explanations are briefly discussed in Chapter 6. Regardless of the precise mechanisms, the current evidence suggests that functional foods supplements might provide a useful means of attenuating EIMD. This was the conclusion of a recent review by Myburgh, (2014), who suggested that the potential benefit of this class of nutrients on EIMD and functional recovery is an avenue worthy of further exploration.

2.7 Beetroot as a recovery intervention

The overarching aim of this thesis is to investigate the effects of a functional food, beetroot, on recovery from strenuous exercise. Therefore, the following sections of this review will focus on the physiological and biochemical effects of beetroot and its various constituents, and how these could be exploited to enhance exercise recovery.

2.7.1 Beetroot: an introduction to its history, constituents and potential benefits

In recent years, the root vegetable *Beta Vulgaris* L. otherwise known as beetroot, has attracted much attention as a health promoting functional food. While scientific interest in beetroot has only gained momentum in the past few decades, reports of its use as a natural medicine date back to Roman times where it was used to treat a variety of illnesses (Nikolic et al., 2012; Ninfali & Angelino, 2013). Intriguingly, the longstanding belief that beetroot has natural healing properties has made it one the most popular alternative treatments amongst cancer patients (Nikolic et al., 2012). Today, beetroot is

grown in many countries worldwide, is regularly consumed as part of the normal diet, and commonly used in manufacturing as a food colouring agent known as E162 (Georgiev et al., 2010; Pietrzkowski et al., 2010).

The recent interest in beetroot was primarily driven by the discovery that foods rich in dietary nitrate may have important implications for managing health and disease. Indeed, recent studies have provided compelling evidence that consuming beetroot, typically acutely as a juice, offers a myriad of beneficial physiological effects (Bailey et al., 2009; Gilchrist et al., 2014; Joris & Mensink, 2013; Kapil, Weitzberg, Lundberg, & Ahluwalia, 2014; Vanhatalo et al., 2010). These effects have been successfully exploited to favourably affect clinical outcomes for several pathologies, such as; hypertension, atherosclerosis, type 2 diabetes and dementia (Gilchrist et al., 2013; Presley et al., 2011; Wootton-Beard, Brandt, Fell, Warner, & Ryan, 2014). There is also now a growing interest in the use of nitrate donors to manage muscle injuries (Rigamonti et al., 2013), and possibly muscular dystrophy (Archer, Vargas, & Anderson, 2006; D'Angelo et al., 2012) since the discovery that nitrate also seems to have a regulatory influence on the phagocytic and regenerative phases of muscle repair (Filippin, Moreira, Marroni, & Xavier, 2009).

The beneficial physiological effects of beetroot juice have been largely attributed to its high inorganic nitrate content (250 $mg \cdot kg^{-1}$ of fresh weight; (Ormsbee, Lox, & Arciero, 2013). Nitrate itself is not considered to mediate any specific physiological function; rather, nitrates beneficial effects are attributed to its *in vivo* reduction to NOx, a multifarious messenger molecule with important vascular and metabolic functions (Hobbs, George, & Lovegrove, 2013). The generation of NOx via nitrate involves a series of sequential steps that have been well described in the literature (Lundberg, Carlström, Larsen, & Weitzberg, 2011; Lundberg, Weitzberg, & Gladwin, 2008). Briefly, ingested nitrate is first absorbed through the upper part of the small intestine into the systemic circulation (Kapil et al., 2014; Lundberg et

al., 2008). It is then estimated that 25% of the circulating nitrate enters the entero-salivary cycle where bacterial species located at the posterior aspect of the tongue bioactivate or reduce salivary nitrate to nitrite (Lidder & Webb, 2013; Lundberg et al., 2011). Because salivary bacteria facilitate the reduction reaction that converts nitrate to nitrite, spitting out saliva or taking oral anti-bacterial treatments, like dental mouthwash for example, has been shown to diminish nitrate-nitrite conversion (Webb et al., 2008). Under normal circumstances, however, salivary nitrite is re-absorbed into the circulation via the stomach, where it is metabolised to NOx and other nitrogen oxides by a variety of reductase enzymes (Hobbs, Kaffa, George, Methven, & Lovegrove, 2012; Lundberg et al., 2008; Webb et al., 2008).

Nitrate is not the only constituent of beetroot proposed to have beneficial effects in health and disease though. Beetroot is a rich source of phytochemical compounds (Figure 2) that includes carotenoids, phenolic acids and flavonoids (Kujala, Vienola, Klika, Loponen, & Pihlaja, 2002; Wootton-Beard & Ryan, 2011). Beetroot is also one of only a few foods that contain a group of highly bioactive pigments known as betalains, which stem from the order Caryophyllales (Kha, 2015; Lee, Wettasinghe, Bolling, Ji, & Parkin, 2005; Vulic et al., 2014). Members of the betalain family are categorised as either betacyanin pigments, that are red-violet in colour, or betaxanthin pigments, that are yellow-orange in colour (Ninfali & Angelino, 2013). A number of investigations have reported betalains to have high AOX and anti-inflammatory capabilities *in vitro* and a *in vivo*, albeit these findings are mostly derived from animal models (Pavlov, Kovatcheva, Georgiev, Koleva, & Ilieva, 2002; Tesoriere, Allegra, Butera, & Livrea, 2004; Vidal, Lopez-Nicolas, Gandia-Herrero, & Garcia-Carmona, 2014; Vulic et al., 2014; Zielinska-Przyjemska, Olejnik, Dobrowolska-Zachwieja, & Grajek, 2009). Interestingly, betalains have also been shown to have adaptogenic effects, at least *in vitro*, in which they act to reinforce the endogenous AOX and xenobiotic defence system by upregulating specific signalling cascades (Esatbeyoglu et al., 2014; Khan, 2015). These findings have sparked interest

in a possible role for beetroot in degenerative pathologies characterised by oxidative stress and chronic inflammation such as liver disease (Ninfali & Angelino, 2013; Vulic et al., 2014), arthritis (Pietrzkowski, 2010) and even cancer (Kapadia et al., 2011; Kapadia et al., 2013).

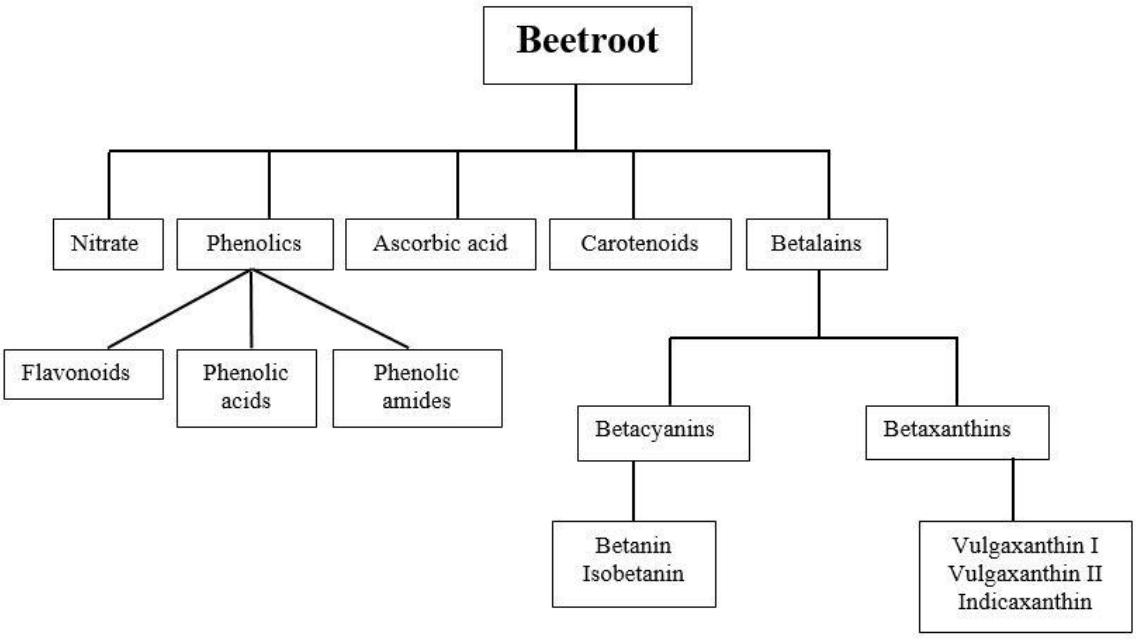

Figure 2 - Overview of potentially bioactive compounds in beetroot (based on data from (Georgiev et al., 2010; Kujala et al., 2002; Ninfali & Angelino, 2013).

The potential benefits of beetroot are not limited to the clinical setting and since the turn of the decade, beetroot has gained popularity as an ergogenic aid for athletic performance. Indeed, a single dose of beetroot juice, taken 2-3 hours before exercise, has been shown to improve cycling (Lansley et al., 2011), running (Murphy, Eliot, Heuertz, & Weiss, 2012) and rowing performance (Hoon et al., 2014), and there are several anecdotal reports of its successful use in the wider sporting community (Jones, 2014b; Ormsbee et al., 2013). Although the precise mechanisms are still being debated, most researchers agree that beetroots ergogenic effects are mediated by NOx and

its influence on metabolic pathways related to exercise efficiency/economy (Jones, 2014a, 2014b; Larsen, Weitzberg, Lundberg, & Ekblom, 2007). A more in depth discussion of the studies investigating beetroot and athletic performance and the potential mechanisms involved are not within the scope of this thesis; for this information, the reader is referred to the following review articles (Jones, 2014a, 2014b).

Given the above discussion, it is clear that beetroot contains several chemical compounds that might exhibit beneficial physiological effects, including for conditions associated with inflammation and oxidative stress. Therefore, its application in the exercise domain may extend beyond enhancement of athletic performance, to include exercise recovery. Indeed, Chapter 2.5, highlighted the accumulating evidence that provison of foods with AOX and anti-inflammatory phytonutrients attenuates some of the deleterious symptoms that arise in the days following strenuous exercise; inflammation, oxidative stress, muscle soreness and loss of muscle function (Bell et al., 2014; Bell et al., 2015; Howatson et al., 2010; McLeay et al., 2012; Trombold et al., 2010; Trombold et al., 2011). Consequently, the aim of the following sections is to discuss the anti-inflammatory and AOX effects of beetroot and its constituents and, thus, aims to provide a rationale for the use of a beetroot supplement as a recovery intervention following muscle-damaging exercise.

It is important to note that this discussion is primarily limited to mechanisms underpinned by an AOX and/or anti-inflammatory effect of beetroot. This is because these are the mechanisms that initially generated the hypothesis for this course of investigation and, thus, were tested in some of the studies in this thesis. However, it is acknowledged that there are other mechanisms afforded by beetroot that, conceivably, could also benefit exercise recovery; namely, increased muscle blood flow, improved contractile function and enhanced satellite cell activity: all of which have been demonstrated with either beetroot juice or nitrate supplementation (and are discussed in

Chapter 10). Obviously there was not scope to investigate all of these possible mechanisms within this thesis; hence, the investigations throughout were based on the premise that the anti-inflammatory or AOX effects of beetroot would serve to enhance exercise recovery. Therefore, in the following sections, after reviewing the evidence examining the bioavailability of the bioactive compounds in beetroot, particularly the betalains, the aim is to evaluate the evidence from experimental studies on the effects of beetroot and its various constituents on inflammation and oxidative stress.

2.7.2 Bioavailability

For a food component to be considered beneficial for health it must be bioavailable *in vivo*, that is, following ingestion, the active compounds are absorbed through the gastro-intestinal tract and made available in the circulation, in sufficient quantities, to be utilized by cells (Toutain & Bousquet-Melou, 2004; Wootton-Beard & Ryan, 2011). However, in order to reach the systemic circulation and exert any physiological functions, a food component must maintain its molecular structure through several phases of digestion that each present a significant metabolic challenge for the molecule and affect its eventual rate and extent of absorption (Rein et al., 2013; Toutain & Bousquet-Melou, 2004). It is therefore critically important that any alleged health benefit of a food source be firstly verified with well-designed bioavailability studies that characterise the extent of its *in vivo* absorption (Rein et al., 2013).

In this respect, the bioavailability of both nitrate and the betalains, the major bioactive components of beetroot, have been considered in the literature. The high bioavailability of inorganic dietary nitrate is well established and there are reports of extremely high absorption and bioavailability following digestion (van Velzen, Sips, Schothorst, Lambers, & Meulenbelt, 2008). The extent to which betalains are absorbed and bioavailable is, however, less clear. Two studies have directly investigated betalain bioavailability by

measuring their appearance in human urine after ingesting a single bolus of homemade BTJ (Frank et al., 2005; Kanner, Harel, & Granit, 2001). Kanner et al. (2001) identified 0.5%–0.9% of the ingested betacyanins (betanin and isobetanin) in volunteer's urine in the 12 h after consuming 300 mL of homemade BTJ. This indicates that although in small amounts, betacyanins can be successfully absorbed and are bioavailable in humans. They also showed that the peak urinary excretion rate of betacyanins (indicative of bioavailability), occurred 2–4 h after ingestion; however, there was a high level of inter-individual variability within this time period. Frank et al. (2005) reported similar findings while investigating betacyanin bioavailability. After providing six healthy participants with 500 mL of homemade BTJ, they identified betacyanins in urine at concentrations equivalent to ~0.3% of the ingested dose over a 24 h period. These studies might be interpreted to suggest low bioavailability; however, it is important to take into account that betacyanins are unlikely to be exclusively excreted via the renal pathway (Frank et al., 2005). Indeed, the use of urinary excretion as a sole indicator of bioavailability has received criticism because it does not account for the distribution of molecules to other tissues, thus underestimating true bioavailability (Khan, 2015; Rein et al., 2013). In addition, the extent to which betalains are metabolised and structurally transformed to secondary metabolites is yet to be characterized, but should be acknowledged when evaluating their bioavailability (Frank et al., 2005; Khan, 2015).

Given these limitations, Tesoriere et al. (2013) employed a different approach to investigate the bioavailability of betalains. Tesoriere and colleagues developed a simulated *in vitro* model of the human intestinal epithelium using Caco-2 cell monolayers to mimic a functional intestinal barrier. This model allowed them to examine whether betalains can be absorbed through a functioning intestinal barrier and hence give an indication of their bioavailability. They demonstrated that two betalains; betanin and to a greater extent indicaxanthin were well absorbed through the simulated model of the intestinal lining (Caco-2 cell monolayer) and mostly in their

unmetabolised form via paracellular transport. The latter finding is particularly important, because it reveals that betalains might be absorbed into the systemic circulation in their unchanged form, allowing them to retain at least some of their molecular structure and high biological activity demonstrated *in vitro* (Ting et al., 2014). This study, along with others (Khan, 2015), also suggests that the betaxanthins are more bioavailable than the betacyanin pigments. Nonetheless, it is important to note that results from *in vitro* experiments, even when designed to mimic the biological milieu of the human GI tract, do not necessarily translate *in vivo*, given that several other factors (i.e., first pass metabolism, interactions with gut microflora and protease enzyme degradation) have a significant influence on the concentration of the nutrient that eventually reaches the circulation (Rein et al., 2013; Ting et al., 2014).

In addition to the betalain family, other aforementioned plant derived AOXs have been identified in beetroot, including epicatechin, rutin, and caffeic acid (Georgiev et al., 2010), which, to varying degrees, appear to be well absorbed and bioavailable in humans (Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Although the bioavailability of these compounds and other phenolic compounds from beetroot has not been individually determined, there are data describing the bioavailability of the total phenolic compounds present in beetroot (Netzel et al., 2005). Netzel et al. (2005) measured the urinary excretion of total phenolic substances following a single 500 mL bolus of BTJ. They identified ~685 mg of phenolic compounds in participant's urine <24 h following beetroot juice ingestion; 97% more than the ~347 mg identified after consuming water (i.e., basal concentrations). While the relative bioavailability from the individual compounds could not be determined, these findings clearly show that beetroot's phenolic constituents are bioavailable and are likely to make a significant contribution to beetroot's *in vivo* AOX power. Taken together, the results of the aforementioned studies provide a good base of evidence that beetroot is a bioavailable source of bioactive compounds (particularly nitrate and phenolic compounds) in

humans. With that said, further work is still required to firstly; elucidate the bioavailability of beetroot's individual bioactive components, especially the different betalains and, secondly; to establish the extent that plasma, biliary, and other metabolic pathways contribute to the excretion of these components.

2.7.3 Effects of beetroot on oxidative stress

There is evidence to suggest that beetroot might serve as a useful strategy to strengthen endogenous AOX defences and help to protect cellular components from oxidative damage (El Gamal et al., 2014). The beneficial effects of beetroot in oxidative stress are based on the fact that beetroot is an exceptionally rich source of biochemical compounds that display AOX functions (Ninfali & Angelino, 2013). The betalain pigments in particular have been shown by several *in vitro* studies to protect cellular components from oxidative injury (Kanner et al., 2001; Reddy, Alexander-Lindo, & Nair, 2005; Tesoriere, Fazzari, Angileri, Gentile, & Livrea, 2008). For example, in the study by Kanner et al. (2001), two betalain metabolites (betanin and betanidin) were shown to reduce linoleate damage induced by cytochrome C oxidase and lipid membrane oxidation induced by H2O2-activated metmyoglobin and free iron (AA-Fe). The authors also reported that betanin, the most abundant betalain found in beetroot (300–600 $mg \cdot kg^{-1}$), was the most effective inhibitor of lipid peroxidation. Betanin's high AOX activity appeared to stem from its exceptional electron donating capacity and ability to defuse highly reactive radicals targeting cell membranes (Kanner et al., 2001). However, as alluded to earlier, betalains are not the only AOX compounds present in beetroot. Beetroot contains several highly bioactive phenolics, such as rutin, epicatechin and chlorogenic acid which are also known to be excellent AOXs (Frank et al., 2005; Georgiev et al., 2010; Manach et al., 2005).

A number of studies report that beetroot, in the form of a juice supplement, protects against oxidative damage to DNA, lipid and protein structures *in vitro* (Esatbeyoglu et al., 2014; Kujawska et al., 2009; Winkler, Wirleitner, Schroecksnadel, Schennach, & Fuchs, 2005). A study by Wootton-Beard and colleagues suggests that a key mechanism by which beetroot juice exerts its AOX effects is by directly scavenging radical species (Wootton-Beard, Moran, & Ryan, 2011). They found that two commercially available beetroot juices inhibited *in vitro* radical formation in the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) and (3-ethylbenzothiazoline-6-sulfonicacid) ABTS• assays by 100% and 92%, respectively. Importantly, when these assays were repeated, but in conditions designed to simulate the human digestive process, the juices still retained ≥55% of their pre-digestion radical scavenging capacity. Furthermore, the AOX capacity of both drinks, as measured by ferric-ion reducing antioxidant power (FRAP), was higher than the other 22 vegetable juice drinks under investigation. In another study (Wootton-Beard & Ryan, 2011), they showed that the FRAP of BTJ actually increases following simulated digestion. This could be the consequence of several compounds being structurally altered to secondary metabolites that possess AOX functions (Wootton-Beard et al., 2011; Wootton-Beard & Ryan, 2011). Further work from this group has shown that the AOX capacity of BTJ is comparable to or higher than a variety of fruit and vegetable juices (see Figure 3 and Figure 4) (Ryan & Prescott, 2010; Wootton-Beard et al., 2011). Interestingly, the AOX capacity of BTJ in both the DPPH• and FRAP assays was far greater than more well-known vegetable juices, such as tomato and carrot, and fruit juices, such as orange and pineapple, with only pomegranate juice displaying a higher AOX capacity in the FRAP assay.

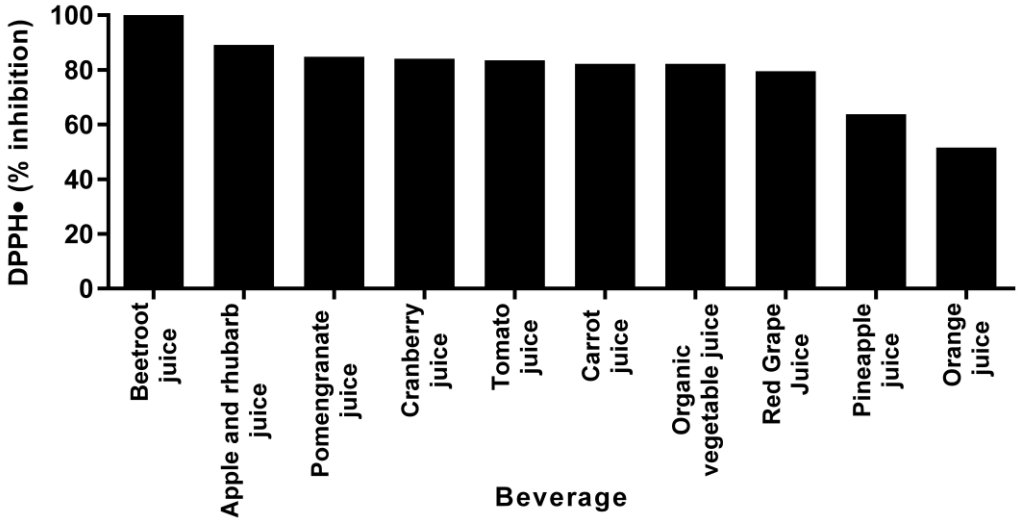

Figure 3 - A comparison of the 2,2-diphenyl-1-picrylhydrazyl (DPPH•) inhibiting capacity (%) exhibited by 10 popular fruit and vegetable beverages available in the UK (values based on data from Ryan & Prescott, 2010; Wootton-Beard et al., 2011).

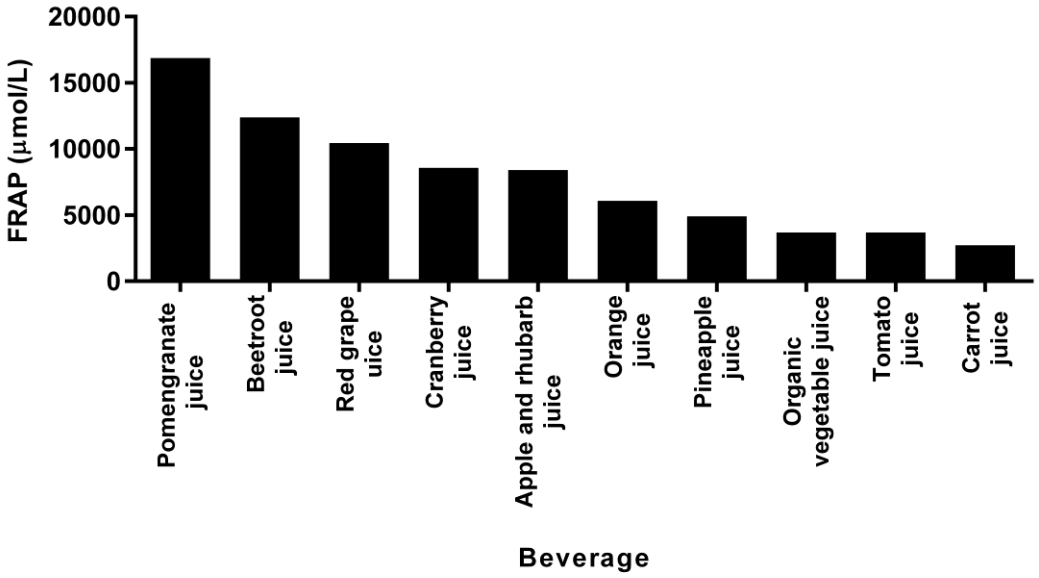

Figure 4 - The ferric-ion reducing antioxidant power (FRAP) of 10 commercially available fruit and vegetable beverages post a simulated *in vitro* model of human digestion (values based on data from Ryan & Prescott, 2010; Wootton-Beard et al., 2011).

In addition to being a source of AOXs *in vitro*, a growing body of evidence using animal models illustrates that beetroot exhibits radical scavenging ability *in vivo* (see Table 2). In a recent study (Vulic et al., 2014), rats were provided with 1–3 mL·kg·bm^{-1} of a beetroot pomace extract for 7 days prior to being exposed to 2 mL·kg·bm^{-1} of carbon-tetrachloride (CCl4), a well-established carcinogen and RONS generator. After CCI4 administration, liver homogenate was removed from rats pre-treated with the beetroot extracts and those acting as controls (i.e., CCl4 only). Rats treated with beetroot extracts expressed significantly lower levels of lipid peroxidation measured as TBARS. Furthermore, the beetroot extracts appeared to maintain endogenous AOX activity (reduced glutathione, glutathione peroxidase and catalase enzymes) at normal cellular concentrations following the oxidative insult. This led the authors to speculate that in response to *in vivo* cellular attack, beetroot may exhibit indirect AOX effects that act to up regulate AOX

defence mechanisms (Vulic et al., 2014). Similar AOX effects have also been reported with studies using BTJ. Providing rat's BTJ (8 mL·kg·bm·day^{-1}) for 28 days was shown to attenuate lipid peroxidation, protein oxidation and DNA damage following xenobiotic induced liver injury (Kujawska et al., 2009). In a more recent study, rats were fed BTJ (8 mL·kg·bm·day^{-1} for 28 days) and treated with the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) on day 27 and 28 of the BTJ feeding period (Szaefer, Krajka-Kuzniak, Ignatowicz, Adamska, & Baer-Dubowska, 2014). Several markers of liver damage and inflammation were significantly increased following the DMBA treatment; however, these were markedly reduced in the rats pre-treated with BTJ compared to the control group that received water only (Table 2). There were no differences in DNA damage between the groups. Intriguingly, rats given BTJ only, as in not treated with DMBA, exhibited increased activity of phase II detoxifying enzymes (GST and NQO1), which play an important role in endogenous AOX defence. The enhanced endogenous AOX activity *in vivo*, by beetroot is a consistent finding in the literature (see Table 2). According to recent *in vitro* data, such effects might be related, in part, to betanin and its effect on signalling pathways that mediate the transcription of AOX genes. Esatbeyoglu et al. (2014) found that betanin (extracted from beetroot) dose dependently (5–15 μm) increased the activity of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), a transcription factor that activates a gene promoter sequence: the antioxidant response element (ARE), responsible for the transcription of several endogenous AOX enzymes (Esatbeyoglu et al., 2014; Nguyen, Nioi, & Pickett, 2009; Satoh, McKercher, & Lipton, 2013). Krajka-Kuźniak, Paluszczak, Szaefer, & Baer-Dubowska, (2013) presented similar findings, showing that betanin (2, 10 and 20 μm concentration) activates the Nrf2-ARE binding sequence in non-tumor human hepatic cell lines. Furthermore, this led to increased activity and mRNA expression of several phase II detoxifying enzymes, including glutathione S-transferases and NAD(P)H:quinone oxidoreductase, which play important roles in host defence against xenobiotics. This raises the

possibility that beetroot's AOX potential is not limited to just scavenging and suppressing RONS, but includes the ability to reinforce the endogenous AOX network. However, whether such effects translate *in vivo*, particularly in humans, is yet to be investigated. Given these findings, it should also be considered that other compounds in beetroot (and their downstream metabolites upon ingestion) possess similar effects to betanin on transcriptional activity. Thus, *in vivo*, these compounds and metabolites could work synergistically to activate the Nrf2-ARE pathway, which, in turn, mediates an increase in endogenous AOX activity, that is independent of their radical scavenging effects.

Table 2 - Overview of studies investigating the effects of beetroot and its derivatives on oxidative stress and inflammation.

Authors	Cohort under investigation	Dosage	AOX capacity of treatment	Duration	Toxic inducing protocol	Inflammation	Oxidative stress	Enzymatic AOX activity
(Kujawska et al., 2009)	48 male wistar rats	Beetroot juice delivered by gavage (8 ml·kg·bm·day^{-1};≈1.92 ml·day^{-1})	23.5 μmol/L Trolox equivalents·ml^{-1}	28 days	Intraperitoneal injection of CCl_4 (2 ml·kg·bm^{-1}) or NDEA (150 ml·kg·bm^{-1})	N/A	TBARS↓ PC ↓ DNA damage ↓	SOD ↑ GPX ↑ CAT ↑ GR ↑
(Krajka-Kuźniak et al., 2013)	80 male ICR mice	Betalains from beetroot delivered orally (0, 5, 20 and 80 mg·kg·bm·day^{-1} -; ≈0 - 1.44 mg·day$^{-1)}$	N/A	30 days	Exposed to cobalt-60-γ-gamma radiation (6.0 Gy, 1.5 Gy min^{-1})	N/A	MDA ↓	SOD ↑ CAT ↑ GSH ↑
(Lu et al., 2009)	24 male wistar rats	Beetroot juice delivered by gavage (8 ml·kg·bm·day^{-1})	N/A	28 days	Intraperitoneal injection of NDEA (150 ml·kg·bm^{-1})	LDH ↓ AST↓ ALT↓	DNA damage ↓	GST ↑
(Pietrzk-owski et al., 2010)	10 osteoarthritic patients	Capsules made from beetroot extract; delivered orally (70-200 mg·day^{-1})	N/A	10 days	N/A	TNF-α ↓ IL-6 ↓ RANTES ↓ GRO-α ↓	AOPP ↓	
(Vulić et al., 2014)	48 albino wistar rats	Beetroot pomace extract delivered intraperitoneally (1–3 ml·kg·bm·day^{-1};≈ 0.2-0.6 ml·day^{-}1)	N/A	7 days	Intraperitoneal injection of CCl_4 (2 ml·kg·bm^{-1})	N/A	MDA ↓	GSH ↑ GSHPx ↑ GR ↑

Authors	Cohort under investigation	Dosage	AOX capacity of treatment	Duration	Toxic inducing protocol	Inflammation	Oxidative stress	Enzymatic AOX activity
(Krajka-Kuźniak et al., 2012)	24 albino wistar rats	Extracts of fresh beetroot delivered orally (250 and 500 $mg \cdot kg \cdot bm \cdot day^{-1}$; ≈45 – 90 $mg \cdot day^{-1}$)	90.1% radical inhibition in the DPPH• assay (500 $\mu g \cdot ml^{-1}$)	28 days	Intraperitoneal injection of gentamicin (8 $mg \cdot kg \cdot bm^{-1}$ for 8 days)	IL-6 ↓ TNF-α ↓ MPO ↓ NF-κB ↓	MDA ↓	CAT ↑ NP-SH ↑
(Szaefer et al., 2014)	24 female sprague–dawley rats	Beetroot juice delivered by gavage (8 $ml \cdot kg \cdot bm \cdot day^{-1}$; ≈1.92 $ml \cdot day^{-1}$)	N/A	28 days	Intraperitoneal injection of DMBA (10 $mg \cdot kg \cdot bm^{-1}$ for 2 days)	LDH ↓ ALT↓	N/A	GST ↑ NQO1 ↑

NDEA, N-nitrosodiethylamine; GST, glutathione S-transferase; RANTES, regulated upon activation normal T cell growth; GRO-α, regulated oncogene-alpha; CCl_4 carbon tetrachloride; GSH, reduced glutathione; GSHPx, glutathione peroxidase; GR, glutathione reductase; GPX, glutathione peroxidase; TNF-α, tumour necrosis factor-alpha; TBARS, thiobarbituric acid reactive species; PC, protein carbonyls; SOD, superoxide dismutase; ICR, Imprinting Control Region; MDA, Malondialdehyde; CAT, catalase; AOPP, advanced oxidation protein products; IL-6, interleukin-6. Gy, gray unit; LDH, lactate dehydrogenase; ALT, alanine aminotransferase; AST,aspartate aminotransferase; MPO, myeloperoxidase. NF-κB, nuclear factor kappa B; NP-SH, non protein sulfhydryl; DMBA, 7,12-dimethylbenz[a]anthracene; NQO1, NAD(P)H dehydrogenase [quinone] 1.

2.7.4 Effects of beetroot on inflammation

Betalains and beetroot extracts have emerged as potent anti-inflammatory agents. At least part of their anti-inflammatory effects seems to be mediated by interfering with pro-inflammatory signalling cascades. The most important of these is the NF-κB cascade, which, as discussed in a previous section, directly activates and transcribes most gene targets that regulate and amplify the inflammatory response (Baker et al., 2011). Consequently, NF-κB activity plays a central role in the inflammatory processes associated with chronic disease (Baker et al., 2011) and strenuous physical activity (Gomez-Cabrera et al., 2005; Xin et al., 2012). In a recent study (El Gamal et al., 2014), NF-κB DNA-binding activity was dose-dependently attenuated in nephrotoxic rats administered a beetroot extract for 28 days (250 mg or 500 $mg \cdot kg \cdot bm^{-1}$). Furthermore, kidney homogenates from the beetroot treated rats had lower inflammation (TNF-α, IL-6 and MPO) and reduced signs of oxidative damage (MDA), which could be directly related to the blunting of the NF-κB pathway. These effects are likely to be mediated, at least in part, by the betalains present in beetroot; recent evidence shows that betanin treatment (25 and 100 $mg \cdot kg \cdot bm^{-1}$ for 5 days) significantly inhibits NF-κB DNA-binding activity in rats induced with acute renal damage (Tan, Wang, Bai,Yang, & Han, 2015). Betalains have also been shown to markedly supress cyclooxygenase-2 (COX-2) expression *in vitro*, which is an important precursor molecule for prostaglandins (Reddy et al., 2005; Vidal et al., 2014; Zielińska-Przyjemska et al., 2009). Reddy et al. (2005), who found that betanin (IC_{50} value 100 $\mu g \cdot mL^{-1}$) inhibited COX-2 enzyme activity by 97%, first illustrated this. It is interesting to note that although a slightly higher concentration of betanin was required, its COX-2 inhibitory effects were comparable or greater than several phenolic compounds (cyanidin-3-O-glucoside, lycopene, chlorophyll, b-carotene, and bixin) and anti-inflammatory drugs (Ibuprofen, Vioxx and Celebrex).

A recent study from Vidal et al. (2014) provided further support for the anti-inflammatory effects of betalains. As well as supressing COX-2 synthesis, betanidin, extracted from beetroot, dose dependently inhibited (to 9% of control activity), lipoxygenase (LOX), a catalytic enzyme vital for the synthesis of pro-inflammatory leukotriene molecules (Ricciotti, & FitzGerald, 2011). Interestingly, these inhibitory effects appeared to be mediated by a blocking action on membrane binding activity, indicating that betalains target cell signalling pathways at the molecular level, acting in a similar fashion to selective COX-2 inhibitor drugs (Ricciotti, & FitzGerald, 2011; Vidal et al., 2014).

A few recent studies have indicated that nitrate, via its reduction to NOx, might exhibit anti-inflammatory effects. This was demonstrated in a study by Jädert and colleagues (2012) that showed administering mice with sodium nitrate for 7 days attenuated the increase in inflammatory mediators (MPO and P-selectin) following small intestinal injury. The principal mechanism by which nitrate-induced NOx generation exerts anti-inflammatory effects is believed to be through suppressing phagocytosis, which has the beneficial effect of reducing the number of pro-inflammatory mediators that invade the cell and perpetuate cell dysfunction (Jädert et al., 2012; Jädert, Phillipson, Holm, Lundberg, & Borniquel, 2014). This role for NOx in modulating leukocyte activity was evidenced by findings from Rigamonti et al. (2013). They found that mice deficient in iNOS, a precursor for NOx in muscle, had a higher infiltration of phagocytic cells in the days after acute skeletal muscle injury compared to non-deficient controls, leading them to speculate that NOx must have a regulatory role in inflammation.

A recent study linked the anti-inflammatory effect of nitrate to improved muscle function. Justice et al. (2015) found that sodium nitrate treatment reduced the expression of several inflammatory cytokines in the skeletal muscle of older mice, effects that were associated with improved grip strength and run time to exhaustion over an 8 week period (Justice et al.,

2015). Similarly, another study suggested that augmenting NOx availability attenuates muscle damage leading to improved function. In this study, rats were given an acute dose of L-arginine, a NOx donor, or a control prior to downhill running. Twenty four h after the exercise bout, intracellular protein degradation and sarcolemma damage in the soleus muscles was attenuated in the L-arginine treated groups and muscle function, as measured by running capacity, was improved (+12% vs. control) (Lomonosova, Shenkman, Kalamkarov, Kostrominova, & Nemirovskaya, 2015). The authors suggested that these effects could have been due to a NOx mediated reduction in local proteolytic activity and inflammation.

The evidence presented above suggests that exogenous NOx donors, like BTJ for example, could be helpful for protecting against the potentially harmful effects that acute inflammation could have on cell function and integrity. As demonstrated in the study by Lomonosova and colleagues (2015), this could have important implications for dampening inflammatory mediated muscle damage, i.e., the secondary damage response to EIMD. With that said, it is important to note that whether the aforementioned effects in animals translates to humans, or are evident with nitrate-rich food supplements has not yet been investigated.

There are a limited number of studies demonstrating that beetroot supplements have anti-inflammatory effects *in vivo*. Pietrzkowski et al. (2010) showed that therapeutic administration of betalain-rich oral capsules made from beetroot extracts alleviated inflammation and pain in osteoarthritic patients. After 10 days of supplementation (100, 70 or 35 mg per day), the pro-inflammatory cytokines TNF-α and IL-6 had decreased from baseline by 8.3%–35.0% and 22.0%–28.3%, respectively. The activity of two chemokines; regulated oncogene-alpha (GRO-alpha) and regulated upon activation normal T cell growth (RANTES) were also markedly inhibited by the beetroot treatment. Furthermore, the moderated inflammatory response coincided with a significant reduction in self-reported pain on the McGill Pain

Questionnaire. Krajka-Kuźniak, Szaefer, Ignatowicz, Adamska, & Baer-Dubowska, (2012) examined the protective effect of BTJ (8 mL·kg·bm·day^{-1} for 28 days) on markers of liver injury and inflammation induced by the toxic chemical N-nitrosodiethylamine (NDEA) in rats. Compared to an untreated control, the BTJ conferred significant hepatic protection against a range of inflammatory markers induced by NDEA administration; LDH, AST, gamma glutamyl transferase (GGT) and ALT were all shown to be markedly attenuated. In a more recent study, El Gamal et al. (2014) fed rats either water (control) or oral doses of beetroot ethanol extract (250 or 500 mg·kg·bm·day^{-1}) for 28 days; from day 20–28 they were treated with the nephrotoxic drug gentamicin (85 mg·kg·bm·day^{-1}). After 28 days, kidney homogenates were removed from both groups and analysed for several markers of renal damage. They found that the beetroot-treated rats had significantly lower concentrations of several pro-inflammatory mediators, including IL-6, TNF-α, MPO (indicating neutrophil infiltration) and the transcription factor NF-κB. They also found reduced oxidative stress (MDA, uric acid) and increased endogenous AOX activity (CAT, NP-SH) in the homogenates removed from the rats pre-treated with beetroot.

The above summary provides evidence that beetroot is an excellent source of AOX and anti-inflammatory compounds. Although there is still a lack of well-conducted human trials, which precludes any definitive conclusions as to its potential benefits, there is enough evidence to suggest that beetroot is capable of protecting cellular components from inflammatory and/or oxidant-mediated damage. This makes the expectation tenable that a beetroot supplement could be exploited to counter secondary muscle damage after strenuous exercise and, as a result, serve as an efficacious means of enhancing exercise recovery.

2.8 Conclusion

Eccentric-heavy exercise has been shown to cause a variety of morphological changes, including sarcolemma rupture, z-line streaming and ECM disruption. These changes evoke a transient but marked inflammatory response that can be observed in the muscle cell, ECM, and circulation. The inflammatory response encompasses an array of complex biochemical and physiological alterations that may evoke secondary muscle damage.

Muscle damage is typically observed as an increased appearance of intramuscular proteins in the blood, muscle soreness and profound deficits in muscle function. In an attempt to alleviate muscle damage and enhance recovery, athletes and exercise enthusiasts often consume nutritional supplements before and after training and/or competitive events. There are several sporting scenarios when nutrition based recovery supplements might be particularly beneficial for athletes, including during congested fixture and intensified training periods, or when immune function might be depressed or there is a risk of nutrient deficiency. With regards to EIMD, an emerging topic of interest is the potential benefits of functional food supplements because many are rich in AOX and anti-inflammatory phytonutrients. Indeed, the evidence to date seems to indicate that functional foods may be of benefit for exercise recovery when taken before and/or immediately post-exercise, ostensibly due to their ability to dampen the secondary damage response.

Beetroot is a functional food that has received enormous attention in recent years for its potential benefits for both general health and athletic performance. Recent studies have provided evidence that beetroot and its constituent’s may afford beneficial effects for disorders characterized by inflammation, oxidative stress, and aberrant vascular function. Given the aetiology and progression of muscle damage, such effects could be exploited for enhancing exercise recovery. Despite this, no study to date has examined the influence of beetroot on EIMD and, thus, established whether it might be

useful as both a pre-exercise and post-exercise ergogenic aid. As such, the primary aim of this thesis is to investigate the efficacy of BTJ as a recovery intervention following muscle damaging exercise.

3 General methods

3.1 General methods

The methods described below are those that were employed in the majority of investigations in this thesis; therefore, descriptions of measures that were unique to individual studies are not included but can be found in the methods section of the relevant Chapter. Institutional ethical approval was granted prior to data collection for each investigation (see Appendix 2 for an example), and written informed consent was sought after a verbal and written briefing of the study procedures (see Appendix 1 for an example of informed consent).

3.2 Participant recruitment

In all but two investigations (Chapters 7 and 8) participants were recruited from a pool of healthy, male University students between the ages of 18-40. In Chapters 4, 5, 6 and 9, participants were required to be at least recreationally active, which for the purpose of these investigations was defined as completing some form of planned exercise at least 2 days per week. The inclusion criteria for Chapter 7 required participants to be regularly playing team-sports, at least at an amateur level or University standard. In Chapter 8, participants were recruited from a pool of marathon runners who expressed an interest in taking part in the Druridge Bay marathon.

In all studies, participants were recruited via an expression of interest email or, if applicable, through poster advertisements placed around the University campus or on the student blackboard website. All participants completed a health-screening questionnaire to assess eligibility prior to study entry (see Appendix 3 for an example). Participants were excluded if they had any known food allergy, routinely used anti-inflammatory medications, were suffering from a musculoskeletal injury, or had previous history of renal, gastrointestinal or cardiovascular complications or any other contraindication to the study procedures. The use of any treatments (i.e., massage, cold water therapy, compression garments), nutritional supplements (i.e., whey

protein, vitamin C), or anti-inflammatory drugs (NSAIDS) proposed to enhance recovery were prohibited for the duration of each study.

3.3 Dietary and exercise control

With regards to exercise, in all studies (with the exception of Chapter 4), participants were instructed to limit their physical activity in the 48 hours prior to and for the duration of the data collection period (other than study requirements).

In Chapters 4, 5 and 6, participants were required to follow a low phenolic, betalainic and nitrate diet. This included avoiding all vegetables, cured meats, fruits and their juices, chocolate, wholegrain breads and grains, caffeinated beverages including all varieties of tea, coffee and alcohol. These dietary restrictions began 2 days before the main trials and continued until the end of data collection. Participants were also instructed to eat the same or similar meals throughout this period. The principal aim of these restrictions was to try and eliminate the potential influence consuming phytonutrients from food sources other than the treatment under investigation might have on the outcome measures. Given these were the first studies to investigate BTJ in this manner, it was felt that it was important, from a proof of concept point of view, to establish that BTJ had the potential to be used as a recovery intervention. These restrictions have been employed in other studies to more accurately establish the bioavailability of functional foods (Keane et al., 2016), as well as their effects on EIMD (Bell et al., 2014; Bell et al., 2015). As in these aforementioned studies, all participants were given food diaries (see Appendix 4 for an example) to record their dietary intake so that compliance with the imposed dietary restrictions could be checked.

In contrast, in Chapters 7, 8 and 9, no dietary restrictions were imposed but participants were still required to record their dietary intake (24 h prior to and until the end of data collection) (see Appendix 5 for example). This change in design was to increase the ecological validity of the findings in this thesis,

and make them more applicable to real-world scenarios. In these latter Chapters (7-9), food diaries were analysed for macronutrient content using Nutritics dietary analysis software (Nutritics LTD, Dublin, Ireland). This was to check that the nutritional compositon of dietary intakes were similar between different treatment groups and therefore did not have a large impact on the study results. Finally, in all studies, participants were asked to avoid using antibacterial mouthwash throughout data collection due its potential interference with nitrate-nitrite conversion (Webb et al., 2008).

3.4 Nutritional supplements

The nutritional compositions of the supplements used in the studies in this thesis are detailed in Table 3. The BTJ supplement was comprised of 99% beet juice concentrate and 1% lemon juice, and was available commercially under the brand Love Beets Super Tasty Beetroot Juice (Gs Fresh Ltd, Cambridgeshire, UK). In Chapter 5, the supplement that contained a lower dose of beetroot (LBT) juice contained the same nutritional composition as the BTJ but provided half the dose (HBT; 125 ml). The PLA used throughout this thesis consisted of a low fruit (<1%) squash (Kia Ora, Coca Cola Enterprises, Uxbridge, UK) with negligible phytochemical and nitrate content. Both the PLA and L-BT were fortified with maltodextrin (Myprotein, Manchester, UK), flavourless protein powder (Arla Foods, Amba, Denmark) and water, to match the H-BT drink as closely as possible for volume and nutrient content. The BTJ (batch used in Chapter 4-6) was also analysed for AOX activity, phenolic and betalainic content. These analyses were also performed with the PLA; details of these results can be found in Table 3.

Double-blinding was achieved by providing the beverages in identically masked bottles, only distinguished by a single letter code, which was assigned by an individual not directly involved in the research. Due to the distinct taste of BTJ, the PLA was not matched for taste and texture, only energy content. While others have used a nitrate depleted BTJ as a PLA so

that the taste is the same, this is not a *true* PLA because it will still contain many other bioactive constituents (i.e., phenolics and betalains) that, as outlined in the introduction, could favourably affect exercise recovery and performance. Thus, a nitrate depleted BTJ was not deemed suitable for the studies in this thesis. Although it could have been used in Chapter 9 in place of the SN drink, it could not be sourced from the manufacturer at the time of the study. Instead, other controls were in place to ensure double-blinding. Firstly, the independent groups design used in Chapters 5-9 ensured that participants only consumed 1 of the supplements provided and were therefore unaware of any differences between the supplements under investigation. Furthermore, the participants were not informed of what the specific drinks being investigated were or the study aims. The only information they received was that they were AOX-containing drinks used for exercise recovery. This ensured that the participants did not know the overall aim of the study. It was hoped that this would eliminate any bias based on pre-conceptions of BTJs potential ergogenic effects from performance studies.

Table 3 - Energy and macronutrient content of the 3 main supplements used throughout this thesis.

Treatment	BTJ	L-BT	PLA
Energy (Kcals)	81	79	77
Volume (ml)	250	250	250
Carbohydrate (g)	16.4	16.4	16.4
Protein (g)	2.8	2.8	2.8
Fat (g)	0.4	0.2	Trace
Nitrate (mg)	~250	~125	N/A

Amounts are per bottle. BTJ = Beetroot juice (also referred to as H-BT in Chapter 5 and 6); L-BT = low beetroot juice; placebo = PLA.

3.5 Muscle damaging exercise

The same exercise protocol was used to induce muscle damage in Chapters 5, 6 and 9. The protocol consisted of 100 drop-jumps from a 0.6 m high steel box; each jump was separated by a 10 s interval and each 20 jumps by a 2 min rest period (5 sets of 20 repetitions). Participants were instructed to drop off the box and land on two feet, immediately descending to a ~90º knee angle followed by a maximal effort vertical jump. Participants were demonstrated this technique on several occasions before performing the bout and, if required, given corrective feedback throughout. Each participant received strong verbal encouragement to ensure maximal effort was maintained. This protocol was selected because it has previously been demonstrated to induce significant muscle damage (i.e., increased muscle soreness, loss of muscle function) in the lower limbs (Howatson et al. 2009; Howatson et al., 2012). Details regarding the other muscle-damaging protocols used in this thesis can be found in the relevant Chapters.

3.6 Muscle function

Maximal isometric voluntary contraction (MIVC) and CMJ were used to test muscle function in Chapters 5-9. Reactive strength index (RSI) was used as an additional marker of dynamic muscle function in Chapter 7, because of its applicability to the participant cohort under investigation (team-sports players); the methods used for this measure can be found in the relevant chapter.

3.6.1 Maximal isometric voluntary contraction

MIVC was measured on the right knee extensors using a portable strain gauge (MIE Medical Research Ltd., Leeds, UK). Participants were seated up right on a purpose built chair and had a strain gauge attached to a bespoke perspex manipulandum, which was fitted to their right ankle, just above the malleoli. Participants were required to exert maximal force by extension of

the knee joint for 3 seconds. All efforts were performed at a joint angle equivalent to 90° knee flexion, as assessed by a goniometer, and were recorded in Newtons (N). Each participant performed 3 contractions separated by 60 s of passive (seated) recovery, with the peak value used for analysis. This procedure had been employed in several previous investigations to quantify MIVC (Howatson et al., 2009; Howatson et al., 2012). A reliability trial conducted before data collection revealed that the inter-day coefficient of variation (CV) for this protocol was <1.5%.

3.6.2 Counter movement jump

CMJ performance was measured from flight time using an optical measurement system (Optojump next, Balzano, Italy), which is considered a valid and reliable tool to evaluate CMJ performance (Glatthorn et al., 2011). To perform the jumps, participants stood with feet shoulder width apart and, when prompted, rapidly descended into a squat (to a 90º knee angle) and jumped vertically with maximum force. Participants were instructed to land in the same position as take off and keep their hands on their hips for the full movement to minimise any influence of arm swing on performance. Participants performed 3 maximal efforts, interspersed by 30 seconds passive (standing) recovery with the mean height of the 3 jumps used for analysis. Inter-jump rest times were kept to 30 s because pilot testing revealed that this was sufficient time to allow for full recovery. The inter-day CV for this protocol was calculated as <2.5%.

3.1.6 Muscle soreness

In Chapters 5, 6, 7 and 9, muscle soreness was measured as PPT. Due to practical constraints, PPT could not be measured in Chapter 8; therefore, a VAS was used to assess muscle soreness (details can be found in the relevant Chapter).

3.6.3 Pressure pain threshold

PPT was measured with the use of a handheld algometer (Wagner Instruments, Greenwich CT, US). In all studies, measurements were performed while the participant lay supine. A cylindrical flat-headed probe (1 cm diameter) was used to apply pressure (at a constant rate of 10 N cm^{-2} s^{-1}) on the muscle belly until the participant verbally signified they felt pain or discomfort; this point was recorded as PPT in N^2. Muscle sites were pre-marked with a permanent marker pen to ensure consistency for each visit. Muscle sites were vastus laterialis; mid-way between the superior aspect of the greater trochanter and head of the tibia, rectus femoris; mid-way between the anterior patella and inguinal fold, and gastrocnemius; most medial aspect of the calf at relaxed maximum girth. The average of two values from each site was used for analysis, unless the difference between the two values was ≥10 N^2 apart, in which case a third recording was taken, and the average of the two closest values used for analysis. To increase the reliability of the measurement, measures were taken by the same individual across days (Nussbaum & Downes, 1998). The inter-day CV for this procedure was calculated as <8% (average CV for the 3 sites measured).

3.7 Blood sampling and analysis

In all studies, blood samples were obtained from a branch of the basilica vein at the antecubital fossa. Blood was drawn from a cannula in Chapter 4, with all other studies using the venepuncture technique. In Chapters 4, 5 and 6, samples were collected into 2 × 10 ml vacutainer tubes treated with di-potassium ethylene diamine tetra-acetic acid (EDTA). In Chapters 7, 8 and 9, 1 × EDTA (10 ml) and 1 × serum (10 ml) tubes were collected. All samples were centrifuged at 3000 *g* (4˚) for 10 min; EDTA tubes were centrifuged immediately, while serum tubes were left to clot for 30-45 min beforehand. In all studies, plasma and serum supernatant were aspirated into a series of

aliquots and stored at −80º for later analysis. In Chapter 8, an additional 4 ml EDTA tube was collected for haematological analysis at a local hospital.

If a specific blood marker was measured in ≥2 Chapters, the analytical methods used have been described below. However, for markers exclusive to each Chapter a description of the analytical procedures involved can be found in the relevant methods section.

3.7.1 Creatine kinase

In Chapters 5 and 6, plasma CK concentrations were determined spectrophotometrically using an automated system (Roche Modular, Roche Diagnostics, UK). In Chapters 7-9 CK was determined from serum instead of plasma but the same equipment and procedure was used. Normal reference values for this assay in males is 10-190 $I \cdot UL^{-1}$ and the CV for this analysis is typically <2%.

3.7.2 High sensitivity C-reactive protein

Serum concentrations of high sensitivity C-reactive protein (hs-CRP) were measured in Chapters 7-9. Analysis was performed with an automated system (Cobas 8000 c702, Roche Diagnostics, UK). The CV for hs-CRP analysis using this system is calculated as <5%.

3.7.3 NOx

NOx bioavailability was determined from nitrate and nitrite concentrations using a standard assay kit (R&D Systems, Minneapolis, Minnesota). Analysis was performed using plasma in Chapter 4 and serum in Chapter 9. Irrespective of the biological tissue, the assay quantifies NOx by measuring total nitrite after nitrate has been enzymatically reduced to nitrite via nitrate reductase using the griess reaction. The inter and intra assay CV for these analyses were <15%.

4 The phytochemical content and antioxidant capacity of beetroot juice, and the plasma bioavailability of nitrate and betanin

Publication arising from this Chapter: Clifford, T., Constantinou, C. M., Keane, K. M., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The plasma bioavailability of nitrate and betanin from *Beta vulgaris rubra* in humans. *European Journal of Nutrition*, 1-10.

4.1 Introduction

In recent years there has been a growing interest in beetroot with a particular focus on its potential health benefits (for review, see Ninfali & Angelino, 2013). The interest in beetroot has been largely driven by its nitrate content, which is proposed to be ~1,459 mg·kg^{-1} DW (Lidder & Webb, 2013). Dietary nitrate may confer beneficial health effects via its sequential reduction to nitrite and NOx, a pleiotropic molecule that plays a key role in the regulation of vascular homeostasis, immune function and metabolism (Lundberg et al., 2008; Weitzberg & Lundberg, 2013). There are now several reports that acute consumption of beetroot can stimulate endogenous NOx production and evoke positive changes in endothelial function, blood pressure and metabolism (Joris & Mensink, 2013; Siervo, Lara, Ogbonmwan, & Mathers, 2013; Webb et al., 2008). Consequently, beetroot is currently purported as a health promoting food that might be useful for reducing the risk of developing cardiovascular diseases (i.e., hypertension, stroke) and immune disorders (i.e., inflammatory bowel disease) (Jädert et al., 2012; Kapil et al., 2014; Lidder & Webb, 2013).

As discussed in Chapter 2.7, beetroot contains several other constituents other than nitrate that may have beneficial effects for health. As well as being a good source of dietary polyphenols, beetroot contains a group of betalaimic acid derivatives known as betalains (Esatbeyoglu et al., 2015). Betalains are water-soluble phytochemicals that have been shown to possess anti-inflammatory, AOX and chemo-preventive activities (Esatbeyoglu et al., 2015; Lechner et al., 2010; Ninfali & Angelino, 2013; Vidal et al., 2014). In recent years there has been a particular interest in the biochemical activity of betanin (betanidin 5-O-b-glucoside; chemical structure depicted by Figure 5), which is the most abundant betacyanin in beetroot (300-600 mg·kg^{-1} uncooked weight) (Esatbeyoglu et al., 2014; Esatbeyoglu et al., 2015; Georgiev et al., 2010; Pavlov et al., 2002). As a cationized compound, betanin is a highly effective scavenger of RONS (Esatbeyoglu et al., 2014;

Kanner et al., 2001). Betanin also possesses anti-inflammatory properties; betanin, and its aglycone, betanidin were shown to modulate LOX and COX activity *in vitro*, indicating that betanin might downregulate pro-inflammatory signalling (Vidal et al., 2014). These findings have led to interest in the role of beetroot in the protection against the potentially damaging effects of RONS and aberrant immune function (Kapadia et al., 2011; Kapadia et al., 2013).

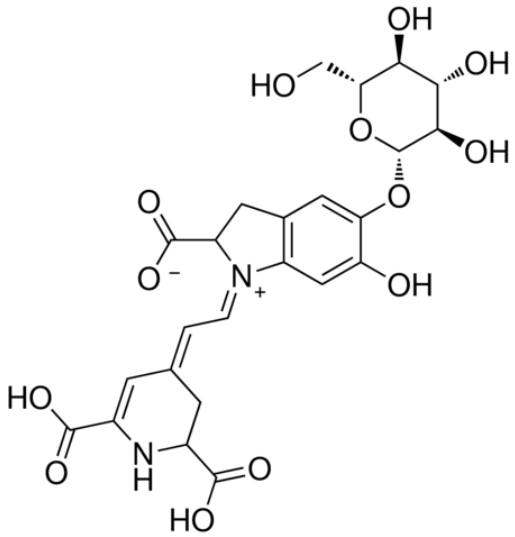

Figure 5 - Chemical structure of betanin (betanidin 5-o-b-glucoside).

An increased awareness of beetroot's potential physiological effects and health benefits, together with an increased demand for convenient health foods, has led to the development of a number of beetroot based juice products. Given the wide array of bioactive compounds present in beetroot, it is possible that regular (or acute) consumption of these juices could have favourable effects for health and wellbeing. However, in order to evaluate the potential usefulness of a commercially available BTJ in the promotion of general health or as a dietary supplement, information on both its phytochemical content and *in vivo* bioavailability in humans is required.

Dietary nitrate is believed to be highly bioavailable (van Velzen, Sips, Schothorst, Lambers, & Meulenbelt, 2008) and increases in the plasma have been observed following ingestion of BTJ (Joris & Mensink, 2013; Wylie et al., 2013). Betalains, in contrast, are thought to have much lower bioavailability (see section 2.7.2); however, no published studies have characterised the bioavailability of betalains in plasma from consuming commercially available BTJ.

Consequently, before beginning to address the main aim of this thesis; whether a commercially available BTJ can attenuate EIMD, it was important to determine the phytochemical content of the BTJ to be used and also the bioavailability of some of its allegedly most bioactive constituents, nitrate and betanin. This information would help to provide a rationale for using BTJ as a recovery intervention for attenuating EIMD. Consequently, this study had two main objectives; 1) to establish the AOX capacity, polyphenol and betalain content of BTJ, and; 2) to determine the bioavailability of betanin, the major betalain in beetroot, and nitrate, a precursor for NOx activity, in human plasma after consuming BTJ.

4.2 Methods

4.2.1 Participants

Ten healthy, non-smoking males (age 23 ± 3 yrs; height 1.82 ± 0.60 m; mass 78.8 ± 6.7 kg) were recruited to participate in this study.

4.2.2 Experimental design

Participants were required to attend the laboratory on 2 occasions, separated by seven days. In the 48 h prior to the first trial, participants were given food diaries and instructed to follow a low phenolic, betalainic and nitrate diet (see Chapter 3.3 for details). They were instructed to replicate this diet as closely as possible in the 48 h before the remaining trial. For each trial, partcipants

attended the laboratory between the hours of 07:00 - 09:00 following a 12 h overnight fast and had a cannula inserted into a vein at the antecubital fossa. After a baseline blood sample, participants were given 1 of 2 treatments; BTJ (250 ml), or an isocaloric PLA (250 ml) in a randomized, crossover fashion. Further blood samples were drawn at 1, 2, 3, 5, and 8 h after ingesting the treatments to ascertain the kinetics of the compounds of interest (see Figure 6 for schematic). Participants were not allowed to consume any food until testing was complete but were allowed water *ad libitum*; the volume of water consumed on the first trial was recorded and replicated in the subsequent trials.

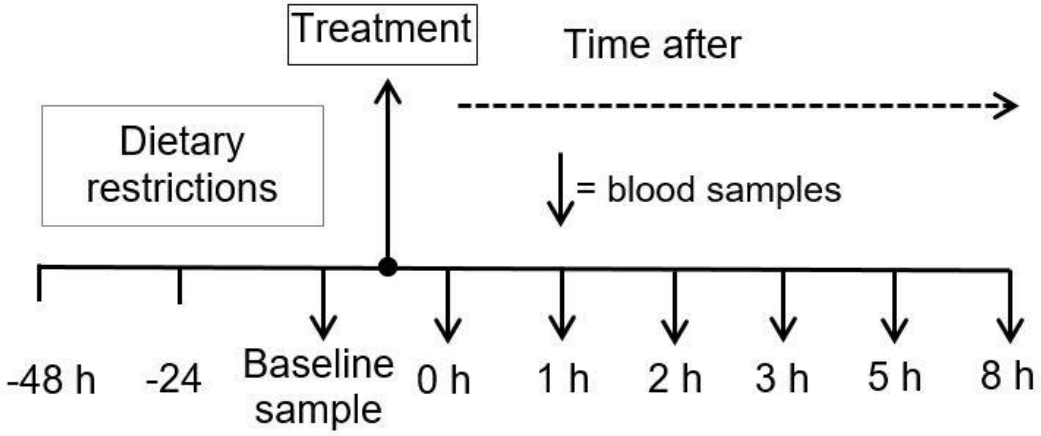

Figure 6 - Schematic outline of bioavailability study procedures.

4.2.3 Blood sampling procedures

For blood sampling procedures please refer to Chapter 3.7.

4.2.4 Supplementation

Information regarding the BTJ and PLA drinks can be found in Chapter 3.4.

4.2.5 AOX activity and phenolic content

Mixtures of PLA and BTJ in the same solvent were used to determine their total phenolic content (TPC) and Trolox equivalent antioxidant capacity (TEAC). A modified 2,2-diphenyl-1-picrylhydrazyl (DPPH•) assay used for AOX activity measurements was adjusted for use in the present study (Brand-Williams, Cuvelier, & Berset, 1995). The DPPH• solution was prepared freshly before the analysis, by dissolving the DPPH• reagent (2.4 mg) in 80% methanol (100 mL). Samples were further diluted in deionised water (1:10 or 1:100) and 10 µL of sample, 40 µL of deionised water, and 200 µL of DPPH• solution were added into each well of the CELLSTAR 96 well plate (Greiner Bio-One, Monroe, USA). Absorbance readings were taken at 515 nm, at 3 min intervals over a 30 min period at 37°C, using a BioTek Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, USA). A calibration curve using Trolox (0-500 µM, R^2=0.99) was plotted. Final values are expressed as means of Trolox equivalents per milligram of sample ± SD for 6 replicants.

TPC was measured using a modified Folin-Ciocalteu colorimetric method (Magalhães et al., 2009; Shahidi & Ho, 2007). Samples were diluted in deionised water (1:10 or 1:100) and 50 µL of the diluted extract. 50 µL of Folin-Ciocalteu reagent diluted in water (1:25) and 100 µL of 6% (w/v) sodium carbonate were then added into corresponding sample wells of a 96 well plate. Absorbance readings were taken at 725 nm, at 5 min intervals, over a 30 min period at 25°C using a BioTek Synergy HT Multi-Mode Microplate Reader. A stock solution of gallic acid (5.9 mM) was prepared in aqueous methanol (80% (v/v)) and quantification was performed on the basis of a standard curve in the range 0-50 mg/mL (R^2=0.99). The analysed samples were measured versus a blank sample. All values are expressed as means of gallic acid equivalents (GAE) per gram of sample ± SD for 6 replicants.

4.2.6 Total betanin and betaxanthin content

The content of betaxanthins and betacyanins in 1:10 juice solutions in aqueous McIlvaine buffer (pH 6.5) was determined at 480 nm and 550 nm with a UV–Vis spectrometer (Ultraspec 2000UV/Vis spectrophotometer, Pharmacia Biotech, Sweden), respectively according to the methods of Cai, Sun, & Corke, (1999) and Moßhammer, Stintzing, & Carle, (2005). Total betalains were quantified using the following equation: betalains [mg/L] = (A × DF × MW × 1000)/(εx1), where A is the absorption value at the absorption maximum, DF the dilution factor, MW the molecular weights and 1 the path length (1 cm) of the cuvette. For quantification of betacyanins and betaxanthins, the MW and molar extinction coefficients (ε) of betanin (MW=550 g/mol; ε=60,000 L/mol cm; λ=538 nm) and indicaxanthin (MW=308 g/mol; ε=48,000 L/mol cm; λ=480 nm) were applied, respectively.

4.2.7 Betanin determination

Betanin standard was purchased from Adooq Bioscience (California, US). Betanin determination of diluted BTJ samples (2.5 mg/mL in 1:1, 0.1% formic acid in water: 2% HCl in MeOH) was carried out on a Dionex UltiMate 3000 RSLC HPLC System (Dionex, Camberly, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000 RS autosampler and a QExactive Quadrupole-Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, USA). Electrospray ionization at negative ion mode was performed with a spray voltage of 2.00 kV and capillary temperature of 280°C. The total ion current (TIC) with a range of 100-1000 m/z and 70000 resolution was measured. The ion m/z 549 was used for quantification of betanin. Sample aliquots (3 µL) were injected on a Phenomenex Luna C18(2) (250 × 2.0 mm, 5 um particle size) reverse-phase column thermostatically regulated at 40°C. The mobile phase consisted of water with 1% acetic acid (solvent A), and acetonitrile with 1% acetic acid (solvent B). After a 6-min equilibration with 20% solvent B, the elution programme was as follows: 0-30 min, 10-100% B,

(0.2 mL/min) followed by a washing stage (100% B, 30-36 min, 0.2 mL/min) and re-equilibration at the initial conditions for 3 min. Betanin with a retention time of 2.56 min was quantified by external standard determination.

4.2.8 Extraction of plasma for betanin determination

Several attempts to extract betanin from plasma samples were performed: a) 1 mL of plasma was mixed with 4 mL oxalic Acid (10 nM) and 0.1 mL HCl (12.6 M) in 15 mL falcon tubes and centrifuged at 826 × *g* for 5 min. The supernatant was absorbed on to a primed solid phase extraction cartridge (Waters Sep-Pak c17 plus short cartridge, 360 mg sorbent per cartridge, 55-105 µm), washed with methanol + 0.2% trifluoroacetic acid (TFA) followed by 2 × 5 mL of water. The sample was eluted with 3 mL of MeOH + 0.2% TFA, dried under N2 at 45°C. Samples were then reconstituted in 400 µl of solvent F: 0.1% formic acid in water: 2% HCl in methanol and filtered through a 0.2 µm polytetrafluoroethylene filter prior to HPLC and LC-MS analyses; b) 1 mL of plasma was extracted with 4 mL 1:1 acetonitrile:water for 10 min and centrifuged for 10 min at 3000 rpm. The supernatant was collected, evaporated to dryness, reconstituted in 1:1 acetonitrile: water and filtered in auto sampler vials. The above two methods analysed various samples at different time points using HPLC/UV/Vis/FLD and liquid chromatography and mass spectroscopy (LCMS) methodologies.

4.2.9 Analysis of plasma NOx

Plasma NOx bioavailability was determined from plasma nitrate and nitrite concentrations. Please refer to Chapter 3.7.3 for further details.

4.2.10 Data analysis

All data are presented as mean ± standard deviation (SD). A two-way, repeated measures analyses of variance (ANOVA) was used to test for between trial differences in plasma NOx concentrations; 2 trials (BTJ vs.

PLA) by 6 time points (baseline, 1, 2, 3, 5 and 8 h post ingestion). In the event of a significant interaction effect (trial*time) Fisher LSD *post hoc* analysis was performed to locate pair-wise differences. Statistical significance was set at $P < 0.05$ prior to analyses. All analysis was performed with IBM SPSS Statistics 20 for Windows (Surrey, UK).

4.3 Results

Inspection of food diaries indicated that participants complied with the imposed dietary restrictions and that their intakes did not significantly differ between trials. No adverse events were reported with any of the supplements.

4.3.1 Betanin content and bioavailability

Betanin was identified in the BTJ with LCMS analysis (Figure 7). Total betanin content for the BTJ is presented in Table 4. Based on these analyses, each bottle of BTJ (250 ml) contained ~194 mg of betanin, No betanin was detected in the PLA used in this study. Betanin could also not be detected in the plasma samples obtained after BTJ and PLA consumption (data not shown).

4.3.2 Antioxidant capacity, phenolic and betalain content

Antioxidant capacity, phenolic content, total betacyanin and total betaxanthin content is presented in Table 4. According to these data, each serving of BTJ (250 ml) had a TEAC of ~3 mmol/L and contained ~405 mg GAE equivalents of phenolic compounds, ~17 mg of betacyanins and ~10 mg of betaxanthins. The PLA (250 ml) contained a small number of phenolic compounds (~43 mg); however, betalains could not be detected and the TEAC was low (<0.5 mmol/L).

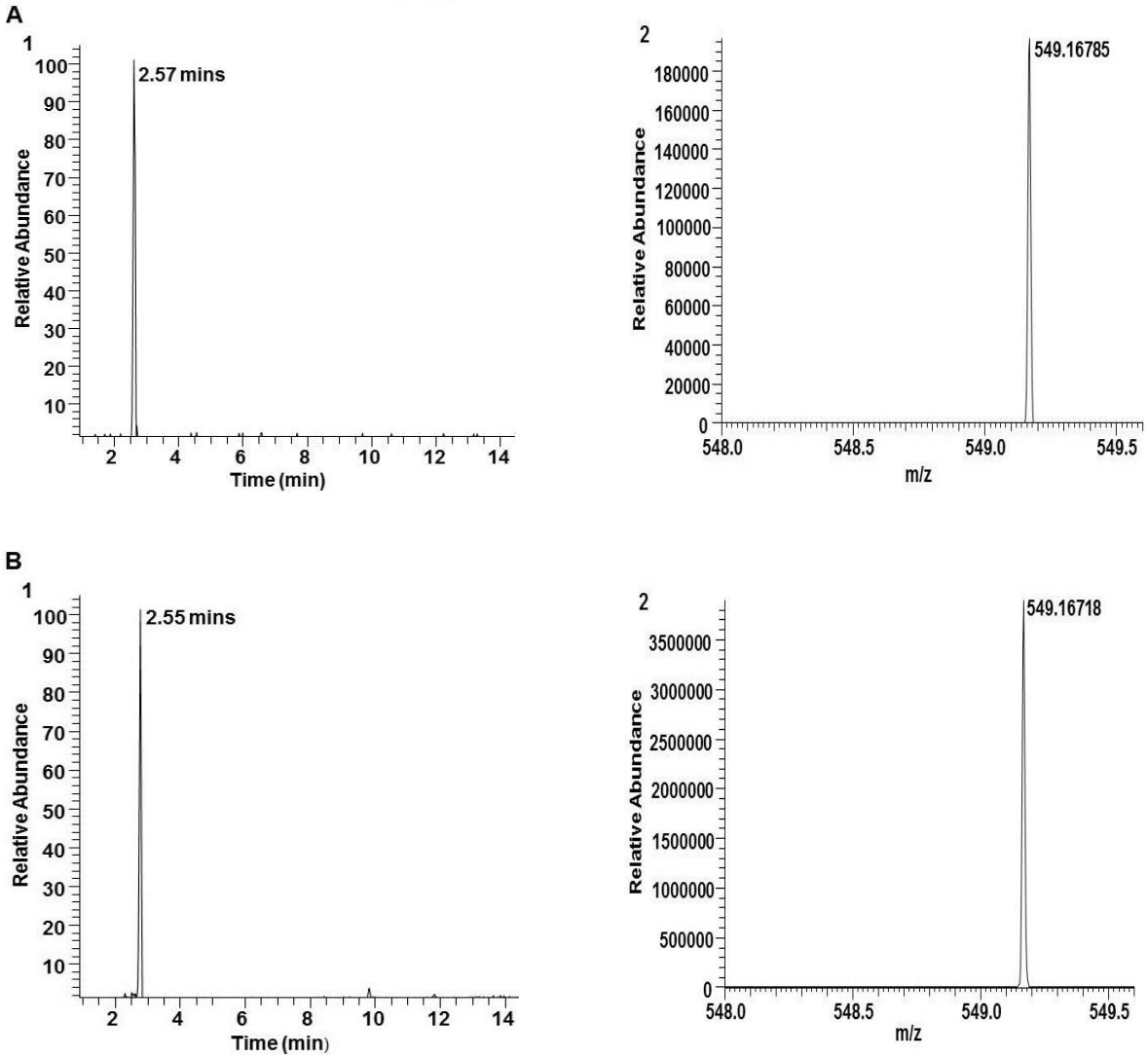

Figure 7- A (1) liquid chromatography mass spectroscopy (LCMS) chromatograms of betanin standard (retention time; RT = 2.57 min) (2) Mass spectroscopy (MS) output of betanin standard (base peak m/z 548.5 – 549.5) B (1) betanin in beetroot juice (BTJ) (RT = 2.55) (2) MS output for BTJ (base peak m/z 548.5 – 549.5).

Table 4 - TEAC, TPC, betanin, betacyanin and betaxanthin content of BTJ and PLA

Treatment	TEAC (mmol/L)	TPC (mg/GAE/ L)	Betanin (mg/L)	Total betaxanthins (mg indicaxanthin equivalents/ L)	Total betacyanins (mg betanin equivalents/ L)
BTJ	11.4 ± 0.2	1606.9 ± 151	777.9 ± 41.3	41.7 ± 0.7	68.2 ± 0.4
PLA	0.25 ± 0.02	172.3 ± 13.3	ND	ND	ND

Values are mean ± SD. GAE, gallic acid equivalent; TEAC, Trolox equivalent antioxidant capacity; TPC, total polyphenol content.

4.3.3 Plasma NOx concentrations

Data are presented in Figure 8. At baseline (0 h), concentrations of plasma NOx were similar between trials ($P > 0.05$). In the PLA trial, there was no change in plasma NOx concentrations at any time point ($P > 0.05$); however, after ingestion of BTJ there was an increase in plasma NOx compared to baseline (time effects; $F_{(5, 45)} = 18.84$; $P < 0.001$) and PLA (drink*time interaction; $F_{(10, 90)} = 5.35$; $P < 0.001$). Plasma NOx reached peak concentrations 2 h post-ingestion in the BTJ group ($P < 0.001$; 163.7 ± 46.9 µmol/L) and was still elevated above baseline values at 8 h post ($P < 0.001$).

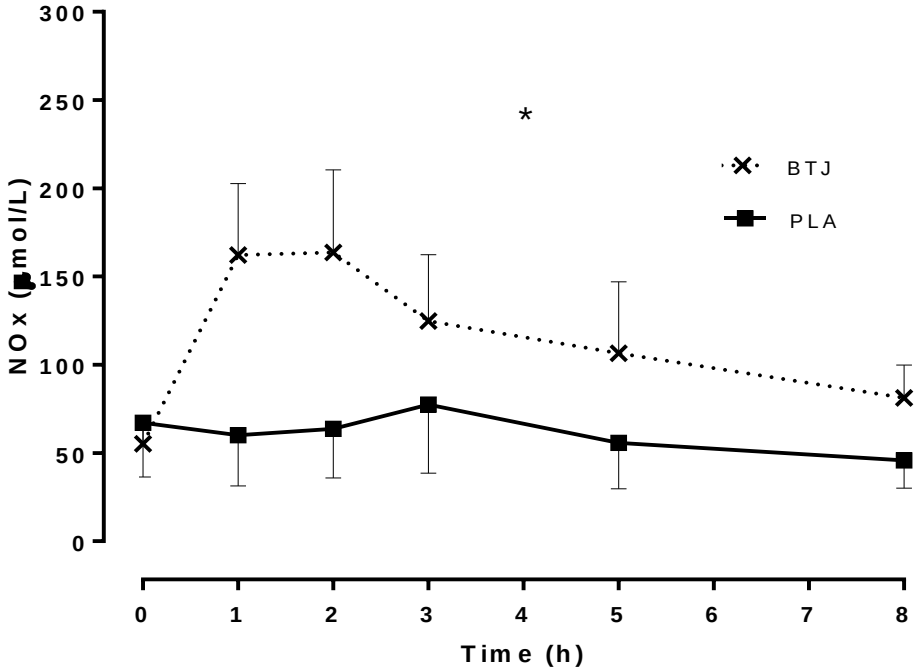

Figure 8 - Plasma nitric oxide (NOx) concentrations after beetroot juice (BTJ) and placebo (PLA) ingestion. Data are means ± SD; *BTJ higher than PLA at all-time points post-baseline ($P < 0.05$).

4.4 Discussion

The present study aimed to determine the phytonutrient content of a commercially available BTJ and the plasma bioavailability of nitrate and betanin in the 8 h after it had been consumed. BTJ was found to be rich in betalains, particularly betanin; however, betanin could not be detected in plasma following consumption. Conversely, and in line with other research (Joris & Menesik, 2013), ingestion of BTJ evoked marked elevations in plasma NOx levels compared to a PLA. BTJ was found to contain significant amounts of polyphenols and AOX compounds. The present findings provide new information regarding the bioavailability and phytochemical content of a commercially available BTJ drink.

The AOX capacity of the BTJ and PLA was measured using the TEAC assay. The TEAC assay estimates AOX capacity by comparing the intervention's scavenging ability to the Trolox standard (van den Berg, Haenen, van den Berg, & Bast, 1999), and is commonly used to provide an index of a food or beverages AOX potential (van den Berg et al., 1999; Pellegrini et al., 2003). The analysis revealed the TEAC for BTJ (~11.4 mmol/L) to be higher than values reported for iced tea, green tea, apple juice, cranberry juice and orange juice (4-10 mmol/L), but lower than acai juice, black cherry juice, blueberry juice and pomegranate juice (12-40 mmol/L) (Seeram et al., 2008). These data indicate that this particular BTJ possesses a good level AOX capacity in comparison to other fruit and vegetable drinks and, therefore, could be a useful supplement for boosting AOX defences and protecting against oxidative damage and inflammation.

The AOX activity of the BTJ can probably be ascribed to the high concentration of polyphenols and betalains it was demonstrated to contain (see Table 4) and also to any synergistic interactions that might occur with these compounds, as suggested previously (Georgiev et al., 2010). As our main focus was on betanin, quantifying individual polyphenols in the BTJ was

beyond the scope of this study. However, according to data from previous investigations, the main polyphenols in beetroot (including other types of BTJ) are phenolic acids (ferulic acid, chlorogenic acid, caffeic acid) and flavonoids (epicatechin, rutin, betagarin) (Georgiev et al., 2010; Kujala, Loponen, Klika, & Pihlaja, 2002; Wootton-Beard et al., 2014), many of which possess high AOX potential (Gülçin, 2012; Velioglu, Mazza, Gao, & Oomah, 1998). Furthermore, the polyphenols in beetroot appear to be well absorbed in humans. Netzel et al. (2005) reported that 51% of the total phenolics (about 338 mg) ingested from a homemade BTJ were detectable in the participant's urine, indicating that several of the polyphenols present in beetroot may be absorbed and made available in the circulation for physiological effects. BTJ was rich in betalain compounds. In accordance with studies on fresh beetroot extracts, the betaxanthin content of BTJ was much lower than the betaycanin content (Stintzing & Carle, 2007). Betacyanin's appear to be stronger AOXs than betaxanthins (Esatbeyoglu et al., 2015; Stintzing & Carle, 2007) and were likely major contributors to the AOX activity demonstrated by the BTJ. This could be because the AOX potential of betanin is believed to be higher than other betalains present in beetroot (Esatbeyoglu et al., 2014; 2015; Kanner et al., 2001).

This is the first study that has characterised the bioavailability of betanin in human plasma following BTJ consumption. Despite the relatively high amount of betanin present in the BTJ, it could not be identified in the plasma at any time point after consumption (1-8 h). These findings conflict with those of a previous study, in which betanin was identified in plasma at relatively high concentrations (~0.2 µmol/l) 2 h after consuming 500 g of fresh cactus pear fruit containing 16 mg of betanin (Tesoriare et al., 2012). However, the discrepant findings between this study and the present investigation could be related to differences in the foods analysed (i.e., cactus pear fruit versus beetroot). This supposition is supported by recent work from Tesoriare and colleagues (2012), which compared the absorption rates of betanin from cactus pear fruit and red beetroot in a simulated *in vitro* model of the

intestinal wall. They showed that epithelial transport was much lower when betanin was derived from red beetroot, speculating that the rate of absorption was inhibited by beetroot's food matrix. This suggests that the bioavailability of betanin may be lower after beetroot consumption compared to other sources of betanin.

Nevertheless, the inability to identify betanin in the plasma in this study suggests that it may be lost or degraded during digestive processes. Previous studies investigating the renal elimination of betalains have indicated that betanin may instead be absorbed as downstream metabolites (Kanner et al., 2001). Kanner and Colleagues (2001) found that after consuming a betanin rich BTJ, isobetanin, but not betanin, could be detected in urine. The authors suggested that betanin undergoes isomerization to isobetanin in the intestinal milieu and may therefore be the major metabolite absorbed after betanin ingestion. In addition to isomerization, there are several other metabolic processes that could degrade betanin and limit its systemic bioavailability, including glycosidase enzyme activity from cellulase (Kanner et al., 2001) or the presence of the pancreatic enzyme amylase (Tesoriere et al., 2008). Such data supports the possibility that betanin is largely metabolized to secondary compounds prior to entering the circulation, which would provide a potential explanation as to why it could not be detected in its intact form in the present study. This raises doubts as to whether the wide array of biological effects displayed by betanin *in vitro* can be extrapolated to *in vivo* conditions. Instead, the *in vivo* biological activity displayed by betanin in some studies (Han, Ma, Zhang, Yang, & Tan, 2015; Tan, Wang, Bai, Yang, & Han, 2015; Tesoriere, Butera, Pintaudi, Allegra, & Livrea, 2004) could be mostly due to the biological effects of secondary betanin metabolites, although this remains to be elucidated. At present, data on the bioavailability of these metabolites or their potential biological activity is not yet available. Unfortunately, it was not possible to unequivocally identify any metabolites of betanin in this study due to appropriate standards for HPLC/LCMS detection not being available. Thus, whether metabolites of

betanin reached the circulation in the present study is purely speculative until clarified with future research. The development of new methodologies, analytical techniques and suitable standards will be required to establish the presence of these compounds. Nonetheless, the fact that betanin does not appear to very bioavailable in BTJ would suggest that perhaps the majority of BTJs AOX effects, at least under *in vivo* conditions, stems from the phenolic compounds present, given their allegedly high bioavailability (Netzel et al., 2005).

Plasma NOx activity was significantly augmented after consumption of BTJ compared to the PLA (Figure 8). These findings agree with a previous study that reported a rapid rise in NOx activity 1-3 h after BTJ ingestion (Joris & Menesik, 2013). These data are important, because an increase in the endogenous NOx pool is associated with a range of physiological effects that might be beneficial to health, such as improved endothelial function, enhanced mitochondrial efficiency, improved metabolic function and immune function (Weitzberg & Lundberg, 2013). Furthermore, as alluded to in section 2.7.4, increased NOx bioavailability might also help to reduce symptoms of EIMD. Thus, these findings support the idea that a commercially available BTJ supplement might help to promote recovery after strenuous exercise.

It is acknowledged that a potential limitation of this study is the absence of urinary measures. Therefore, it cannot be ruled out that betanin would have been detectable in urine voids had these samples been collected after BTJ consumption. Because the urinary excretion of betanin had been described before (Kanner et al., 2001; Netzel et al., 2005), the focus of this investigation was specifically on plasma bioavailability, which had not been characterised after BTJ consumption. It is also acknowledged that restricting the analysis to 8 h post-consumption is also a possible limitation because betanin might have appeared in the plasma at later time points. However, it was felt that this was improbable given that previous work in cactus pear fruit

showed plasma betanin concentration peaked at 3 h post consumption and was undetectable at 8 h post (Tesoriere et al., 2004a).

Despite the aforementioned limitations, the data presented in this study provides new information on the bioavailability and phytochemical content of commercially available BTJ, which is of use to practitioners interested in the potential health benefits of these products. Future research on the bioavailability of betanin metabolites and their potential for biological activity is required to further exude the potential benefits of BTJ for supplemental use.

4.5 Perspectives

The overall aim of this thesis is to determine the efficacy of BTJ supplementation on recovery following muscle-damaging exercise. However, before testing the efficacy of BTJ as a recovery aid, it was important to establish that it contained the compounds shown to exert beneficial biological effects, especially those that could attenuate EIMD. This study succeeded in showing that BTJ contained a number of active compounds in amounts comparable to other functional foods, and perhaps most importantly, to those that have been shown to favourably affect recovery and oxidative stress after exercise such as cherry juice (Howatson et al., 2010), pomegranate juice (Trombold et al., 2010; 2011) and green tea (Jówko et al., 2011).

In line with our hypothesis, the nitrate in BTJ was found to be highly bioavailable in plasma. The large increases in plasma NOx after BTJ suggests that nitrate could afford some beneficial biological effects *in vivo.* In contrast, the poor bioavailability of betanin suggests that the beneficial effects of betalain or BTJ ingestion on inflammation or other biological actions demonstrated *in vivo* might not be attributable to betanin *per se*, as often alleged. Rather, these findings could be due to the biological actions of other compounds in BTJ, such as betaxanthins, polyphenols and nitrate, or metabolites of betanin. It is also important to recognise that the relative

contribution of each compound to the biological actions of BTJ is likely to be very complex under *in vivo* conditions and that additive and synergistic effects between these compounds are probable. Nonetheless, this investigation supports the hypothesis that BTJ has a favourable phytonutrient composition and, thus, like other functional food based supplements, might be of benefit for recovery following strenuous exercise. Thus, the aim of the following Chapter is to investigate whether BTJ can accelerate recovery after eccentric exercise.

5 The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise

Publication arising from this Chapter: Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. (2016). The effects of beetroot juice supplementation on indices of muscle damage following eccentric exercise. *European Journal of Applied Physiology*, *116*(2), 353-362.

5.1 Introduction

The previous Chapter demonstrated that BTJ is rich in a number of phytonutrients shown to exhibit potent AOX and anti-inflammatory effects. Importantly, these findings help provide a rationale for the main aim of this thesis: to investigate the efficacy of BTJ as a recovery intervention. Accordingly, this Chapter examined the effects of acute BTJ supplementation on indices of muscle damage following eccentric-heavy exercise. As alluded to in Chapter 2.3, EIMD is often accompanied by an acute phase inflammatory response that might prolong muscular dysfunction (Pizza et al., 2005; Zerba et al., 1990). Briefly, phagocytic immune cells such as leukocytes, and signalling molecules such as growth factors and cytokines, accumulate at the damaged site in order to repair the tissue; however, the phagocytic phase of the regenerative process may exacerbate cytoskeletal degradation via the release of cytotoxic RONS and other proteolytic molecules (Pizza et al., 2005; Toumi & Best, 2003). This inflammatory response might cause additional physical damage to myofibrils (Lapointe et al., 2002; Pizza et al., 2005; Zerba et al., 1990), and is proposed, at least in part, to contribute to the muscle soreness, and prolonged loss of muscle function typically observed 24–96 h after muscle damaging exercise (Howatson and van Someren, 2008; Toumi & Best, 2003).

Consequently, attempts to attenuate the deleterious effects of muscle damage have focused on nutritional supplements that target the inflammatory response, thereby reducing the potential for any secondary further muscle damage (Bowtell et al., 2011; Bell et al., 2014; Howatson et al., 2010; Trombold et al., 2010). The previous Chapter demonstrated that BTJ is rich in several anti-inflammatory and AOX containing compounds, indicating that it might be of use in attenuating any potential secondary muscle damage. Of these compounds, betacyanins have generated particular interest because of their purported AOX, anti-carcinogenic and anti-inflammatory functions both *in vitro* (Esatbeyoglu et al. 2014; Lechner et

al. 2010; Vidal et al. 2014) and *in vivo* (El Gamal et al. 2014; Szaefer et al. 2014) and these have been discussed in detail in Chapter 2.7. While Chapter 4 raised questions as to whether betanin, at least in its intact form, exhibits these effects in humans, secondary metabolites of betanin, or other phytonutrients (i.e., phenolic compounds) in beetroot could exert similar effects. This possibility is supported by previous studies, in which betalain supplementation demonstrated AOX anti-inflammatory effects in humans (Pietrzkowski et al. 2010; Pietrzkowski et al. 2014).

In addition, the nitrate in BTJ may also be beneficial for attenuating EIMD. A number of studies have demonstrated that nitrate supplementation, via its sequential reduction to NOx, can reduce leukocyte and cytokine activity; effects that, conceivably, might help to dampen an inflammatory response (Jädert et al., 2012; Justice et al., 2015). These data, coupled with the known AOX and inflammatory effects of betalains, make the expectation tenable that BTJ could be used to attenuate EIMD and, potentially holds promise as a beneficial recovery beverage following exercise. Therefore, the main purpose of this Chapter was to establish whether an acute dose of BTJ would help aid recovery following a strenuous bout of eccentric-heavy exercise in the form of 100 drop jumps. Also, given that this was the first study to investigate BTJ in this manner, it seemed prudent to evaluate the potential for dose-response effects with acute supplementation. This data would enable more cognizant decisions regarding the optimal dose required to elicit effects for future investigations in this thesis. Thus, the effects of BTJ when administered in both a high (H-BT) and low dose (L-BT) was examined compared to a PLA. It was hypothesized that given its high nitrate, phenolic, and betalain content, BTJ would, in a dose dependent manner, help to protect against the potentially harmful effects of acute exercise-induced inflammation, thereby facilitating a faster recovery of muscle function.

5.2 Methods

5.2.1 Participants

A power calculation was conducted to determine an adequate sample size for this investigation. Using the findings of previous studies that examined group differences in isometric strength (Bell et al., 2015; Howatson et al., 2010), it was estimated that a ≥10% group difference (SD: 7.5%, based on % change from baseline data) in one of the primary outcome variables (MIVC), would be required to detect significant changes. Thus, with a power of 0.80 and two tailed α level of 0.05, the estimated number of participants required was n = 9 per group. Consequently, 30 healthy, recreationally active males met the inclusion criteria (see Chapter 3.2) and were recruited to participate in the study (characteristics presented in Table 5). None of the participants had previously completed the protocol used to induce muscle damage in this study.

Table 5 - Descriptive data for the three supplement groups; high beetroot (H-BT), low beetroot (L-BT) and placebo (PLA).

Group	Baseline MIVC (N)	Age (Years)	Height (m)	Weight (kg)
H-BT	602 ± 144	22 ± 6	1.80 ± 0.57	74 ± 6
L-BT	596 ± 112	21 ± 3	1.75 ± 0.87	76 ± 8
PLA	602 ± 133	21 ± 3	1.79 ± 0.84	77 ± 11

Values are mean ± SD (n = 10 per group). No significant differences were detected between groups for any variable (P > 0.05).

5.2.2 Experimental design

This study employed a double blind, independent groups design with three experimental treatment arms; participants were allocated to receive a HBT, a L-BT drink or a PLA for 3 days after a bout of muscle-damaging exercise.

Treatment groups were matched according to MIVC scores recorded at familiarisation. One week after familiarisation participants were required to attend the laboratory for 4 consecutive days. Each visit was at the same time of day and was preceded by an overnight fast. On their first visit, dependent measures were taken pre and immediately post muscle-damaging exercise in the following order: muscle soreness (PPT), venous blood draw, CMJ and MIVC. The muscle-damaging bout consisted of 100-drop jumps. Following the post-exercise measures, participants were given one serving of their assigned supplement and a standardized breakfast meal, followed by a further blood draw 90 min post ingestion. The breakfast meal consisted of 2 slices of toasted bread with butter, providing 246 kcal, 32.8 g carbohydrate, 5.6 g protein and 10.3 g fat. During the 90 min rest period, participants were allowed to consume water *ad libitum* but were required to avoid consuming any other foods until the final blood draw. On the following 3 days (24, 48 and 72 h post muscle-damaging exercise) participants returned to the laboratory to repeat the dependent measures and to consume their allocated supplements (24 and 48 h post only). On each visit participants performed a 5 min warm up on a treadmill at a self-selected pace that corresponded to 12 on the rate of perceived exertion (RPE) scale (Borg, 1982).

5.2.3 Supplementation and dietary control

The macronutrient compositions of the supplements used in this investigation are presented in Chapter 3.4. Participants consumed 3 servings (250 ml per serving) of their assigned beverage on the day after exercise (H-BT, L-BT or PLA); one 30 min post-exercise alongside a breakfast meal, one 90 min later, and a third with their evening meal. Participants consumed 2 more servings at 24 and 48 h post (one within 30 min of leaving the laboratory and another with their evening meal). The rationale for providing 2 daily servings of each supplement (one in the morning and one in the evening) was based on previous studies with cherry and pomegranate juice that showed such a strategy was effective in the prevention of some aspects of EIMD (Bell et al.,

2014; 2015; Howatson et al., 2010; Trombold et al., 2010). The rationale for providing an additional serving on day 1 (2 h post-exercise) was based on observations that inflammation is more pronounced ≤24 h after an eccentrically-damaging bout of exercise (Pizza et al., 2001; Chatzinkolaou et al., 2010) and thus it was speculated there may be an amplified requirement for exogenous phytochemicals at this time. Please refer to Chapter 3.4 for details regarding the double-blind procedure. Participants were also required to avoid foods with a high phytochemical, nitrate or nitrite content 48 h prior to and during data collection (see Chapter 3.3 for further details and appendix 4 for an example food record diary).

5.2.4 Muscle damaging exercise

The muscle-damaging bout of exercise consisted of 100 drop jumps from a 0.6 m high box. Please refer to Chapter 3.5 for further details.

5.2.5 Muscle soreness

Muscle specific soreness was assessed as PPT. See Chapter 3.6.3 for further information.

5.2.6 Maximal isometric voluntary contraction

Please refer to general methods Chapter 3.6.1 for details on MIVC measurement.

5.2.7 Counter movement jump

Please refer to general methods Chapter 3.6.2 for details on CMJ measurement.

5.2.8 Blood sampling and analysis

See Chapter 3.7 for blood sampling procedures. Determination of plasma CK is described in section 3.7.1. Plasma IL-6, IL-8, IL-1β and TNF-α were analysed on a multiplex plate using an electrochemiluminescence sandwich immunoassay (Meso Scale Discovery, Sector Imager 2400, Rockville, USA). IL-1β concentrations could only be detected in <5% of samples and were therefore excluded from analysis. Intra assay CV's were <12%, <4% and <6% for IL-6, IL-8 and TNF-α, respectively; samples for individual participants were analysed on the same plate to avoid inter plate variation.

5.2.9 Data analysis

All data are expressed as mean ± SD and were analysed using IBM SPSS Statistics 21 for Windows (Surrey, UK). To test for differences between participant group characteristics, one-way independent analyses of variance (ANOVA) were performed. As is typical in most studies examining the effects of an intervention on EIMD (Bowtell et al.,2011; Howatson et al., 2009; Howatson et al., 2009; Kirby et al., 2012; Trombold et al., 2010; 2011) data analysis for functional (also primary) outcome measures (MIVC, CMJ and PPT) was conducted on data corrected to percentage change from baseline. This was to account for the large inter-participant variability typically seen with these markers in response to muscle-damaging exercise (Sayers & Clarkson, 2002). For consistency, this same method for data analysis was used throughout this thesis; however, where relevant, absolute values have been provided alongside data corrected to percentage change from baseline for illustrative purposes. All dependent variables were measured using a mixed model ANOVA; 3 group levels (H-BT vs. L-BT vs. PLA) by 5 time levels (pre-exercise, post-exercise, 24, 48 and 72 h post-exercise). In the event of a significant interaction effect (treatment*time) Fisher LSD *post hoc* analysis was performed to locate where the significant differences occurred. Were relevant, Cohen's *d* effect sizes (ES) were calculated with the

magnitude of effects considered small (0.2-0.49), medium (0.5-0.79) and large (≥0.8). Statistical significance was set at $P < 0.05$ prior to analyses.

5.3 Results

There were no significant differences in physical characteristics (height, weight, age), and baseline MIVC values between treatment groups ($P > 0.05$, see Table 5). Close inspection of the food diaries revealed that all participants complied with the imposed dietary restrictions. All muscle function (CMJ, MIVC) and soreness (PPT) measures showed time effects ($P < 0.05$) indicating that the exercise protocol was effective at inducing muscle damage.

5.3.1 Muscle function

Immediately following the muscle-damaging bout, CMJ height (relative to baseline) declined to a similar extent in all 3 groups (H-BT: 17 ± 9.7% vs. L-BT: 17.5 ± 7.5% vs. PLA: 15.4 ± 6.5%). There was however, a treatment*time interaction effect ($F_{(2,27)} = 2.87$; $P = 0.031$) with *post hoc* analysis revealing that CMJ height recovered faster with H-BT vs. PLA at 48 ($P = 0.009$; ES = 1.00) and 72 h post exercise ($P = 0.046$; ES = 1.25). In the H-BT group, CMJ height was 91.7 ± 12.4 and 93.4 ± 7.6% of baseline values at 48 and 72 h respectively, while at the same in the PLA group, CMJ height was 75.3 ± 17.3 and 86.1 ± 5.9%, respectively (Figure 9). There were no group or interaction effects for MIVC ($P > 0.05$) (Table 6).

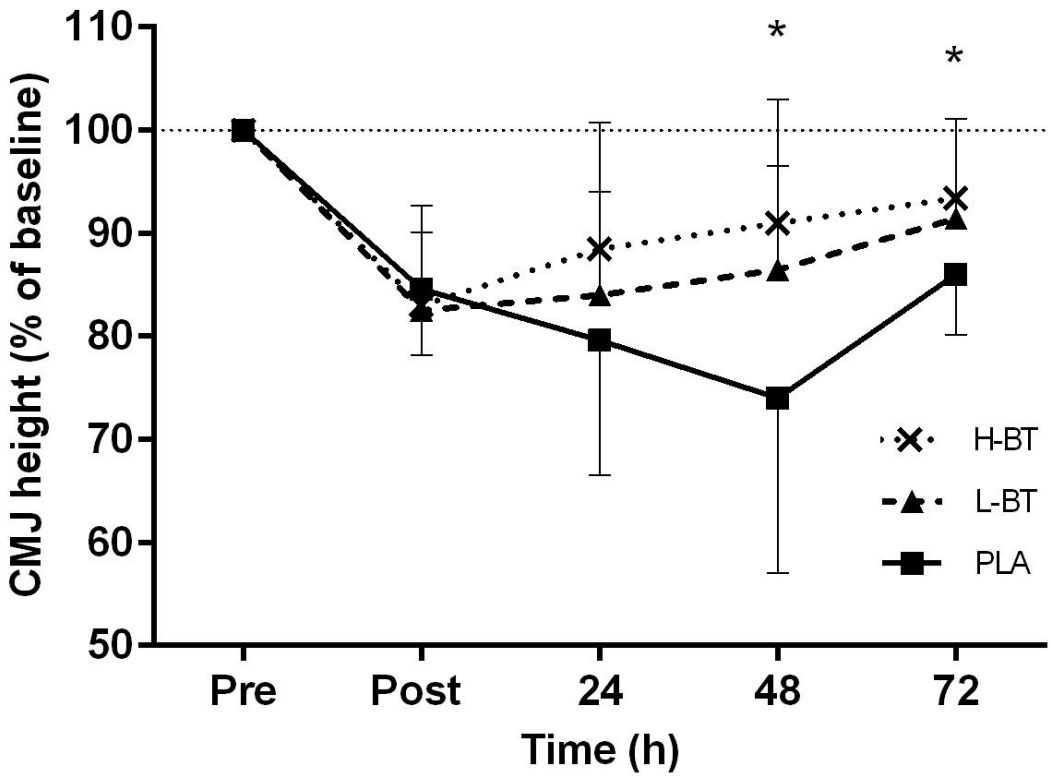

Figure 9 - Percentage changes in counter movement jump (CMJ) height before and after muscle damaging exercise. *Represents between group difference (high beetroot juice; H-BT vs. placebo; PLA, $P < 0.05$). Values are means ± SD ($n = 10$ per group).

5.3.2 Muscle soreness

Muscle soreness, as measured by PPT (sum of all 3 muscle sites), showed interaction effects ($F_{(7, 308)} = 7.83$; $P < 0.001$); *post hoc* analysis revealed a reduction in PPT in the PLA group vs. both the H-BT and L-BT groups at 24, 48 and 72 h post exercise ($P < 0.001$; Figure 10). At 72 h post exercise, PPT had returned to baseline values in the H-BT and L-BT groups (103.5% and 103% of initial values, respectively) but remained significantly reduced in the PLA group (80% of initial value; $P < 0.001$).

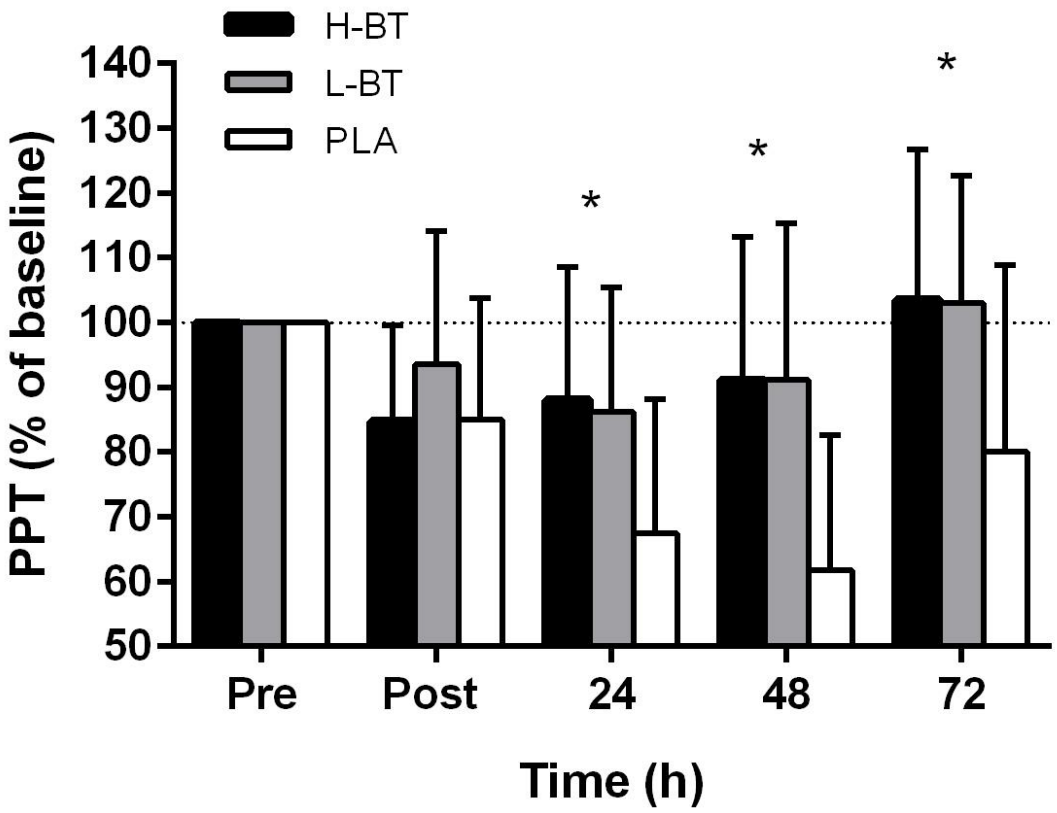

Figure 10 - Percentage changes in pressure pain threshold (PPT) before and after muscle damaging exercise. Values presented are average of the three sites measured (calf, CF; rectus femoris, RF; vastus laterialis, VL). *Indicates between group difference for high beetroot juice (H-BT) and low beetroot juice (L-BT) vs. placebo (PLA); $P < 0.001$. Values are means ± SD (n = 10 per group).

5.3.3 Blood markers

Plasma CK demonstrated main effects for time ($F_{(2, 52)} = 9.99$; $P < 0.001$), peaking at 24 h post exercise in all groups; however, no group or interaction effects were detected ($P > 0.05$; Table 6). Main effects for time were also observed for IL-6 ($F_{(2, 47)} = 6.47$; $P = 0.003$), which was elevated immediately and 2 h post exercise but returned to baseline values by 24 h post; no group or interaction effects were detected ($P > 0.05$; Table 6). TNF-α did not

demonstrate a main effect for time ($F_{(3, 58)} = 1.04$; $P = 0.374$); a time*group interaction effect was found ($F_{(5, 58)} = 2.55$; $P = 0.035$), but no significant group differences were detected with *post hoc* analysis. IL-8 was not significantly altered by the exercise protocol, showing no time, group or interaction effects ($P > 0.05$).

Table 6 - MIVC, CK, IL-6, IL-8 and TNF-α values pre and post muscle damaging exercise.

Variable	Pre exercise	Post exercise	2 h post exercise	24 h post exercise	48 h post exercise	72 h post exercise
MIVC (N)*#						
H-BT	594(100) ± 134(0)	489(81) ± 162(13)		510(84) ± 183(15)	533(89) ± 202(19)	551(93) ± 174(15)
L-BT	555(100) ± 102 (0)	460(83) ± 102(7)		462(85) ± 93(10)	499(92) ± 100 (8)	525(96) ± 120(9)
PLA	601(100) ± 152(0)	463(79) ± 126(12)		482(82) ± 141(15)	491(83) ± 138(16)	528(89) ± 138(16)
CK ($IU \cdot^{-1}$)*						
H-BT	183 ± 102	218 ± 110	320 ± 158	572 ± 442	431 ± 260	319 ± 217
L-BT	263 ± 152	297 ±165	370 ± 156	597 ± 400	335 ± 222	236 ± 101
PLA	295 ± 248	350 ± 28	437 ± 424	679 ± 512	533 ± 384	543 ± 443
IL-6 ($pg \cdot ml^{-1}$)*						
H-BT	0.46 ± 0.26	0.87 ± 0.55	0.79 ± 0.53	0.45 ± 0.23	0.27 ± 0.29	0.25 ± 0.24
L-BT	0.52 ± 0.20	0.75 ± 0.35	0.65 ± 0.31	0.53 ± 0.21	0.50 ± 0.22	0.45 ± 0.26
PLA	0.38 ± 0.18	0.56 ± 0.18	1.19 ± 0.97	0.55 ± 0.30	0.59 ± 0.50	0.66 ± 0.32
IL-8 ($pg \cdot ml^{-1}$)						
H-BT	4.01 ± 2.53	4.62 ± 1.87	3.63 ± 1.65	4.09 ± 2.51	3.59 ± 1.57	3.53 ± 2.29
L-BT	3.42 ± 1.50	3.72 ±1.54	3.47 ± 1.09	3.63 ± 1.42	3.26 ± 1.30	3.00 ± 1.25
PLA	2.80 ± 0.62	3.15 ± 0.81	2.91 ± 0.57	2.99 ± 0.76	3.09 ± 1.35	3.58 ± 1.99
TNF-α ($pg \cdot ml^{-1}$)						
H-BT	2.98 ± 1.08	2.90 ± 1.31	2.77 ± 1.03	2.97 ± 1.13	2.76 ± 0.98	2.24 ± 1.01
L-BT	2.39 ± 0.39	2.30 ± 0.49	2.26 ± 0.44	2.26 ± 0.44	2.34 ± 0.49	2.23 ± 0.48
PLA	2.45 ± 0.72	2.29 ± 0.56	2.46 ± 0.70	2.57 ± 0.67	2.76 ± 1.09	2.79 ± 1.16

Values are mean ± SD (*n* = 10 per group). *Denotes time effect*; P* < 0.05. #Values in brackets represent % change from baseline data. MIVC, maximal isometric voluntary contraction; CK, creatine kinase; IL-6, interleukin-6; IL-8, interleukin-8; TNF-α, tumor necrosis factor-alpha.

5.4 Discussion

The main findings of the present study were that; 1) three days of supplementation with H-BT facilitated a more rapid recovery of CMJ performance 48 and 72 h following exercise, and; 2) consuming both a lower dose (125 ml) and higher dose (250 ml) of BTJ reduced muscle soreness 24–72 h post-exercise. Other markers of functional recovery, muscle damage, and inflammation were unaffected by BTJ.

Although a group difference in MIVC was not detected, CMJ performance recovered quicker in H-BT vs. PLA at 48 and 72 h post exercise, suggesting that BTJ conferred a protective benefit against the acute loss in neuromuscular function. Group differences (vs. PLA), at least stastically, were only evident with H-BT, suggesting that a higher dose (250 ml) might be more beneficial for attenuating acute losses in muscle function. This may be explained by the higher quantity of AOX and anti-inflammatory compounds presumably within H-BT; however, further research is required to clarify the precise mechanisms involved. It is important to note however, that although the differences in CMJ performance between L-BT and PLA was not statistically different at these time points post-exercise, there was still a trend towards a large improvement with L-BT (Figure 9), and perhaps with greater statisctical power significant differences would have also been detected with this dose. It therefore cannot be ruled out that similar benefits might be afforded by the L-BT dose as well. Studies that specifically assess the minimal effective dose of BTJ on recovery are needed in the future to help inform practical use of this supplement. Nevertheless, this is the first study to report that an acute BTJ intervention can enhance the recovery of CMJ performance following a damaging bout of eccentric exercise. While several studies have reported improved recovery of isometric strength after consuming fruit containing beverages, such as cherry (Bell et al., 2015; Bowtell et al., 2011; Howatson et al., 2010), pomegranate (Trombold et al.,

2010; Trombold et al., 2011) and blueberry juice (McLeay et al., 2012), these studies did not include measures of CMJ.

Unlike the aforementioned studies that reported improved recovery of isometric strength, the present study did not detect any group differences for MIVC. This was a somewhat surprising finding and difficult to explain given the improved recovery observed for CMJ performance. This finding doesn't appear to be related to differences in reliability between these measures as both were calculated to have similar CV values ~2% (see section 3.6.2). It is conceivable however, that the disparity is an artefact of the mechanical differences between these variables. In accordance with the principle of specificity, tests of muscle function are more sensitive to detect performance changes if they closely simulate the movement patterns associated with the exercise task in question (Baker, Wilson, & Carlyon, 1994; Gathercole et al., 2015). In this study, the drop jump protocol used to elicit damage required participants to perform a countermovement (descend to a 90° knee angle) prior to each jump, and therefore the kinematic characteristics (i.e., angular velocity, involved muscles, speed of contraction) closely resembled that of the CMJ. In comparison, the movement patterns and mode of contraction required to perform a MIVC are less analogous to the drop jumps; MIVC measures force production when muscle length is unchanged and requires no dynamic movement (Byrne & Eston, 2002). Thus, given that the movements for drop jumps and counter movement jumps are similar, the latter might have been a more sensitive and specific test for detecting subtle performance changes than MIVCs in the present study. It would be useful for future studies to include measures of both dynamic and isometric performance in response to EIMD to shed more light on the potential differences between these two measures.

Although the maintenance of CMJ performance by BTJ points towards a potential blunting of the secondary damage response, large between group changes in indices of inflammation were not observed. However, perhaps

this is not unsurprising given that the alteration in inflammatory indices was small; there was a modest increase in IL-6 immediately and 2 h post exercise, but TNF-α and IL-8 were not elevated above baseline values at any time points or in any of the groups. This would suggest that either the exercise protocol was not sufficiently damaging to induce an inflammatory response (or one large enough to be detected systemically) or that these markers are not representative of the inflammatory response to eccentric exercise. With regards to the first point, it was anticipated that the drop jump protocol would induce a systemic inflammatory response, given that analogous bouts of plyometric exercise has evoked marked inflammatory responses in previous studies (Chatzinkolaou et al., 2010; Dousset et al., 2007), including significant elevations in leukocyte numbers and IL-6 up to 24 h post-exercise (Chatzinkolaou et al., 2010). The discrepancy in findings between these studies and the present study could therefore be due to differences between participant cohorts and/or analytical methods used. With regards to the second point, it was recently proposed that exercise induced changes in TNF-α and IL-8 concentrations are much greater in the muscle and interstitial cells than the circulation (Paulsen et al., 2012; Peake et al., 2015a). As a result, the systemic concentrations of these markers might not have adequately reflected the inflammatory response elicited by the exercise protocol, and therefore the ability to detect an anti-inflammatory effect with BTJ was limited. Notwithstanding, there was a strong rationale for examining these cytokines, since the only previous study measuring the anti-inflammatory effects of beetroot in humans found a marked reduction in plasma TNF-α and IL-6 concentrations after taking a beetroot based supplement (Pietrzkowski et al., 2010); thus, it was felt they might represent a potential mechanism by which beetroot mediates anti-inflammatory effects. Furthermore, several previous studies examining the influence of AOX rich foods on exercise-induced inflammation detected changes in these markers (Bell et al., 2015; Howatson et al., 2010). Perhaps measuring the level of inflammation in the exercised muscles through biopsies, or with a wider array

of systemic inflammatory markers would have provided a greater insight into the exercise-induced inflammatory response in this study.

There was a substantial reduction in PPT in the 72 h period post exercise, indicating that the drop jumps effectively induced muscle pain. These findings are in accordance with previous research that also noted a decrease in PPT following a single bout of strenuous eccentric exercise (Bowtell et al., 2011; Connolly et al., 2006). In this study, PPT recovered to baseline values more rapidly with H-BT and L-BT compared to PLA. Furthermore, the recovery of PPT did not differ between H-BT and L-BT groups, indicating that the lower dose was equally as effective in reducing muscle pain. These findings concur with two recent studies that showed a betalain-rich supplement derived from whole beetroots dampens joint pain from chronic knee discomfort (Pietrzkowski et al., 2014) and osteoarthritis (Pietrzkowski et al., 2010). Although the precise events leading to muscle pain still remain to be fully elucidated, the most recent evidence indicates that intramuscular generation of noxious stimuli such as nerve growth factor (NGF), bradykinin, and prostaglandin E2 (PGE_2), are likely to play important roles (Hyldahl & Hubal 2014; Mizumura & Taguchi, 2015; Murase et al., 2010). These substances can be synthesized by immune cells and act to excite and sensitize local muscle nociceptors. This can result in feelings of soreness and pain in the muscle belly and connective tissues (Hyldahl & Hubal 2014; Mizumura & Taguchi, 2015; Murase et al., 2010). Unfortunately, it was not possible to determine the presence of these substances (i.e., PGE_2 or NGF) or the local inflammatory responses in this study, and thus whether changes in these indices contributed to the findings of the present study is unclear and needs to be clarified in future research.

In the present study, there were no group differences in plasma CK after exercise, which is consistent with previous studies that report CK concentrations were unaffected by cherry juice (Bell et al. 2015; Howatson et al. 2010) and pomegranate juice (Trombold et al., 2010). This suggests that

BTJ and AOX rich foods in general might not be beneficial for attenuating damage to the cell membrane resulting from eccentric exercise. However, a large inter-individual variability in CK efflux could have limited our ability to detect any group differences (Paulsen et al., 2012).

It is important to acknowledge the limitations in this study that might affect the ecological validity of these findings. Firstly, the participants in the present study were recreationally active exercisers, which potentially limit the application of these findings to well-trained athletic populations. Multiple sprint sport athletes, for example, are likely to be more accustomed to high force eccentric contractions and therefore less susceptible to muscle damage and changes in neuromuscular function. Secondly, it is acknowledged that instructing the participants to follow a diet low in AOX, phenolic, and nitrate containing foods throughout the study could have influenced the results. Therefore, future studies are needed to establish whether the present results can be replicated when participants consume their habitual diet.

In summary, the present study demonstrated that supplementing with BTJ for 3 days following a damaging bout of eccentric exercise improved certain aspects of exercise recovery, specifically, muscle soreness and the decrement in CMJ performance. These findings suggest that BTJ supplementation might serve to protect against the negative effects of EIMD, which might be of particular benefit to individuals required to perform several bouts of potentially damaging exercise in a short time frame.

5.5 Perspectives

The overall aim of this thesis is to test the efficacy of BTJ as a recovery aid following strenuous exercise. This study addressed the second question posed in the introduction of this thesis: does acute BTJ supplementation attenuate indices of muscle damage and enhance recovery from eccentric-heavy exercise, and are these effects dose-dependent. Findings show that

BTJ provided relief from muscle pain in the days after exercise and helped to maintain some aspects of muscle function.

While muscle pain was attenuated with both the lower and higher dose of BTJ, muscle function, as measured by CMJ height, was only improved with the higher dose. Therefore, this study indicated that a larger serving (1 full 250 ml bottle) of BTJ was a more effective strategy for attenuating EIMD, and provides rationale for its use in future investigations in this thesis.

Because a systemic inflammatory response, as measured by changes in cytokines, was largely absent after exercise, the beneficial effects of BTJ could have been due to mechanisms at the local level. Alternatively, it could be that these markers did not provide an adequate reflection of the inflammatory response in this model. Therefore, other markers may be more appropriate for future investigations in this thesis. Notwithstanding, it is important to consider these findings in view of the potential negative effects of AOX containing supplements on exercise-induced adaption. Although to date, there are no reports of a functional food supplement interfering with exercise adaptation, this could be because such a possibility is yet to be adequately investigated. Consequently, the following Chapter will examine the effects of BTJ on the RBE, in an attempt to help elucidate whether the dose of BTJ used in this study negatively affects the acute adaptive response to eccentric-heavy exercise.

6 Effects of beetroot juice supplementation on acute adaptation following eccentric exercise

Publication arising from this Chapter: Clifford, T., Bell, O., West, D. J., Howatson, G., & Stevenson, E. J. (2016). AOX-rich beetroot juice does not adversely affect acute neuromuscular adaptation following eccentric exercise. *Journal of Sports Sciences*, 1-8.

6.1 Introduction

In the previous Chapter, the fact that some aspects of EIMD were attenuated with BTJ suggests that it possibly had some influence on the degradative and regenerative processes in the muscle (i.e., inflammation, oxidative stress, proteolysis, and myogenesis) after an acute bout of strenuous exercise. Given the weight of evidence suggesting that interfering with these processes might impact on subsequent adaptation (see Chapter 2.5), this Chapter addresses whether BTJ could negatively affect the acute adaptive response to eccentric exercise.

AOX supplementation is purported as a strategy to attenuate the signs and symptoms of muscle damage (i.e., soreness, loss of muscle function, inflammation) that result from exercise involving strenuous exercise, particularly that has a heavy eccentric component (Bloomer, 2007; Peake & Suzuki, 2004). However, while an excess production of RONS and the associated inflammatory events might initially harm the muscle cell, they are also fundamental to the regenerative process, and are now widely considered to be a necessary stimulus for both acute and chronic cellular adaptations to exercise (Close et al., 2006; Michailidis et al., 2013; Paulsen et al., 2014b). With regards to acute adaptations, RONS and other immune cells that transiently increase after a bout of eccentric exercise have been proposed to act as signalling molecules for molecular changes that reinforce cell defences in the muscle and ECM (Hubal et al., 2008; Hyldahl et al., 2015; McHugh, 2003; Pizza et al., 2002; Xin et al., 2014). Such adaptations could make cells more resistant to damage during similar exercise bouts in the future. This acute adaptive response to eccentric, muscle-damaging exercise is classically illustrated by the RBE, in which the magnitude of muscle damage (i.e., force deficits) evoked by a single exercise damaging stimulus is attenuated in a subsequent bout performed many weeks later (McHugh, 2003; Nosaka & Clarkson, 1995).

Evidence that post-exercise inflammation and RONS production might afford beneficial effects for physiological and functional adaptation has sparked some debate within the literature (Gomez-Cabrera, Ristow and Viña, 2012; Higashida et al., 2011). Specifically, the question arises whether use of AOX-rich supplements to blunt oxidative stress and inflammation could have detrimental effects on the regeneration and adaptive responses that might translate to muscle function and performance enhancement (Close et al., 2006; Gomez-Cabrera et al., 2012; Paulsen et al., 2014b). However, very little attention has been given as to the effect of AOXs or any nutritional recovery intervention on the acute adaptive effects associated with eccentric exercise. The fact that the adaptive response from a just single bout of eccentric exercise results from (at least in part) cellular changes, it would be anticipated that AOX supplementation might attenuate the magnitude of the RBE. To date, this possibility has only been addressed by one recent study, which showed that 2 weeks of supplementation with a very high AOX dose of vitamin C (1000 $mg \cdot d \cdot^{-1}$) and E (400 $I \cdot U^{-1}$) did not blunt the RBE in response to a bout of downhill running (He et al., 2015). However, these findings are limited by the absence of any measures of muscle function, which are widely considered the best indicators of skeletal muscle damage (Paulsen et al., 2012; Warren et al., 1999).

In the previous Chapter, consuming AOX-rich BTJ attenuated some indices of muscle damage after a bout of eccentric exercise, suggesting that BTJ may hold promise as a recovery aid. However, given the above discussion, whether such a strategy would have deleterious effects for acute cellular adaptations is unknown. Consequently, as a follow up to the previous Chapter, a sub-set of the participants repeated the same bout of eccentric exercise 3 weeks later, without supplementation. The aim of this study was to examine the effects of BTJ supplementation on the acute adaptive response (represented by the RBE) after muscle damaging exercise. It is important to make the distinction that this study specifically tested whether BTJ would adversely affect the adaptive response to a *single* bout of

exercise, not a series of exercise bouts, and therefore the primary outcomes were the acute functional adaptations typically seen after a single bout of exercise (i.e., improved muscle recovery, lowered muscle soreness), as opposed to more chronic adaptations (increased hypertrophy and strength).

A secondary aim of this investigation was to establish whether a higher or lower dose of BTJ would differentially affect the RBE, given that previous reports have suggested that although higher AOX doses might be harmful (i.e., vitamin C ≥1000 mg·day^{-1}) (Close et al., 2006; Paulsen et al., 2014b), more moderate doses are not (500 mg mg·day^{-1}) (Yfanti et al., 2010; 2011). It was hypothesised that participants who consumed BTJ after the initial bout would have a blunted RBE compared to those who consumed a PLA beverage, and the magnitude of these responses might be exacerbated after a higher dose of BTJ.

6.2 Methods

6.2.1 Participants

Twenty-nine participants completed all study procedures. All participants were recreationally active (see Chapter 3.2 for definition), healthy males (age 21 ± 3 years; height 1.77 ± 0.80 m; body mass 75.6 ± 8.8 kg) who had no prior experience with the bout of muscle-damaging exercise.

6.2.2 Experimental overview

The study employed a randomised, double blind, PLA controlled, independent groups design. Excluding familiarisation, participants were required to attend the laboratory on 8 occasions. After familiarisation, participants were stratified into three supplement groups according to their baseline MIVC scores: H-BT; *n* = 10, L-BT; *n* = 9 or a PLA; *n* = 10. Supplements were consumed immediately, 2 h post-exercise, and at set times 24 and 48 h following a first bout of muscle damaging exercise (see

Chapter 5.2.3 for details). Muscle function, muscle soreness and CK were measured pre, post, 24, 48 and 72 h post-exercise. Fourteen to twenty-one days after the first bout of exercise (bout 1), participants returned to the lab to repeat an identical bout of exercise (bout 2) but no supplements were provided on this occasion. All testing was conducted in the morning following an overnight fast at the same time of day (within participants). Participants were familiarised with all equipment and study procedures prior to testing.

6.2.3 Muscle damaging exercise

The muscle-damaging bout of exercise consisted of 100 drop jumps from a 0.6 m high steel box. See Chapter 3.5 for further details.

6.2.4 Supplementation and dietary control

Participants were provided with the same H-BT and L-BT drinks outlined in the previous Chapter; the nutritional content of both drinks can be found in Chapter 3.4. For information regarding the timing of supplement intake and blinding procedures please refer to Chapters 5.2.3 and 3.4, respectively. Details of the imposed dietary restrictions can be found in Chapter 3.3.

6.2.5 Maximal isometric voluntary contraction

Please refer to general methods Chapter 3.6.1 for details on MIVC measurement.

6.2.6 Counter movement jump

Please refer to general methods Chapter 3.6.2 for details on CMJ measurement.

6.2.7 Muscle soreness

Muscle specific soreness was assessed as PPT with a handheld algometer. See Chapter 3.6.3 for further information.

6.2.8 Blood sample collection and analysis

Blood samples were collected according to the methods outlined in Chapter 3.7. For details of CK analysis refer to Chapter 3.7.1.

6.2.9 Data analysis

Data were analysed using IBM SPSS statistics version 21 and expressed as mean ± SD. A mixed model ANOVA with 3 treatment levels (H-BT vs. L-BT vs. PLA) × 2 bouts (bout 1 vs. bout 2), × 5 time points (pre, post, 24, 48 and 72 h post-exercise) was used to test for significant differences between the dependent variables. Group differences in height, weight, age, and baseline MIVC scores were analysed using one-way independent group ANOVAs. If significant group and interaction effects were observed, Fisher LSD *post hoc* tests were performed to locate where the differences occurred. Significance was set at $P < 0.05$ prior to analyses.

6.3 Results

There were no differences in participant characteristics (age, height, mass) and baseline MIVC between groups ($P > 0.05$). Data relating to group differences after bout 1 can be found in Chapter 5.3. Main effects for time were observed for all dependent variables (CMJ, MIVC, CK and PPT; $P < 0.05$) following bout 1, indicating that the drop jumps effectively induced muscle damage. The decrement in MIVC was less in bout 2 compared to bout 1 ($F_{(1, 26)} = 4.49$; $P = 0.04$; Figure 11), providing evidence of an RBE. The percentage decrease in MIVC in bout 1 (average across groups) was 16.1%, 12.2% and 17.3% at 24, 48 and 72 h post exercise, respectively,

whereas after bout 2, decrements were attenuated to 9.5%, 6.2%, and 2.3% at 24, 48 and 72 h post-exercise, respectively. There were no bout*group interactions ($F_{(2, 26)}$ = 0.20; P = 0.815), indicating that strength loss was similarly attenuated in all groups. At 72 h post bout 2, MIVC had recovered to 98.0 ± 9.0, 97.8 ± 6.0 and 97.1 ± 7.0% of baseline values in the H-BT, L-BT and PLA groups, respectively.

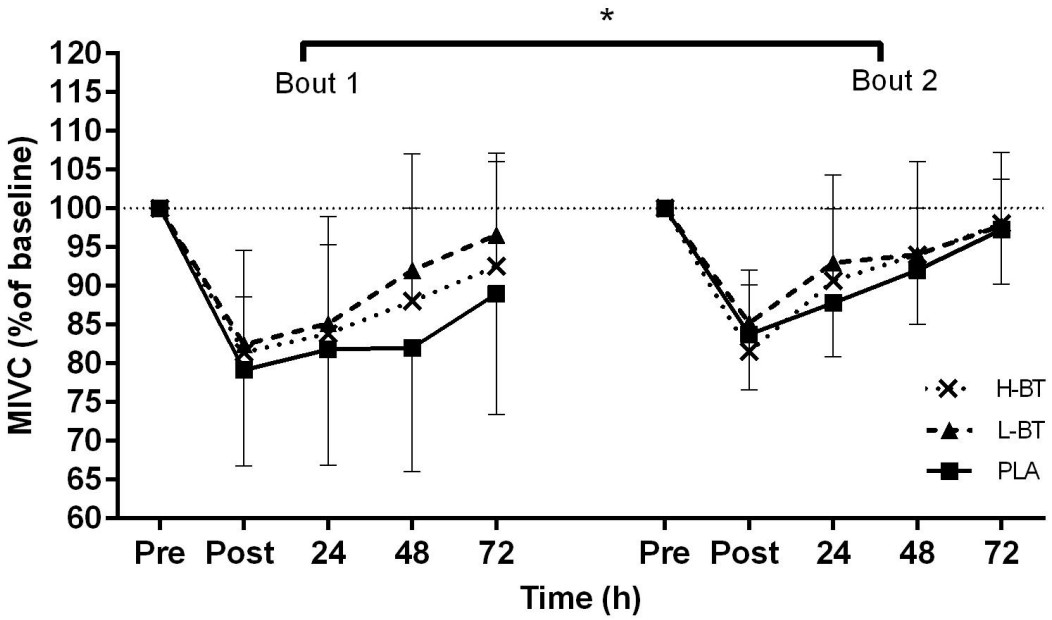

Figure 11 - Changes (% of baseline) in maximal isometric voluntary contraction (MIVC) pre and up to 72 h after exercise bout 1 and bout 2 in all 3 groups; high beetroot juice (H-BT), low beetroot juice (LBT) and placebo (PLA). *Attenuation of muscle force in bout 2 compared to bout 1(P = 0.040); n = 29.

The decrease in CMJ height was also attenuated in bout 2 compared to bout 1 (bout effect; $F_{(1, 26)}$ = 25.43; P <0.001) and there were no differences between the groups (bout *group interaction; $F_{(2, 26)}$ = 0.71; P = 0.501). Seventy two h post bout 1 CMJ height recovered in the H-BT, L-BT, and PLA

groups to 91.7 ± 9.7, 93.2 ± 7.1 and 87.4 ± 7.3% of baseline values, respectively, whereas 72 h following bout 2, CMJ height recovered to 101.4 ± 5.9, 96.9 ± 7.4 and 96.9 ± 4.8% of baseline values, respectively (Figure 12).

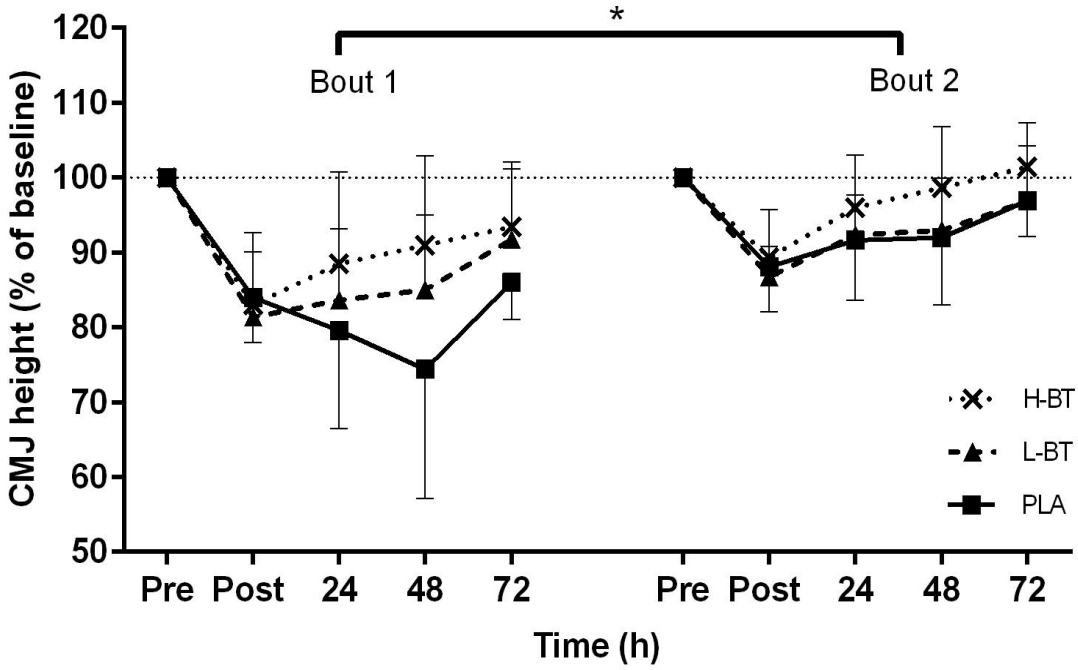

Figure 12 - Changes (% of baseline) in counter movement jump (CMJ) height pre and up to 72 h after exercise bout 1 and bout 2 in all 3 groups; high beetroot juice (H-BT), low beetroot juice (LBT) and placebo (PLA). *Attenuation of jump height in bout 2 compared to bout 1(P = 0.001); n = 29.

A main effect for bout showed that plasma CK was lower in bout 2 compared to bout 1 ($F_{(1, 24)}$ = 15.20; P = 0.001; Figure 13); there were no differences between the 3 groups (bout*group interaction; $F_{(2, 21)}$ = 2.42; P = 0.113).

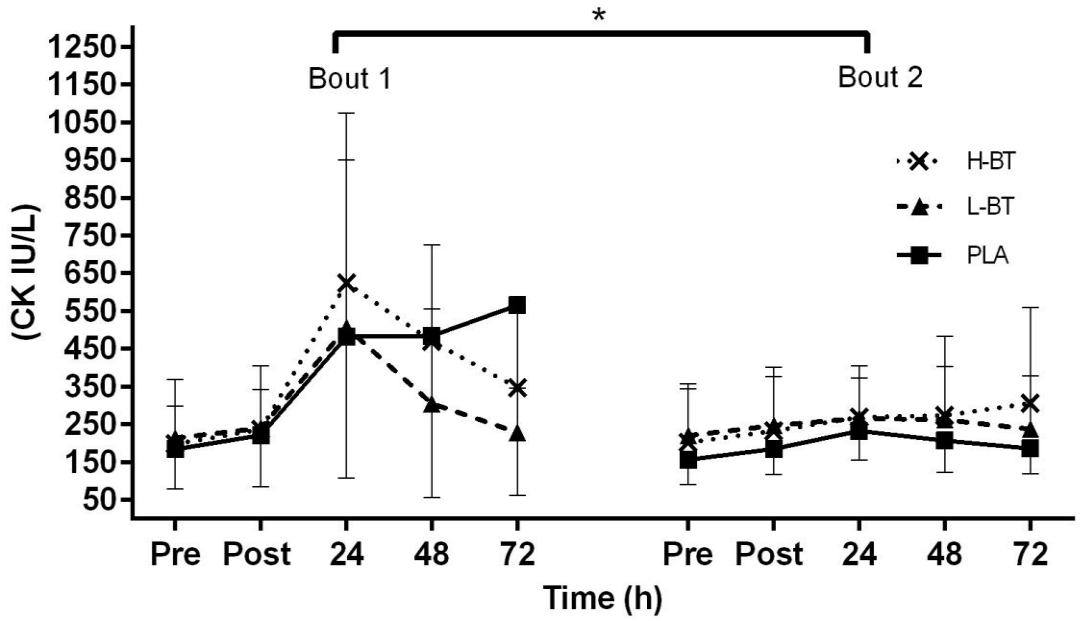

Figure 13 - Plasma creatine kinase (CK) concentrations pre and up to 72 h after exercise bout 1 and bout 2 in all 3 groups; high beetroot juice (H-BT), low beetroot juice (LBT) and placebo (PLA). *Decrease in CK efflux in bout 2 compared to bout 1(P = 0.001); n = 29.

PPT did not show an overall bout effect ($F_{(1, 84)}$ = 1.68; P = 0.198); but a bout*group interaction was observed ($F_{(2, 84)}$ = 4.00; P = 0.022), with *post hoc* analysis revealing that PPT was improved in bout 1 compared to bout 2 in PLA only (Table 7; P = 0.001).

Table 7 - Percentage change from baseline in PPT for the 3 supplement groups.

			Time Post exercise				
			Pre	**Post**	**24 h**	**48 h**	**72 h**
PPT	**HBT**	1	100 ± 0	84.7 ± 14.9	88.1 ± 20.5	91.1 ± 22.0	103.5 ± 23.2
		2	100 ± 0	90.6 ± 11.3	86.0 ± 16.8	93.2 ± 16.1	95.7 ± 12.9
	L-BT	1	100 ± 0	93.2 ± 21.4	87.1 ± 19.7	92.4 ± 24.0	104.5 ± 20.1
		2	100 ± 0	90.3 ± 10.7	86.6 ± 12.5	91.2 ± 13.7	99.8 ± 12.4
	PLA*	1	100 ± 0	85.01 ± 18.8	67.4 ± 20.8	61.7 ± 20.8	80.0 ± 28.9
		2	100 ± 0	87.5 ± 14.4	79.5 ± 15.1	81.5 ± 14.9	90.5 ± 17.9

*Different from bout 1; $P < 0.05$, $n = 29$.

6.4 Discussion

The aim of the current study was to examine whether the beneficial effects demonstrated by BTJ in the previous Chapter would negatively impact the RBE, as illustrated by an attenuation of functional recovery in a subsequent exercise bout. The principal finding of the current study was that 3 days of BTJ supplementation, at either a higher or lower dosage, did not interfere with the RBE to a single bout of eccentric exercise.

There were significant reductions in MIVC and CMJ height in all three groups after bout 1; however, consistent with a RBE, these reductions were significantly attenuated in bout 2, irrespective of supplementation and dose. These findings are in agreement with a previous study that observed similar attenuations in strength loss when a series of drop jumps were repeated 14-21 days after an initial bout (Howatson et al., 2009). CK activity was also significantly lower following bout 2 than bout 1, an effect typical of the RBE, and which has been observed in previous investigations using repeated bouts of eccentric exercise (Paulsen et al., 2010a; Stupka et al., 2001). The fact that there were no group differences in MIVC, CMJ or CK activity after bout 2, would imply, albeit indirectly, that BTJ supplementation did not

interfere with the cellular mechanisms postulated to underpin the RBE to a single bout of exercise.

While the precise cellular mechanisms that contribute to the RBE are not clear, they are postulated to include an increased expression of inflammatory related genes in muscle (Hubal et al., 2008; Xin et al., 2013), a blunted inflammatory and oxidative stress response (Hirose et al., 2004; Nikolaidis et al., 2007; Pizza, Baylies and Mitchell, 2001; Pizza et al., 2002) that, together, drive extensive cytoskeletal and ECM remodelling (Hubal et al., 2008; Hyldahl et al., 2015) to protect the muscle from damage when exposed to a similar stimulus in the future (McHugh, 2003). Although the aforementioned mechanisms were not directly measured in the present study, it is highly likely that they are at least partly responsible for the RBE we observed (i.e., faster resolution of force deficits, reduced CK efflux). Therefore, the magnitude of the RBE would have intuitively been altered if this cascade of events had been negatively affected by BTJ. Furthermore, the similar responses to bout 2 in both the L-BT and H-BT groups suggest that no dose-response effects were evident in terms of the magnitude of the RBE experienced. Notwithstanding these findings, it is important to acknowledge that the effects of BTJ (and at different doses) on longer term adaptive responses remains to be elucidated.

In contrast, only in the PLA group was the decrement in PPT, used as a measure of muscle soreness, attenuated in bout 2 versus bout 1. However, PPT did not differ between the PLA and BTJ groups in the 72 h period following bout 2. Therefore, this discrepancy is likely explained by the fact that PPT was significantly improved with BTJ supplementation after bout 1 (Table 7), but not PLA. Thus, after bout 2, while no further improvements in PPT were evident in the BTJ groups (probably because no BTJ was provided on this occasion), there was a significant improvement (or attenuation in muscle soreness) in the PLA group, as normally expected with an RBE.

The most pertinent new question posed in this study is the examination of exercise-induced adaptation after supplementation with an AOX-rich food (BTJ), whereas previous studies have predominately focused on the potential effects of high dose AOXs vitamin C and E (He et al., 2015; Nikolaidis et al., 2012a; Sousa et al., 2013). Furthermore, studies examining the effects of AOXs on adaptation to a single bout of eccentrically biased exercise, where muscle damage is principally induced via mechanical stress, are scarce. Nevertheless, these findings are in agreement with those of a recent study, in which 2 weeks of vitamin C and E supplementation did not have any adverse effects on adaptation to repeated bouts of downhill running (He et al., 2015). Furthermore, they also concur with the study of Theodorou et al. (2011) who although not measuring the RBE *per se*, reported that 11 weeks of supplementation with vitamin C and E had no effect (positive or negative) on the recovery of muscle function following an acute exercise bout performed after 4 weeks of resistance training. Nevertheless, a few studies have suggested that functional measures of exercise-induced adaptations might be blunted by AOX supplementation and these cannot be ignored. For instance, Close et al., (2006) reported that vitamin C supplementation (1000 $mg \cdot day^{-1}$) for 14 days following a bout of 30 min of downhill running impaired the acute regeneration (within 2 weeks) of isokinetic muscle strength compared to a PLA. Deleterious effects with AOX supplementation were also demonstrated in a long term trial, where combined ingestion of vitamin C (1000 $mg \cdot day^{-1}$) and E (235 $mg \cdot day^{-1}$) for 12 weeks impaired resistance training-induced gains in muscle strength and lean muscle (Bjørnsen et al., 2015).

The fact that the aforementioned studies showing negative effects were not designed to specifically assess the RBE precludes any direct comparisons to the current study. Nevertheless, it is important to highlight that unlike previous work, the present study investigated acute adaptive responses after consuming a phytochemical rich food in BTJ, not highly concentrated doses of vitamin C or E AOXs. It has been proposed that AOX molecules derived

from plant sources, such as polyphenols, are likely to elicit distinct physiological effects to nutritional AOX supplements, which are typically formulated in highly concentrated doses (Nikolaidis et al., 2012a). This is possibly due to the fact that 1) AOX-rich functional foods are less likely to provide isolated molecules in supra-physiological doses (Sousa et al., 2013), which is perhaps due to differences in absorption, bioavailability and biotransformation, and; 2) many contain a diverse range of molecules, each of which might possess additional biochemical effects beyond just AOX (Nikolaidis et al., 2012a). For instance, the BTJ used in the present study contains a range of bioactive molecules, such as nitrate, phenolics and betalains, which, in addition to being AOXs, have demonstrated anti-inflammatory, anti-proliferative, chemo-preventive (El Gamal et al., 2014; Jädert et al., 2012; Justice et al., 2015; Lechner et al., 2010) and hormetic/adaptogenic effects on the endogenous AOX network (Esatbeyoglu et al., 2014). This data would suggest that BTJ might possess distinct biochemical effects to concentrated AOX sources such as vitamin C and E, and this may result in different physiological outcomes in terms of functional recovery and the RBE. Further research is needed to clarify the potential differing effects of these two supplements on acute adaptive responses to eccentric exercise.

The main limitation of this study is the inability to ascertain the inflammatory and oxidative stress response to both bouts of exercise. Thus, it cannot be ruled out that the present findings are due to the fact that BTJ had little, if any, influence on the level of inflammation and oxidative stress following bout 1, and that other mechanisms were responsible for the enhanced functional recovery observed with BTJ in Chapter 5. With that said, because the potential implications of AOX supplementation on exercise performance are of most concern for athletes and practitioners, this study purposely limited its outcomes to the acute changes in functional recovery markers. Nonetheless, it is stressed that based on the present data alone, it cannot be conclusively ruled out that BTJ does not influence the RBE at the cellular level or would

not influence more long term adaptations to eccentric-heavy exercise training.

Despite these limitations, this is the first study to suggest that acute supplementation with an AOX-rich BTJ does not adversely affect the RBE to a single bout of exercise. These preliminary findings suggest that athletes seeking strategies to increase their AOX intake might favour the use of BTJ or other AOX-rich functional foods over high doses of vitamin C and E supplements that might interfere with exercise-induced adaptations. Nonetheless, future work with higher participant numbers is needed to not only corroborate these conclusions, but to examine the chronic use of BTJ in the adaptive process to elucidate its influence in longer-term adaptive training responses.

6.5 Perspectives

This study addressed the 3rd aim of this thesis, which was to investigate the effects of acute BTJ supplementation on the RBE to eccentric-heavy exercise. There were no differences in the recovery of several indices of muscle damage between the BTJ and PLA groups when the exercise bout was repeated, which would indicate that BTJ did not negatively affect acute adaptation, as reflected by the RBE.

The results from Chapter 5 suggested that BTJ might serve as a useful strategy to alleviate muscle damage after a single bout of exercise. However, because of studies proposing that supplements with AOX or anti-inflammatory functions might adversely affect exercise-induced adaptations (Pasulsen et al. 2014a; Paulsen et al. 2014b), it was important to establish, albeit acutely, if such effects were evident with BTJ. The fact that no adverse effects were demonstrated after BTJ supplementation suggests that athletes and recreational exercises might favour its use over high doses of vitamin C and E or NSAID supplements.

It is important to note however, that the finding in this Chapter should not be interpreted to reflect the potential long-term consequences of BTJ supplementation on exercise-adaptations. As the aim of this thesis is to focus on the short-term provision of BTJ, it is beyond the scope of this thesis to determine the long-term effects chronic use might have on exercise-adaptation.

Given the findings of Chapter 5, in which BTJ enhanced some indices of exercise recovery in recreationally active individuals, and this Chapter, showing that BTJ did not adversely affect acute adaption, the next Chapter will examine whether acute BTJ intake could aid short-term recovery in a more athletic population. In addition to a range of recovery indices, this study will also consider whether BTJ can influence subsequent performance.

7 Effects of beetroot juice on recovery of muscle function and performance between bouts of repeated sprint exercise

Publication arising from this Chapter: Clifford, T., Berntzen, B., Davsion, W. G., West, D. J., Howatson, G., & Stevenson, E. J. (2016). Effects of beetroot juice on recovery of muscle function and performance between bouts of repeated sprint exercise. *Nutrients*, *8*(8), 506 – 523.

7.1 Introduction

Chapter 5 demonstrated that BTJ can minimise some aspects of EIMD and help to accelerate exercise recovery after eccentric-heavy exercise. However, this study was performed with recreationally active participants, which limits the applicability of the findings to more athletic populations, for whom the need to optimise recovery is undoubtedly more important. Team-sport athletes for example, are often required to complete multiple training sessions and, in some instances, competitive matches in a weekly cycle. In these circumstances, recovery time is likely to be insufficient and subsequent performance could be impaired. Thus, identifying recovery strategies that could help to minimise the magnitude of EIMD is of great interest in this group of athletes. In view of this, this study aimed to address the 4th aim of this thesis, which was to investigate the effects of BTJ on muscle damage and recovery in team-sport athletes between bouts of repeated sprint exercise.

Repeated sprint exercise (RSE), in which a number of short-duration maximal effort sprints (2-6 s) are completed intermittently with brief recovery periods (≤60 sec), places a great deal of stress on the physiological and musculoskeletal systems (Girard, Mendez-Villanueva, & Bishop, 2011). The high energy turnover during RSE induces significant metabolic stress, triggering rapid perturbations in the nervous, immune, and endocrine systems (Girard et al., 2011), as well as an increased formation of RONS (Bogdanis et al., 2013; Jówko, Długołęcka, Makaruk, & Cieśliński, 2014). In addition, the high-force eccentric muscle contractions required to accelerate and decelerate during RSE places a great deal of mechanical stress on the musculoskeletal system, particularly the quadriceps and hamstring muscle groups (Howatson & Milak, 2009). It is therefore not surprising that team-sport players, who routinely engage in RSE in training sessions and matches, often display symptoms of muscle damage (i.e., muscle soreness and reduced muscle function) that can persist for several days (Duffield et

al., 2010; Howatson & Milak, 2009). Because the typical time between training sessions and or matches is often not sufficient for full recovery, athletes and coaches are continually seeking strategies that could help minimise the negative effects of muscle damage, particularly the decrements in muscle function (Barnett, 2006; Nédélec et al., 2013).

The exact mechanisms to explain the causes of muscle damage after RSE are not fully understood, but a host of factors such as muscle membrane damage, sarcomere disorganization, EC coupling dysfunction, contractile protein damage, and inflammation, are all likely to play a role (Hyldahl & Hubal, 2014). Furthermore, as alluded to in Chapter 2.3, it has been suggested that the generation of RONS in the day's post-exercise, likely a consequence of inflammatory mediated repair processes, might exacerbate the existing muscle damage by degrading components of the cytosol that are integral to force production (Pizza et al., 2005; Toumi & Best, 2003). A number of studies have provided evidence of oxidative stress in the hours and days following RSE (Bogdanis et al., 2013; Jówko et al., 2015), suggesting that the endogenous AOX system is unable to cope with excess RONS production under these conditions. Thus, it would be reasonable to assume that the prolonged decrement in muscle function might be, at least in part, attributable to excessive oxidant production. This also makes the expectation tenable that interventions attempting to combat the excess production of RONS and control oxidative stress, such as AOXs, could help accelerate the rate of muscle recovery following RSE.

While the nutritional AOXs vitamin C and E have proven largely ineffective at attenuating muscle damage (Nikolaidis et al., 2012a), there is growing support for the use of AOX-rich fruit and vegetable beverages as recovery aids (see Myburgh et al., 2014; Sousa et al., 2013 and discussion in Chapter 2.6). Moreover, in Chapter 5, it was demonstrated that BTJ was able to protect against some of the symptoms of EIMD, effects that could have been mediated by a dampening of oxidative stress. At present however, the effects

of BTJ (or any AOX-rich food) on muscle damage, recovery and oxidative stress after activity incorporating RSE has not been investigated. Additionally, the effectiveness of such an intervention on subsequent performance has not been considered. Therefore, the main aim of this study was to examine whether BTJ can attenuate losses in muscle function and performance between two repeated sprint tests (RST) performed 72 h apart. We also examined the effects of BTJ on biochemical markers associated with muscle damage, specifically oxidative stress. Based on the findings from Chapter 5, it was hypothesized that; 1) BTJ would attenuate muscle function deficits and oxidative stress between and after the two repeated sprint tests, and; 2) that performance during the second RST would be preserved with BTJ compared to a PLA.

7.2 Methods

7.2.1 Participants

A power calculation was conducted to determine an adequate sample size for this study. Based on the findings from Chapter 5, with a power of 0.80 and two tailed α level set at 0.05, the minimum number of participants required to detect an 8% (ES = 1.25) difference in CMJ performance between groups (SD: 6%) was estimated as *n* = 10 per group. As such, twenty male participants were recruited and gave written informed consent for participation in this study (characteristics presented in Table 8). All participants were University standard or amateur level team-sports players, competing in either soccer (*n* = 10), rugby (*n* = 5), basketball (*n* = 2) hockey (*n* = 2) or handball (*n* = 1); all testing was performed at the end of the competitive season.

Table 8 - Descriptive data for participants in the BTJ and PLA groups.

Group	Age (Years)	Height (m)	Mass (kg)
BTJ	23 ± 3	1.83 ± 0.90	76.8 ± 9.5
PLA	21 ± 2	1.77 ± 0.51	73.4 ± 12.4

Values are mean ± SD (*n* = 10 per group). No significant differences were detected between groups for any variable (*P* > 0.05).

7.2.2 Experimental design

This study employed a double-blind, PLA controlled, independent groups design. Participants were required to attend the laboratory for 6 visits over a 2-week period. The first visit was to familiarise the participants with the study procedures and allocate them to either a BTJ or an isocaloric PLA group. As in previous Chapters, their baseline MIVC scores were used to match the groups. The next 5 visits were performed on consecutive days in the same laboratory, at the same time of day, and preceded by an overnight fast. For the main trials, participants performed two repeated sprint tests separated by 72 h (visit 2 = RST1 and visit 5 = RST2) (see Figure 14 for schematic outline). A range of dependent variables were taken pre, immediately post, 24, 48 and 72 h after RST1, and immediately post and 24 h after RST2 to monitor recovery. On each occasion, dependent variables were performed in the following order: PPT, venous blood draw, CMJ, RSI and MIVC. After completing the post-exercise measures participants consumed 1 serving of their allocated treatment, and returned to the lab 2.5 h post-ingestion for a further blood sample.

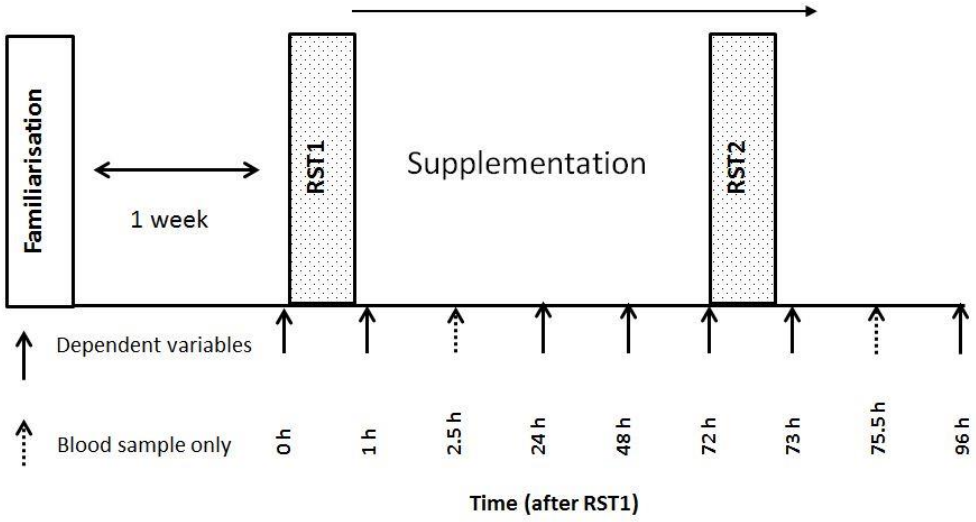

Figure 14 - Schematic outline of study procedures.

7.2.3 Repeated sprint test

The RST consisted of 20 maximal-effort 30 m sprints, interspersed by 30 seconds of passive recovery. A 10 m deceleration zone was marked out at the end of each 30 m sprint, in which participants were required to stop within; the 30 second rest period commenced when participants had come to a halt. The RST was adapted from previous studies that showed repeated sprints with forced decelerations induce substantial muscle damage and fatigue in team-sport trained participants (Howatson & Milak, 2009; Keane, Salicki, Goodall, Thomas, & Howatson, 2015). Furthermore, the muscle damage induced by a similar RST (Howatson & Milak, 2009), seems to cause reductions in muscle function comparable to those observed after intermittent sport simulations, such as the Loughborough Intermittent Shuttle Test (LIST) (Thompson et al., 2001) and competitive matches (Andersson et al., 2008). Before performing each RST, participants undertook a standardized warm up as per previously described methods (Howatson & Milak, 2009). Briefly, participants completed 400 m of self-selected jogging, a series of dynamic stretches, and sprints at 60 and 80% of maximal effort. Participants were then given a further 5 min to complete their own stretching.

Timing gates (Brower Timing Systems, Draper, UT, US) were positioned at 0 and 30 m to record sprint times. Participants were instructed to give maximal effort for each sprint and provided with strong verbal encouragement throughout. All testing took place in an air conditioned sprint track in similar environmental conditions.

7.2.4 Maximal isometric voluntary contractions

Please refer to general methods Chapter 3.6.1 for details on MIVC measurement.

7.2.5 Counter movement jump

Please refer to general methods Chapter 3.6.2 for details on CMJ measurement.

7.2.6 Reactive strength index

Reactive strength index (RSI) was used to measure the impact of muscle damaging exercise on participant's ability to utilize the stretch shortening cycle and perform explosive actions. In a similar fashion to previous studies (Cockburn, Stevenson, Hayes, Robson-Ansley, & Howatson, 2010; Cockburn et al. 2012), participants performed a drop jump from a 30 cm box and, upon landing, immediately jumped vertically, with instructions to minimise ground contact time while maximising jump height. RSI was calculated as jump height divided by ground contact time (cm/ms) recorded from an optical measurement system (Optojump next, Bolzano, Italy). Participants performed 3 maximal efforts separated by 30 seconds of passive (standing recovery) with the mean height of the 3 jumps used for analysis. The CV for this protocol was calculated as <2%.

7.2.7 Treatments and dietary control

Participants consumed 2 bottles (250 ml per bottle) of their assigned treatment (BTJ or PLA) immediately post, 24, and 48 h after RST1 and immediately post RST2, equating to 8 servings in total. One bottle was consumed immediately after each trial, and one with an evening meal. Macronutrient and phytochemical information for these treatments can be found in Chapter 3.4. Participants were provided with food dairies to record their intake 24 h prior to RST1 up until data collection was complete (24 h post RST2; 5 days in total) (see Chapter 3.3 for further details). Average energy and macronutrient intake for each group is presented in Table 9.

Table 9 - Average intake and macro nutrient composition of participant's diets (average of 5 days).

	Mean dietary intake (5 days)	
	BTJ	**PLA**
Energy (Kcal)	2554 ± 682	2448 ± 390
Carbohydrates (%)	41 ± 5	43 ± 6
Protein (%)	21 ± 3	24 ± 7
Fat (%)	38 ± 7	33 ± 9

Values are mean ± SD (*n* = 10 per group). No significant differences were detected between groups for any variable (*P* > 0.05).

7.2.8 Muscle soreness

Site specific muscle soreness was assessed as PPT with a handheld algometer (see Chapter 3.6.3 for details).

7.2.9 Blood sampling

Venous blood was obtained as described in Chapter in 3.7.

7.2.10 Biochemical analysis

Determination of serum CK and serum hs-CRP is described in section 3.7.1 and 3.7.2, respectively. Plasma PC was measured according to the manufactures instructions with a commercially available assay kit (Cayman Chemical, Ann Arbor, Michigan, USA). Samples were analysed in duplicate, with inter and intra assays CVs calculated as <5% and <8%, respectively. Lipid hydroperoxides (LOOH) were measured in serum using the ferrous iron/xylenol orange (FOX) assay (Wolff, 1994). The FOX assay determines the susceptibility to iron-induced LOOH formation in blood. Absorbance was read at 560 nm using a spectrophotometer (U-2001, Hitachi, England) (range 0–5 μmol.L^{-1}). Samples were analysed in duplicate, with inter and intra assays CVs calculated as <6% and <4%, respectively. Ascorbyl free radical ($A^{\bullet-}$) determination was quantified at room temperature using a Bruker EMX series X-band EPR spectrometer (Bruker, Karlsruhe, Germany). One ml of plasma was mixed thoroughly with 1 ml of dimethyl sulfoxide (DMSO) and slowly flushed into an aqua X multiple bore cavity cell. The EMX parameter settings were frequency, 9.785 GHz; microwave power, 20 mW; modulation frequency, 100 kHz and modulation amplitude, 1.194 G. All EPR spectra were subjected to 3 scans, identically filtered, and analysed using WinEPR software (Version 3.2, Bruker WinEPR). The average spectral peak-to-trough line amplitude was used to determine free radical concentration. The intra assay CV for this analysis was <5%.

7.2.11 Data analysis

All data are expressed as mean ± SD and were analysed using IBM SPSS Statistics 22 for Windows (Surrey, UK). Please refer to Chapter 3.3 for details on the analysis of participant food diaries. Differences between participant group characteristics were analysed with an independent samples t-test. CMJ, RI, MIVC and PPT were measured (after being corrected for % change from baseline) using a mixed model ANOVA; 2 group levels (BTJ vs.

PLA) by 7 time levels (pre, post, 24, 48, 72, 73 and 96 h post RST1). The same ANOVA was used to analyse all blood indices but with 2 additional time levels (2.5 h post RST1 and 2.5 h post RST2). A separate ANOVA was used to measure for differences between RST1 and RST2; 2 group levels (BTJ vs. PLA) by 2 time levels (pre and post). In the event of a significant interaction effect (group*time) Fisher LSD *post hoc* analysis was performed to locate where the significant differences occurred. Statistical significance was set at $P < 0.05$ prior to analyses. To estimate the magnitude of the supplements effects, Cohen's *d* ES were calculated with the magnitude of effects considered either small (0.20-0.49), medium (0.50-0.79) and large (>0.80).

7.3 Results

There were no between group differences in age, height or mass (Table 8; $P > 0.05$), indicating that the groups were well matched prior to testing. Furthermore, there were no differences in participant's energy and macronutrient intake 24 h prior to and throughout the duration of the study (Table 9; $P > 0.05$).

7.3.1 Repeated sprints

RPE showed no bout ($P = 0.925$) or interaction effects ($P = 0.584$) between RST1 and RST2, indicating that perceived exertion was similar for both bouts (Table 10). This was reflected in the sprint data, as fastest sprint time and fatigue index were also similar between repeated sprint bouts, showing no main effects of time, bout, or bout*group interactions ($P > 0.05$).

Table 10 - Sprint and RPE data for the BTJ and PLA groups in the first and second repeated sprint test (RST1 and RST2, respectively).

Group	Average sprint time (s)	Fastest sprint time (s)	Fatigue index (%)	RPE
BTJ				
RST1	4.65±0.25	4.41±0.23	5.60±2.13	15±1
RST2	4.66±0.24	4.38±0.17	6.48±2.66	15±1
PLA				
RST1	4.70±0.15	4.48±0.14	4.91±1.51	14±2
RST2	4.77±0.20	4.53±0.15	5.19±3.21	14±2

7.3.2 Functional measures

All tests of neuromuscular function (CMJ, MIVC, RSI), and PPT, showed main effects for time ($P < 0.05$), indicating that the RST induced muscle damage. Immediately post RST1, CMJ height was reduced by 11.8 ± 8.9% and 9.6 ± 4.8% (of baseline values) in the BTJ and PLA groups, respectively. A group effect showed that CMJ height appeared to recover quicker in BTJ vs. PLA throughout the remainder of the testing period ($P = 0.048$; Figure 15). Although no group*time interaction effects were present ($P = 0.176$), there was a large ES (1.86) at 72 h post RST1, whereby CMJ height in the BTJ group was 7.6% higher than in the PLA group. A group effect for RSI ($P = 0.030$) showed that the maintenance of RSI performance was also greater in BTJ vs. PLA throughout the trial (Figure 16). As with CMJ, a large ES (1.43) was evident at 72 h post RST1 where RSI had returned to 95.8 ± 9.5% of baseline values in BTJ compared to 82.0 ± 9.5% in PLA. There were no group effects for PPT ($P = 0.368$); however, an interaction effect was observed ($P = 0.013$). *Post-hoc* analysis revealed a group difference at 96 h post RST1 ($P = 0.012$; ES = 0.57); in the BTJ group, PPT had recovered to 104.7 ± 12.5% of baseline values, while in the PLA group, PPT was 94.3 ±

18.0% of baseline values (Figure 17). There were no significant group or interaction effects for MIVC ($P > 0.05$).

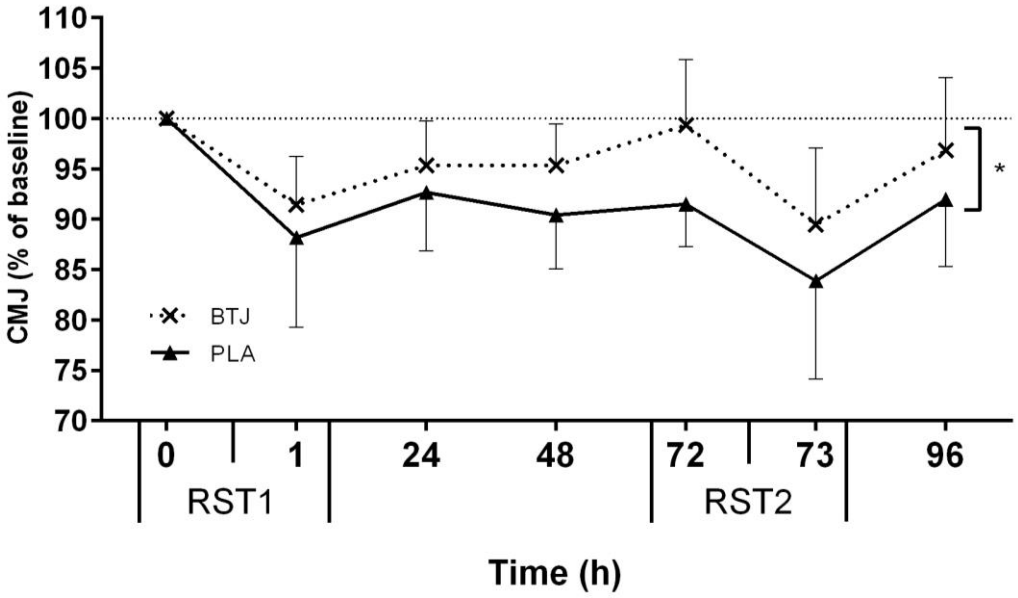

Figure 15 - Percentage changes in counter movement jump (CMJ) height between repeated sprint tests (RST1 and RST2). *Represents group difference (beetroot juice (BTJ) vs. placebo (PLA); $P < 0.05$). Values are mean ± SD ($n = 10$ per group).

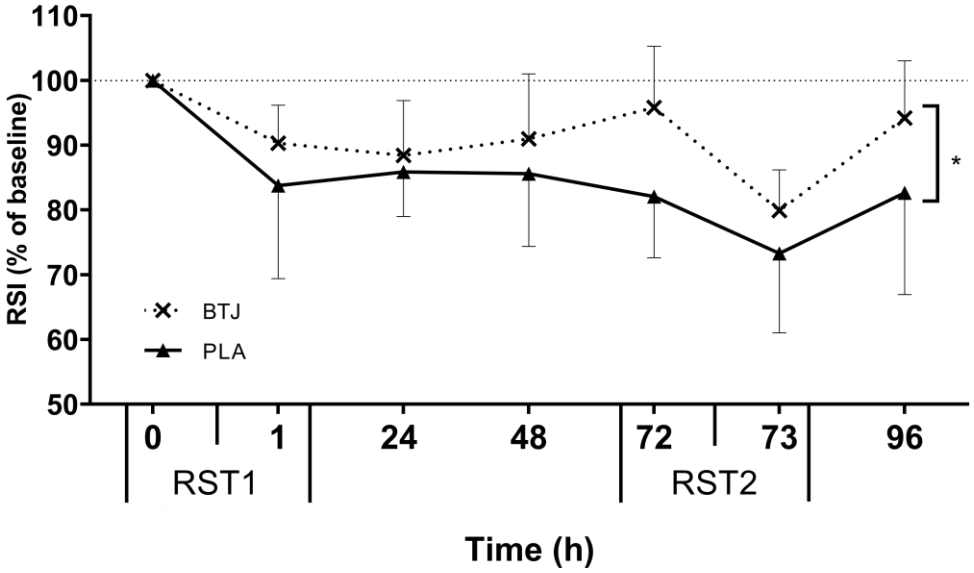

Figure 16 - Percentage changes in reactive strength index (RSI) between repeated sprint tests (RST1 and RST2). *Represents group difference (beetroot juice (BTJ) vs. placebo (PLA); $P < 0.05$). *Values are mean ± SD (n = 10 per group).

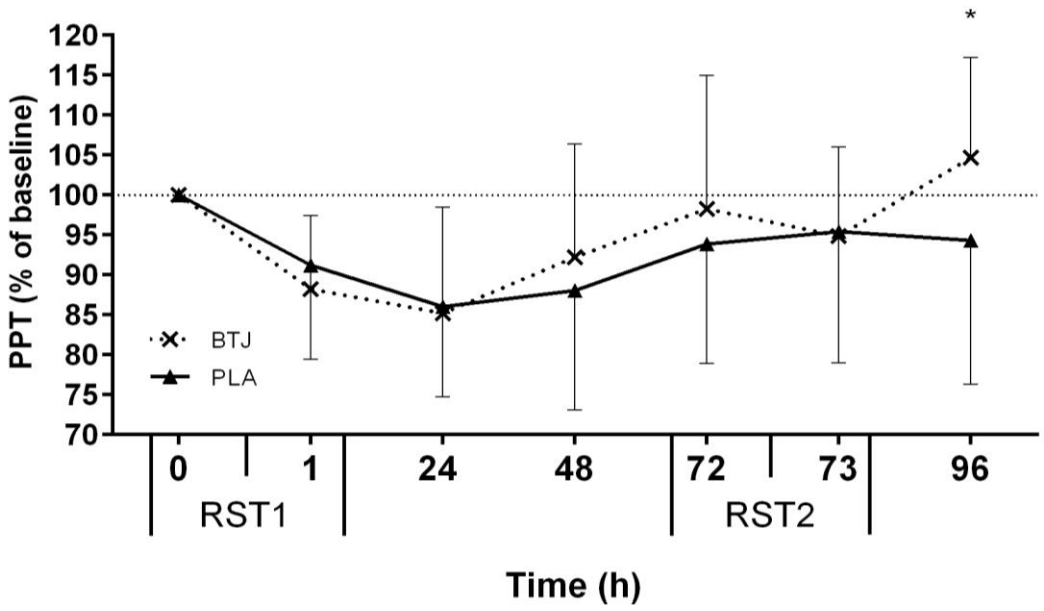

Figure 17 - Percentage changes in pressure pain threshold (PPT) between repeated sprint tests (RST1 and RST2). Values presented are average of the three sites measured (calf, CF; rectus femoris, RF; vastus laterialis, VL). *Indicates interaction effect beetroot juice (BTJ) vs. placebo (PLA), $P < 0.05$. Values are mean ± SD ($n = 10$ per group).

7.3.3 Biochemical indices

Serum concentrations of hs-CRP remained close to baseline values throughout the trial, showing no time, group or interaction effects ($P > 0.05$). Serum CK showed main effects for time ($P < 0.001$), with the greatest increases observed 2.5 and 24 h post RST1 and RST2 in both groups ($P > 0.05$; Table 11). However, no group of interaction effects were observed ($P > 0.05$). A main effect for time was observed for serum LOOH ($P < 0.001$); LOOH was elevated immediately and at 2.5 h post-RST1 in both groups before returning to baseline 24 h post. A transient increase in LOOH was also evident at 75.5 h (2.5 h post RST2) but by 96 h had recovered to pre-exercise values. No group or interaction effects were present for LOOH ($P > 0.05$). PC remained largely unchanged after both sprint bouts and showed no group or interaction effects ($P > 0.05$). Likewise, $A^{\bullet-}$, as measured by EPR, showed no time, group or interaction effects throughout the trial ($P > 0.05$).

Table 11 - MIVC, hs-CRP, CK, LOOH, PC and $A^{\bullet-}$ for the BTJ and PLA group's pre (0 h) - 96 h post RST1.

	0 h	1 h	2.5 h	24 h	48 h	72 h	73 h	75.5 h	96 h
MIVC (N)*									
BTJ	601(100)±89(0)	554(92)±75(6)		545(91)±92(10)	558(93)±94(10)	579(96)±121(9)	516(85)±101(8)		558(93)±75(8)
PLA	590(100)±123(0)	541(92)±128(7)		535(91)±120(6)	543(92)±130(5)	544(92)±132(6)	511(86)±128(6)		536(91)±121(6)
Hs-CRP ($mg \cdot L^{-1}$)									
BTJ	0.60±0.72	0.61±0.75	0.57±0.69	0.64±0.56	0.64±0.58	0.52±0.52	0.52±0.49	0.46±0.50	0.48±0.50
PLA	0.44±0.39	0.46±0.45	0.44±0.42	0.52±0.52	0.4±0.25	0.34±0.21	0.35±0.21	0.38±0.22	0.38±0.24
CK ($IU \cdot L^{-1}$)*									
BTJ	188±62	219±68	383±197	542±461	406±252	310±145	349±163	474±246	516±210
PLA	318±145	362±154	518±274	592±321	435±255	387±273	433±290	623±423	749±423
LOOH($mmol \cdot mL^{-1}$)*									
BTJ	1.49±0.25	1.68±0.23	1.69±0.37	1.33±0.36	1.46±0.19	1.47±0.17	1.53±0.40	1.74±0.31	1.44±0.21
PLA	1.53±0.14	1.79±0.23	1.77±0.40	1.54±0.16	1.57±0.14	1.53±0.12	1.69±0.23	1.94±0.75	1.44±0.14
PC ($\mu mol \cdot L^{-1}$)									
BTJ	14±6	16±0	15±6	17±5	18±7	14±5	16±5	17±10	15±6
PLA	16±8	19±6	14±6	13±5	14±5	18±9	16±7	15±5	15±4
$A^{\bullet-}$ (AU)									
BTJ	5567±1898	6111±2145	5883±2044	6247±1846	6202±1911	5809±2238	5752±1854	5422±2002	5862±1802
PLA	6372±1454	6730±1337	6559±2027	5674±1716	5225±1088	6013±671	5433±1521	6354±1315	5819±975

AU = Arbitrary units. *Denotes time effect ($P < 0.05$). MIVC, maximal isometric voluntary contraction; hs-CRP, high sensitivity C-reactive protein; CK, creatine kinase; LOOH, lipid hydroperoxides; PC, protein carbonyls; $A^{\bullet-}$, ascorbyl radical.

7.4 Discussion

The main finding of the present study was that BTJ, when compared to a PLA, was able to accelerate the recovery of CMJ and RSI performance and reduce muscle pain after a muscle-damaging RST, but had no influence on sprint performance. Markers of systemic oxidative stress or other biochemical indices associated with muscle damage were unaffected by BTJ supplementation.

In both the BTJ and PLA groups CMJ and RSI significantly decreased after RST1, indicating the presence of muscle damage; however, both CMJ and RSI recovered quicker with BTJ (vs. PLA) during the following +96 h. This was most evident at 72 h after the first sprint bout (RST1), whereby CMJ and RSI were still significantly lower than baseline values, but restored close to pre-exercise values in BTJ group. These findings are consistent with Chapter 5, in which 3 days of BTJ supplementation enhanced the recovery of CMJ performance 72 h after high intensity plyometric activity.

Interestingly, although BTJ appeared to enhance the recovery of dynamic muscle function (CMJ and RSI), isometric strength (MIVC) was unaffected by BTJ supplementation. As suggested in Chapter 5, perhaps this discrepancy can be explained by the different movement patterns (static vs. dynamic function), specific abilities each test measures (power vs. isometric strength) and primary mode of contraction (eccentric vs. concentric). Both CMJ and RSI are arguably more ecologically valid tests of functional recovery than MIVC, particularly for team-sports players as their movement patterns more closely reflect the activity required for performance (Gathercole et al., 2015). Thus, it would be reasonable to assume that CMJ and RSI are likely to be more sensitive tests for detecting decrements in muscle function after RSE in team-sports players.

It was initially hypothesized that sprint performance would be reduced in RST2 compared to RST1; a consequence of muscle damage, and that this

reduction would be attenuated with BTJ supplementation. However, contrary to this hypothesis, aside from a non-significant decrease in average sprint time, sprint performance was largely unaffected in RST2 compared to RST1 (Table 10). The reductions in muscle function were similar ≤24 h after both sprints tests though, suggesting that the participants did not become accustomed to the sprint test after the first bout. Additionally, BTJ had no influence on any aspect of sprint performance, although perhaps our ability to detect any differences between groups was limited by the lack of change in performance between the two sprint tests. Nonetheless, the fact that sprint performance was unchanged seems to contrast with other studies, which reported sprint times to still be slower than pre-exercise values up to 72 h after an RST similar to the present study (Brown, Howatson, Keane, & Stevenson, 2015; Keane et al., 2015; Woolley, Jakeman, & Faulkner, 2015). Because the muscle-damaging RST was similar between these studies (in fact, the one in the present study was designed to be more challenging), perhaps the divergent findings between these studies and the present one is due, in large part, to the different training status of the participants. The participants in the present study were experienced team-sports players who regularly perform RSE as part of training and matches and, thus, may have been less vulnerable to prolonged decrements in sprint performance than recreationally active participants tested in some of the other studies (Brown et al., 2015; Wooley et al., 2014).

In addition, the fact that CMJ and RI were still significantly depressed at 72 h post-RST1, but sprint performance was not, suggests that there is dissociation between jump based tests and repeated sprint tests. Indeed, previous literature appears to be equivocal on how well sprint and jump tests correlate. Some studies demonstrate that the time course of recovery for CMJ and sprint performance are similar after muscle-damaging RSE (Brown et al., 2015; Wooley et al., 2014), while others agree with the present study (Gathercole et al., 2015; Semark, Noakes, Gibson, Lambert, 1999), and have found that reductions in CMJ are more prolonged than sprint decrements. A

recent study attempted to address this issue by comparing CMJ, drop jumps (DJ) and a 20 m sprint test after intermittent exercise and concluded that sprint performance seemed to recover more rapidly than both CMJ and DJ performance, both of which were still below pre-exercise values 72 h post-exercise (Gathercole et al., 2015). This led the authors to suggest that CMJ and DJ are more sensitive tests of changes in neuromuscular function, which could provide an explanation for the dissociation between the jump and sprint tests in the present study.

Due to the fact that oxidative stress has been associated with muscle damage after eccentric-heavy exercise (Zerba et al., 1990), and that beetroot and its constituents have been shown to act as AOXs (El Gamal et al., 2014; Esatbeyoglu et al., 2014; Tesoriere et al., 2004b), it was hypothesized that BTJ could attenuate muscle damage by protecting cells against oxidative stress. However, the findings of this study do not support this contention. There was no evidence that BTJ attenuated oxidative stress as both indirect markers (LOOH and PC) and a direct marker of free radical production ($A^{\bullet-}$) were similar between the BTJ and PLA groups at all-time points (Table 11). These data are in contrast to a number of previous studies that found AOX-rich food supplements reduced oxidative stress after high intensity sprint exercise (Bell et al., 2014; Jówko et al., 2015). However, unlike these studies, there was no evidence of oxidative stress throughout the trial, apart from a small increase in LOOH immediately and 2.5 h after both sprint tests (Table 11). The modest increase in these markers was unexpected, as previous studies reported large systemic elevations in oxidative stress up to 48 h after high intensity intermittent cycling exercise (Bogdanis et al., 2013; Jówko et al., 2015), an activity which, in comparison to running, typically results in less oxidative stress because of the absence of an extensive eccentric component (Nikolaidis et al., 2008). The divergent findings in oxidative stress response between the present and aforementioned studies could, therefore, be explained by the different biochemical markers examined and/or analytical techniques used. Jówko et al. (2014) for instance, noted

systemic increases in total AOX capacity (TAC), superoxide dismutase, and glutathione peroxidase (GPX) 24 h after exercise and Bogdanis et al. (2013) noted increases in TAC, PC, and GPX; thus, neither study measured LOOH or $A^{\bullet-}$ formation, as in the present study. Although this study and Bogdanis et al. (2013) both measured PC, different analytical methods were used, which could account for the discrepant results. Nonetheless, EPR spectroscopy is a very sensitive method for detecting excessive free radical production (Buettner & Jurkiewicz, 1993; Pietri, Seguin, Darbigny, & Culcasi, 1994) and the fact that there was no evidence of an increase with this marker in the present study perhaps draws into question the reliability of the biomarkers in other studies using a similar protocol.

The fact that muscle damage was clearly evident in the days after both RST tests but oxidative stress was not, suggests that muscle damage occurred independent of any systemic changes in oxidative stress. This would perhaps suggest that RONS have a limited role in the muscle damage process post-exercise. However, it cannot be ruled out that oxidative stress occurred, but was confined predominately to muscle cells and surrounding tissues. Unfortunately, muscle samples could not be obtained in this study, and as such, this supposition is speculative. A recent review however, concluded that skeletal muscle is a prime producer of RONS following exercise; so, intuitively, oxidative stress would perhaps be expected to be greater in muscle than the circulation (Jackson et al., 2016). Alternatively, the muscle damage observed could have been unrelated to oxidative stress. Instead, the muscle damage could have been caused by other biochemical changes within muscle, such as increased inflammation and calpain activity (Belcastro, Shewchuk, & Raj, 1998; Paulsen et al., 2005) or damage to components involved in the EC coupling pathway, as previously suggested (Warren et al., 2002).

Serum CK concentrations, incorporated as a surrogate marker of sarcolemma damage, were raised to a similar extent in both groups after

exercise (Table 11). The increase in CK after the RST was similar to previous reports (Howatson & Milak, 2009; Wooley et al., 2015), as was the lack of a suppressive effect with an AOX-rich food beverage (Bell et al., 2014; Howatson et al., 2010). These data suggest that improved sarcolemma integrity cannot explain the enhanced rate of recovery by BTJ in this study.

Because there were no changes in oxidative stress between groups, the beneficial effects of BTJ on the recovery of CMJ and RSI cannot be attributed to an AOX effect of the juice. This suggests that mechanisms other than AOX effects were possibly involved. It was beyond the scope of this study to examine the role of other mechanisms by which BTJ could attenuate muscle damage, but owing to the seemingly pleotropic nature of the chemical compounds in BTJ (NOx, phenolics and betalains) there are a number of possible candidates. For instance, other effects associated with phenolic compounds and NOx donors akin to BTJ are anti-inflammatory (El Gamal et al., 2014; Jädert et al., 2012) and regenerative, insofar as they appear to have a regulatory role in phagocytosis and promote satellite cell proliferation in skeletal muscle (Kruger & Smith, 2012; Rigamonti et al., 2013; Sakurai et al., 2013). Increasing *in vivo* NOx availability has also demonstrated additional biochemical and physiological effects that, conceivably, could contribute to improved functional recovery after exercise, such as reduced calpain activity (Lomonosova et al., 2014), increased muscle blood flow (Ferguson et al., 2012) and enhanced muscle power potential, possibly via improved Ca^{2+} handling (Coggan et al., 2014; Hernández et al., 2012; Hoon, Fornusek, Chapman, & Johnson, 2015). Thus, there are a number of potential physiological mechanisms that could explain why BTJ supplementation was able to enhance the recovery of muscle function, independent of AOX effects. However, since none of these mechanisms were measured *per se*, it can only be speculated on what role, if any, they might have had in the present study's findings (and those of

Chapter 5). Clearly, the potential role of these additional mechanisms and their relative contributory effects are worthy of further exploration.

Participants in the BTJ group reported a significantly higher PPT than the PLA group 24 h after the second sprint test (Figure 17). Reduced muscle soreness when other AOX-rich food supplements are taken after muscle-damaging exercise has been reported previously (Connolly et al., 2006; Drobic et al., 2014). Chapter 5 also found evidence of improved PPT with BTJ after muscle-damaging exercise. The mechanism(s) by which BTJ might attenuate muscle pain is unclear, however. Previous reports suggest that the betalains in beetroot are responsible for its analgesic effects, most likely via an anti-inflammatory related mechanism (Pietrzkowski et al., 2010). The possibility that an anti-inflammatory mechanism would be involved is supported by data that suggests muscle pain after exercise may stem from the release of inflammatory and noxious stimuli (i.e., bradykin and NGF) due to tears at the ECM (Crameri et al., 2007; Murase et al., 2010). Perhaps BTJ acts to dampen these responses or desensitize pain receptors, as has been suggested with curcumin supplements (Drobnic et al., 2014); however, whatever the precise mechanisms, they are likely to occur at the skeletal muscle level, and thus could not be tested in the present study.

It is also unclear why BTJ only improved PPT 24 h after RST2 in the present study and not at earlier time points, as was shown in Chapter 5. A previous study did observe greater reductions in pain scores after participants took betalain-rich beetroot supplements for 5-10 days compared to 1 day (Pietrzkowski et al., 2010), which, coupled with our data, suggests that the analgesic effects of BTJ might be augmented with longer-term dosage regimens. Such a possibility needs to be investigated in future studies.

In conclusion, this study demonstrates that consuming BTJ for 4 days after a muscle damaging RST attenuated muscle pain (96 h later) and decrements in dynamic muscle function, as measured by CMJ and RSI. These effects did

not translate to improved recovery of isometric strength or sprint performance however. These data suggest BTJ could be applied as a post-exercise recovery strategy to help restore some aspects of dynamic muscle function in team-sports players between bouts of RSE. However, because actual sprint performance was unchanged, which is arguably more meaningful to team-sports players, how transferable these findings are to real-world team-sport competition is unclear. Future studies are needed to clarify the underlying cellular mechanisms, as the beneficial effects of BTJ were shown to be unrelated to systemic changes in oxidative stress or other biochemical markers of muscle damage.

7.5 Perspectives

This study addressed the 4th aim of this thesis, which was to examine the effects of BTJ on muscle damage following RSE in team-sports players. Findings show that the rate of recovery for some markers of functional performance (CMJ and RSI) was improved with BTJ but others (isometric strength and subsequent sprint performance) were unaffected. BTJ also showed signs of attenuating muscle pain, consistent with the findings of Chapter 5.

The RST proved to be a good model to investigate muscle damage in team-sport players, given the large decrements in functional performance in the ensuing days. However, sprint performance had largely recovered by 72 h after the RST, which could explain why markers such as CMJ and RSI that were still depressed at this time point were positively influenced by BTJ supplementation, but sprint performance was not. This finding also suggests that 72 h was sufficient to allow full restoration of repeated sprint performance in this cohort of athletes. Perhaps limiting recovery to 48 h between repeated sprint tests would have shed more light the potential effects of BTJ on the recovery of sprint performance.

Another important finding of this study was that oxidative stress, as measured by systemic changes in PC and the $A^{-\bullet}$, was largely absent after exercise. There was an increase in LOOH immediately and at 2.5 h post-exercise but BTJ did not attenuate this rise compared to a PLA, suggesting that BTJ did not exhibit any AOX effects in this study. This would suggest that any beneficial effect of BTJ on recovery might be unrelated to its AOX potential but instead due to other mechanisms. Irrespective of the precise mechanisms, this study indicates that BTJ could be a useful strategy for mitigating some of the negative symptoms associated with RSE in team-sports players, notably muscle pain and loss of power generating capacity. Coupled with the findings of Chapter 5, the present findings raise the possibility that BTJ could be an effective recovery aid after other types of muscle-damaging exercise.

8 The influence of beetroot juice on muscle damage and inflammation following a marathon race

8.1 Introduction

In Chapters 5 and 7, BTJ was demonstrated to attenuate some of the negative symptoms associated with EIMD, which would indicate that it has the potential to be an effective recovery aid following muscle-damaging exercise. However, in both these studies, the exercise protocols were performed under well-controlled laboratory conditions, which potentially limit the applicability of the findings to real-world athletic performance. Furthermore, the exercise protocols used to inflict muscle damage in these studies (drop jumps and repeated sprints) were relatively short duration (<20 min) and, thus, may not reflect the muscle damage resulting from more prolonged strenuous activities known to precipitate muscle damage, such as long-distance running. Thus, in an attempt to widen the applicability of the findings from Chapters 5 and 7 to other sporting scenarios, the present Chapter sought to investigate the effects of BTJ on muscle damage following a marathon running race.

It has long been established that running a marathon can cause marked and prolonged damage to skeletal muscle fibres and the surrounding connective structures (Hikida, Staron, Hagerman, Sherman, & Costill, 1983; Warhol et al., 1985). From a functional perspective, the ultrastructural damage often manifests as a loss in muscle force generating capacity and feelings of muscle pain and tenderness in the lower limbs (Areces et al., 2014; Howatson et al., 2010; Hill et al., 2014). Typically, these symptoms, particularly muscle soreness, become more apparent in the days after the marathon (Hill et al., 2014; Howatson et al., 2010); hence, soreness is usually referred to as DOMS (Smith, 1992). Such prolonged impairments in functional capacity can hinder an individual's ability to perform exercise and even daily activities that incorporate the affected muscle groups. Furthermore, muscle soreness, coupled with a restricted range of movement, and attenuated muscle function, might increase the propensity for injury (Smith, 1992). It is therefore of great interest to identify recovery strategies

that could minimise the muscle damage associated with running a marathon. Based on the findings in Chapter 5 and 7, BTJ supplementation might offer an effective and practical modality for alleviating the deleterious symptoms that accompany marathon running.

A marathon can also evoke a profound inflammatory response, which, as alluded to in Chapter 2.2.3, is proposed to be a key driver of secondary muscle damage. Evidence for the marked perturbations in immune function after marathon running is provided by a number of studies, in which large systemic increases in cytokine and leukocyte activity in the hours and days were observed following a race (Howatson et al., 2010; Laupheimer, Perry, Benton, Malliaras, & Maffulli, 2014; Scherr et al., 2011; Suzuki et a., 2003). Consequently, the marathon also serves as useful model to study the acute inflammatory response and its relationship with muscle-damaging exercise. Additionally, because the increase in inflammation is generally greater than after other exercise paradigms, it is perhaps one of the most appropriate models for studying the anti-inflammatory potential of an intervention. Thus, this study also provided an opportunity to gain a deeper understanding of the anti-inflammatory potential of BTJ; an aspect that has received limited attention in this thesis so far (see Chapter 5). To test this, a comprehensive array of inflammatory makers proposed to play a role in the secondary muscle damage process were measured alongside the primary outcome measures (muscle function), in the hope that it would shed some light on the potential mechanism(s) by which BTJ might alleviate muscle damage and facilitate recovery. It was hypothesized that BTJ would be more effective than a PLA for attenuating muscle damage and the systemic inflammatory response in the 48 h after the marathon.

8.2 Methods

8.2.1 Participants

Participants were recruited from a pool of runners taking part in the 2016 Druridge Bay Marathon, Northumberland, UK, which took place on April the 17th 2016. Based on a previous study that examined recovery after a marathon race (Howatson et al., 2010), it was estimated that at 0.80 power and 0.05 significance level, the minimum number of participants required to detect a ≥10% group difference (SD: 7.5%) in one of our primary outcomes, MIVC, would be n = 10 per group. We surpassed this target, with 36 runners initially volunteering to participate in this investigation. Two runners dropped out during the race after sustaining injuries unrelated to the study procedures, thus, thirty-four runners completed all study requirements. A summary of their characteristics is presented in Table 12. Female participants completed a menstrual cycle questionnaire in order to determine menstrual cycle phase. Of those still menstruating, testing fell during the early/mid luteal phase. Information regarding eligibility criteria can be found in Chapter 3.1.

Table 12 - Participant characteristics and training history for the BTJ and PLA groups.

	BTJ (*n*=17)	**PLA (*n*=17)**
Age (yrs)	42±10	39±12
Sex (M/F)	10/7	11/6
Height (m)	1.71±0.09	1.72±0.08
Mass (kg)	69.7±10.4	71.0±11.3
Yrs running	13±11	7±6
Average weekly mileage	34±11	32±10
Longest training run (miles)	20±2	21±2
Previous marathons	12±14	19±38
Predicted finish time	04:04:06	04:09:24
Actual finish time	04:05:39	04:28:29
Pre-post change in body mass (kg)	1.3±0.9	1.3±0.8
Pre-post change in plasma volume (%)	0.5 ± 5.3	−1.9 ± 5.7

Values are mean ± SD. There were no differences between groups for any variable (P> 0.05).

8.3 Study design

This study employed a double blind, placebo-controlled, independent group design with two experimental arms. Participants were allocated to receive either BTJ; n = 17 or a PLA; n = 17 for 3 days after a marathon race, with the first supplement taken immediately after a series of post-race marathon tests had been completed. All participants recorded their dietary intake throughout the trial (24 h prior to until 48 h after the marathon) (See Chapter 3.3 for further details). The groups were matched according to predicted marathon finish times and contained a similar number of females and males (Table 12). Participants attended the lab on 3 occasions in total: the first was in the week leading up to the marathon to collect baseline measures, and the other two on consecutive days after the marathon. Pre-marathon (baseline), immediately post-marathon, and on two days after the marathon (day 1 and day 2) participants had a blood sample taken, rated their muscle soreness,

and completed a CMJ and MIVC test. Participants were fully familiarised with these procedures on their baseline visit.

8.3.1 Supplementation

Please refer to general methods Chapter 3.4 for further details on the supplements used in this study. With regards to timings, in the present study, participants consumed 3 bottles (250 ml each) of their assigned supplement on the day of the marathon (immediately post-marathon, 3 h later and at 20:00); another 3 bottles on day 1 post (upon waking, with lunch and at 20:00) and 1 bottle (upon waking) on day 2 post. The rationale for providing 3 daily servings after the marathon was based on the findings from Chapter 5 that showed 3, 250 ml servings taken after muscle-damaging exercise attenuated muscle pain and muscle function deficits in the ensuing days. Furthermore, because it has been shown that systemic inflammation is still significantly elevated in the days after a marathon, 3 servings were also provided on day 1 post-marathon in an attempt to counteract this stress (Hill et al., 2015; Scherr et al., 2011). Participants were given written instructions on when to consume their supplements. They were also asked to complete a checklist, which was to be returned to the principle investigator at the conclusion of the study to ensure compliance.

8.3.2 Marathon race

The race consisted of 4 laps of the Druridge Bay Country Park situated on the Northumberland coast (Morpeth, UK). The course is mostly flat and across a mix of grassy or concrete terrain; however, approximately 1 mile of each lap was on soft sand (see Figure 18 for race route). At 09:00, when the race started, the air temperature was 3.8˚C, humidity 82%, barometric pressure 1013 hpa and wind speed 9 $km \cdot h^{-1}$. Towards the end of the race (13:00-14:00) there was an increase in air temperature (8.5°C) and wind

speed (14 km·h^{-1}) with the humidity dropping (62%). It remained dry and mostly overcast for the duration of the race with intermittent sunny spells.

Figure 18 - Marathon race route.

8.3.3 Maximal voluntary isometric contraction

Please refer to general methods Chapter 3.6.1 for details on MIVC measurement.

8.3.4 Countermovement jump

Please refer to general methods Chapter 3.6.2 for details on CMJ measurement.

8.3.5 Muscle soreness

Muscle soreness was measured as per previously described methods (Howatson et al., 2009). After performing a squat (at ~90˚ knee flexion), participants rated their perceived level of muscle soreness (lower limbs only) by drawing a vertical line on a VAS, in which 0 represented 'no soreness' and 200 mm represented 'unbearably painful'. The line placement was measured with a ruler and recorded in mm.

8.3.6 Blood sampling and biochemical analysis

For details of blood sampling procedures please refer to general methods, Chapter 3.7. Haemoglobin, haematocrit, and leukocytes were measured in whole blood using an automated haematology system (Sysmex XE-2100, Illinois, US). According to data provided by the laboratory, the CV's for this procedure are typically <10%. Haemoglobin and haematocrit were used to calculate pre-post marathon changes in plasma volume according to the methods of Dill & Costill (1974). Serum concentrations of cytokines and growth factors were measured according to the manufacturer's instructions using a multiplex sandwich chemiluminescent immunoassay kit (Evidence Investigator, Randox Laboratories, Northern Ireland, UK). Inter and intra assay CV's were below 5%.

CK, hs-CRP and AST were measured in serum using an automated system based on an electrochemiluminescence method (Roche Modular, Roche Diagnostics, UK). According to data provided by the laboratory, the typical CV for these measures with this procedure is <5%.

8.3.7 Data Analysis

All data are expressed as mean ± SD and statistical significance was set at $P < 0.05$ prior to analyses. Please refer to Chapter 3.2 for details on the analysis of participant food diaries. Differences in participant group characteristics were analysed with multiple student t-test's. All blood variables were adjusted for plasma volume changes prior to analyses. Dependent variables (MIVC, CMJ, VAS and all blood indices) were analysed using a mixed model ANOVA with 2 independent group levels (BTJ vs. PLA) and 4 repeated measures time points (pre, post, day 1 and day 2 post). If the ANOVA indicated a significant interaction effect (drink*time) Fisher LSD *post hoc* analysis was performed to locate where the significant differences occurred. Homogeneity of variance was checked with Mauchly's test of sphericity and in the event of a significant result, Greenhouse-Geisser adjustments were used. All data were analysed using IBM SPSS Statistics 22 for Windows (Surrey, UK).

8.4 Results

There were no between group differences for training history and personal characteristics suggesting that the groups were well matched before the marathon (Table 12). Mass decreased to a similar extent in both groups after the marathon. Changes in plasma volume from pre-post marathon were modest and did not differ between groups ($P > 0.05$).

Average energy intake did not differ between groups (BTJ, 2488 ± 607 vs. PLA, 2308 ± 480 kcal), nor did the proportion consumed from carbohydrates (BTJ, 49.88 ± 0.07 vs. PLA, 46.76 ± 0.05%) and protein (BTJ, 16.38 ± 0.02

vs. PLA, 14.71 ± 0.04%) (P > 0.05). The proportion of calories from fat was slightly higher in the PLA vs. BTJ group (P = 0.032; 36.12 ± 0.04 and 32.06 ± 0.06%, respectively).

8.4.1 Muscle function and muscle soreness

All muscle function tests (CMJ and MIVC) and muscle soreness showed a main effect of time (P < 0.001), indicating the presence of muscle damage after the marathon (Figure 19 and Figure 20). Relative to baseline values, CMJ decreased to a similar extent in the BTJ and PLA groups immediately post-marathon (71.4 ± 18.5 vs. 69.2 ± 20.5%, respectively) and remained similarly depressed at day 2 (94.5 ± 8.86 vs. 95.3 ± 5.9%, respectively). No group or interaction effects were present at any time point (P > 0.05). MIVC was less affected by the marathon than CMJ and had recovered to pre-marathon values by day 2 (BTJ: 100.6 ± 13.5 vs. PLA: 99.1 ± 10.6% of baseline). MIVC recovery was independent of treatment with no group or interaction effect observed (P > 0.05). Muscle soreness was greatest immediately after the marathon and was largely absent at day 2 (Figure 20). No group or interaction effects were present (P > 0.05).

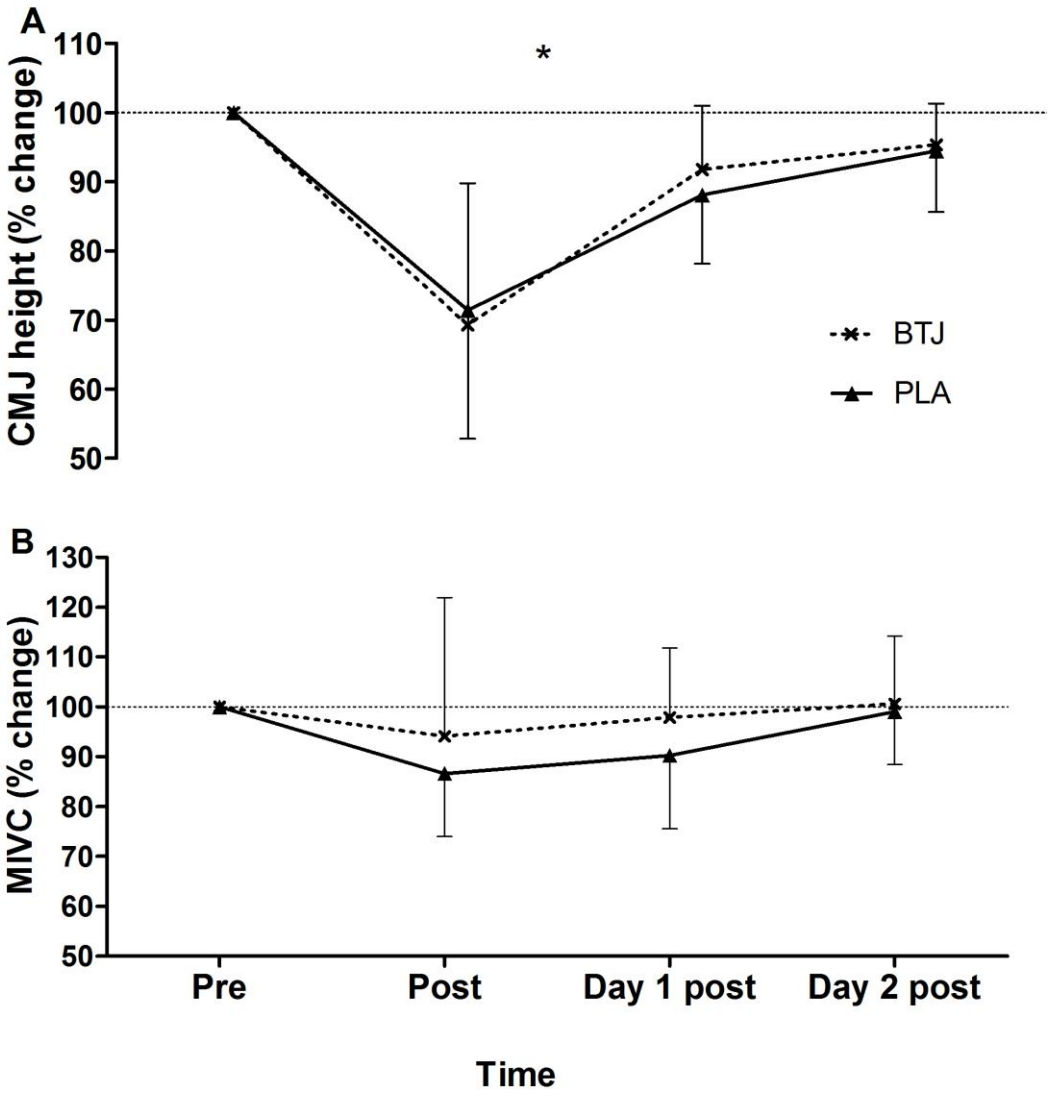

Figure 19 - (A) Percentage change from baseline in counter movement jump (CMJ) height before and after the marathon (beetroot juice; BTJ, placebo; PLA). (B) Percentage change from baseline in maximal isometric voluntary contractions (MIVC) before and after the marathon. *Represents time effect ($P < 0.05$). Values are mean ± SD ($n = 17$ per group).

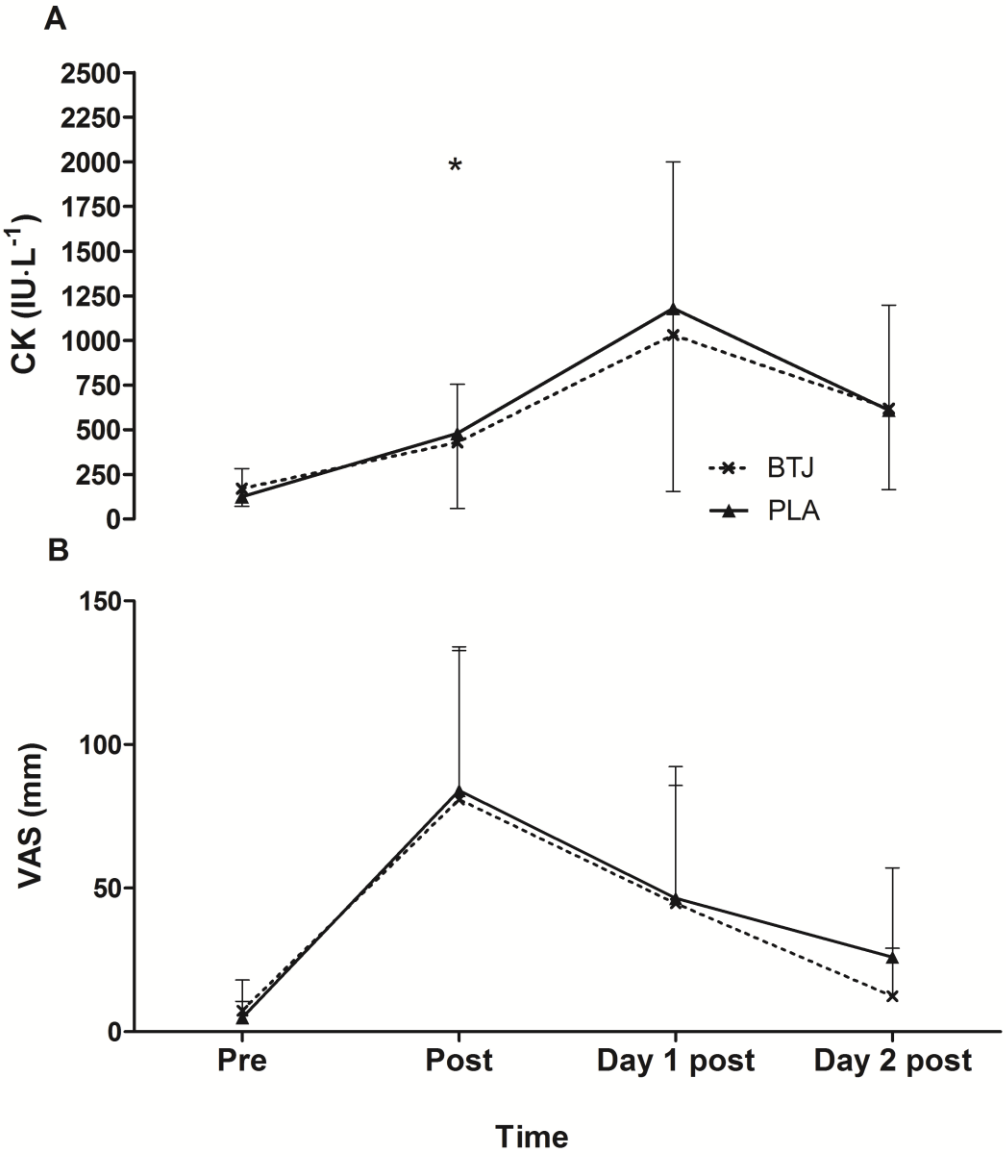

Figure 20 - (A) Serum creatine kinase (CK) concentrations before and after the marathon (beetroot juice; BTJ, placebo; PLA). (B) Muscle soreness (VAS) before and after the marathon. *Represents time effect ($P < 0.05$). Values are mean ± SD (n = 17 per group).

8.4.2 Leukocytosis, inflammation and liver function

Total blood leukocyte counts demonstrated main effects for time ($F_{(3, 90)}$ = 200.16; $P < 0.001$) increasing 1.7 fold (average across groups) immediately after the race. Leukocytes were still raised above pre-marathon levels on day 1 ($P < 0.001$) and day 2 post-marathon ($P < 0.05$); however, no group or interaction effects were present ($P > 0.05$; Table 13). Neutrophils and monocytes followed the same pattern, peaking immediately after the marathon and not returning to baseline by day 2 post-marathon in both groups ($P < 0.05$). No group or interaction effects were present at any time point for these measures ($P > 0.05$).

Serum CK and AST demonstrated main effects for time ($P < 0.001$) but no group or interaction effects were present ($P > 0.05$). In both groups CK and AST increased immediately after the marathon, peaked day 1 post, and although were attenuated on day 2, still remained higher than pre-marathon values (Figure 20 and Table 13, respectively). Serum hs-CRP also demonstrated main effects for time ($F_{(3, 87)}$ = 45.30; $P < 0.001$) and was elevated on day 1 and day 2 post-marathon (Table 13). There were no group or interaction effects for hs-CRP ($P > 0.05$).

Table 13 - Serum inflammation and liver function response before and after the marathon in the BTJ and PLA groups.

Biomarker	Pre race	Post race	Day 1 post	Day 2 post
Leukocytes (10^{*9} cells·L^{-1})				
BTJ	6.26 ± 1.11	15.24 ± 2.90*	7.46 ± 2.02*	7.28 ± 1.94*
PLA	5.25 ± 1.5	15.22 ± 3.60*	7.10 ± 1.44*	6.47 ± 1.47*
Neutrophils (10^{*9} cells·L^{-1})				
BTJ	3.55 ± 1.01	12.55 ± 2.55*	4.21 ± 1.23*	4.04 ± 1.13*
PLA	2.67 ± 1.07	12.52 ± 3.49*	3.88 ± 1.13*	3.38 ± 0.89*
Monocytes (10^{*9} cells·L^{-1})				
BTJ	0.51 ± 0.11	1.00 ± 0.38*	0.65 ± 0.21*	0.68 ± 0.16*
PLA	0.50 ± 0.17	1.06 ± 0.27*	0.61 ± 0.13*	0.62 ± 0.20*
Hs-CRP ($mg \cdot L^{-1}$)				
BTJ	0.88 ± 1.16	0.62 ± 0.62	12.22 ± 9.03*	7.18 ± 4.49*
PLA	0.70 ± 0.82	0.57 ± 0.71	11.37 ± 10.10*	6.10 ± 4.90*
AST ($IU \cdot L^{-1}$)				
BTJ	21.66 ± 7.43	30.68 ± 9.25*	49.97 ± 29.44*	41.16 ± 23.5*
PLA	23.25 ± 12.47	31.24 ± 7.36*	60.28 ± 48.80*	48.94 ± 31.79*

Values are mean ± SD, n = 17 per group. *Elevated above baseline values ($P < 0.05$). hs-CRP, high sensitivity-C-reactive protein; AST, aspartate aminotransferase, CK, creatine kinase.

8.4.3 Cytokines, growth factors and chemokines

Changes in cytokine, growth factor, and chemokine activity pre to post marathon are displayed in Table 14. Immediately post-race, the serum cytokines IL-6, IL-8, IL-10 and TNF-α all increased (time effect; $P < 0.001$). IL-6 was the only cytokine still significantly elevated on day 2 (~0.9 fold change in BTJ and ~0.5 fold in PLA) but no significant group or interaction effects were present ($P > 0.05$). Other cytokines measured (IL-ra, IL-2, IL-4 and IFN-γ) did not rise appreciably at any time point after the marathon in either group ($P > 0.05$). VEGF was unchanged after the marathon but EGF

showed main effects for time ($F_{(3, 90)} = 23.47$; $P < 0.001$), initially decreasing after the marathon before increasing at day 1 and day 2; however, no group or interaction effects were observed ($P > 0.05$). The chemokine, MCP-1, demonstrated a main effect for time ($F_{(3, 90)} = 151.88$; $P < 0.001$) increasing in BTJ (~2 fold-change) and PLA (~1.9 fold change) immediately after the marathon. MCP-1 was still elevated on day 2 post marathon but to a similar extent in both groups with no group or interaction effects present ($P > 0.05$).

Table 14 - Serum cytokine, growth factor, and chemokine response before and after the marathon in the BTJ and PLA groups.

Biomarker (pg/ml)	**Pre race**	**Post race**	**Day 1 post**	**Day 2 post**
IL-1_{ra}				
BTJ	0.38 ± 0.27	0.31 ± 0.12	0.29 ± 0.09	0.30 ± 0.09
PLA	0.39 ± 0.27	0.37 ± 0.24	0.38 ± 0.22	0.37 ± 0.31
IL-1β				
BTJ	1.44 ± 0.72	3.09 ± 1.41*	1.76 ± 0.60	1.63 ± 0.77
PLA	1.79 ± 1.03	2.29 ± 1.21*	2.26 ± 1.70	2.45 ± 2.31
IL-2				
BTJ	2.89 ± 3.32	4.96 ± 8.72	2.85 ± 2.57	2.87 ± 2.52
PLA	4.19 ± 3.00	3.84 ± 2.97	4.34 ± 3.16	6.75 ± 10.03
IL-4				
BTJ	2.15 ± 0.61	2.13 ± 0.41	2.25 ± 0.45	2.56 ± 0.81
PLA	2.52 ± 0.78	2.32 ± 0.73	2.36 ± 0.68	2.38 ± 0.78
IL-6				
BTJ	1.15 ± 0.48	31.12 ± 15.93*	2.26 ± 1.89*	1.64 ± 0.73
PLA	1.02 ± 0.51	31.42 ± 25.12*	1.53 ± 0.58*	1.05 ± 0.47
IL-8				
BTJ	7.60 ± 3.57	19.34 ± 8.95*	7.00 ± 3.06	7.78 ± 4.03
PLA	8.38 ± 5.83	19.22 ± 10.91*	6.94 ± 4.93	6.83 ± 6.40
IL-10				
BTJ	0.94 ± 0.35	17.55 ± 16.42*	0.90 ± 0.18	1.34 ± 1.74
PLA	1.62 ± 1.01	16.58 ± 19.01*	1.36 ± 0.72	1.50 ± 1.02
TNF-α				
BTJ	2.98 ± 1.13	3.61 ± 1.34*	2.94 ± 0.84	3.17 ± 1.15
PLA	2.81 ± 0.70	3.17 ± 0.77*	2.70 ± 0.58	2.95 ± 1.01
VEGF				
BTJ	127.41 ± 86.69	131.57 ± 112.48	144.87 ± 101.78	144.74 ± 94.74
PLA	100.66 ± 77.58	97.24 ± 102.34	121.54 ± 99.59	120.5 ± 100.09
INF-γ				
BTJ	0.52 ± 0.51	0.51 ± 0.65	0.43 ± 0.61	0.61 ± 0.77
PLA	0.42 ± 0.32	0.34 ± 0.24	0.43 ± 0.31	0.66 ± 0.86
EGF				
BTJ	22.55 ± 20.36	17.68 ± 13.25$^{\#}$	59.24 ± 47.00*	72.21 ± 45.06*
PLA	27.68 ± 34.97	10.37 ± 10.15$^{\#}$	50.94 ± 37.99*	58.35 ± 43.95*
MCP-1				
BTJ	178.49 ± 85.24	537.22 ± 107.60*	235.82 ± 60.80*	225.92 ± 76.46*
PLA	172.75 ± 52.12	486.49 ± 190.83*	222.19 ± 78.08*	198.28 ± 69.59*

Values are mean ± SD, n = 17 per group. *Elevated above baseline values (P < 0.05); $^{\#}$Decreased below baseline levels (P > 0.05). IL-1-ra, interleukin 1-receptor agonist; IL-1β, interleukin-1beta; IL-2, interluekin-2; IL-4, interleukin-4; IL-6, interleukin-6; IL-8, interleukin-8; IL-10, interleukin-10; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; INF-γ, interferon-gamma; MCP-1, monocyte chemoattractant protein 1.

8.5 Discussion

It was hypothesized that BTJ would enhance the recovery of muscle function and attenuate muscle soreness after a marathon race, possibly by reducing the acute inflammatory response shown to accompany long-distance running. However, contrary to this hypothesis, BTJ did not favourably affect the recovery of muscle function or attenuate muscle soreness in the 2 days after the marathon. In addition, biochemical markers of inflammation or muscle damage measured before and up to 2 days after the race did not differ between the BTJ and PLA groups, suggesting that BTJ was ineffective for attenuating the acute inflammatory response after marathon running.

The findings in the present study are in contrast to a previous study that examined the effects of a phytonutrient rich drink (cherry juice) on EIMD after a marathon (Howatson et al., 2009). In this study, cherry juice supplementation was shown to attenuate biomarkers of inflammation (IL-6) and oxidative stress (TBARS) in the 48 h following the marathon; effects that were associated with an accelerated recovery of MIVC (Howatson et al., 2010). There are, however, some differences in study design that could account for these discrepant findings. Apart from the obvious difference in that cherry juice was used as opposed to BTJ and, therefore, the biological activities of the phytonutrients in each drink likely differ, Howatson and colleagues (2010) also provided supplements in the 5 days leading up to the marathon, as well as the 3 days after. By contrast, in the present study, supplementation only began after the marathon race; thus, the discrepancy in findings between these two studies could be due, at least in part, to the different dosage strategies used. Speculatively, a more long-term prophylactic dosage strategy could have been required to mediate similar recovery benefits with BTJ after the marathon.

The lack of benefit with BTJ in the current study is also in direct contrast to the findings in previous Chapters, in which BTJ was shown to enhance the

recovery of CMJ performance and PPT in the days following strenuous plyometric exercise (Chapter 5) and RSE (Chapter 7). The differences in exercise protocol (marathon race), participant cohort (experienced marathon runners) and techniques used to assess EIMD (VAS vs. PPT) between the present and previous studies are all factors that could provide a potential explanation for these disparate results. Another possible explanation is related to the magnitude of the muscle damage response, which, in the present study, was markedly lower, and largely absent by 2 days post. Additionally, in contrast to the previous studies (Chapter 5 and 7), there was no further loss in muscle function in the post-exercise period. It is therefore possible that the small magnitude of muscle damage in this study might have limited our ability to detect any subtle differences between groups. Indeed, it would be reasonable to assume that any recovery benefits associated with BTJ or any recovery intervention would only be evident when muscle damage is still present. The present data suggests that the marathon runners in this study might have already been sufficiently well protected from marked and prolonged symptoms of muscle damage and, thus, BTJ conferred no benefits for recovery.

After strenuous exercise, leukocytes, mostly neutrophils and monocytes, migrate from the circulation into damaged muscle in order to degrade structural components and, thus, are thought to play a major role in secondary muscle damage after exercise (Butterfield et al., 2006; Pizza et al., 2005). As alluded to in earlier Chapters (2.7.4), several of the phenolic compounds and pigments in BTJ have been shown to exhibit ant-inflammatory activity (El Gamal et al., 2014; Vulic et al., 2014), and there is also evidence that NOx, which can be endogenously generated from the nitrate in BTJ (Chapter 4; Wylie et al., 2012), is a critical regulator of inflammation (Waltz, Escobar, Botero, & Zuckerbraun, 2015; Weitzberg & Lundberg, 2013). As such, it was hypothesized that BTJ might inhibit leukocytosis, which, in turn, might reflect a preservation of muscle cell integrity and functional strength. However, contrary to this hypothesis,

leukocyte accumulation in the circulation was unaffected by BTJ supplementation. As shown in Table 13, while the marathon provoked a large inflammatory response, with total leukocyte counts, neutrophils and monocytes still elevated above pre-marathon values 2 days' post, the magnitude of change was not different in the PLA and BTJ groups. These results suggest that BTJ, at least at this dose and in marathon runners, did not modulate the systemic leukocyte response after exercise. Based on these data, perhaps it is unsurprising this study did not find any beneficial effect of BTJ on muscle soreness and muscle function.

BTJ also had no effect on AST, hs-CRP or a host of cytokines in the days after the marathon race. Several cytokines were increased immediately after the marathon race (IL-6, IL-8 and TNF-α), which is in agreement with previous studies that examined the early cytokine response after long distance running events (Howatson et al., 2010; Nieman et al., 2002; Shanely et al., 2013; Scherr et al., 2011). However, some of the cytokines measured did not change at all after the marathon (IL-1β, IL-1ra, IL-2, IL-4, IFN-y) and only IL-6 was elevated above pre-exercise values in the days after the marathon. These findings are in contrast to a previous study that found experienced runners still exhibited elevated levels of TNF-α and IL-10 >1 day after a marathon race (Scherr et al., 2011). This study did contain a larger cohort of participants than the present study however (n = 105), so perhaps this study was underpowered to detect such small changes in these markers. Nonetheless, the present findings suggest that cytokines, at least at the systemic level, might not play a significant role in the secondary muscle damage process in the days after the marathon. Instead, their activity might be primarily limited to the muscle and surrounding tissues, as previously suggested after other types of exercise (Peake et al., 2015a). It is possible that had blood samples been taken at additional time points however, specifically between the end of the marathon and day 1, several of these cytokines would have still been elevated. This might have been a better time in the post-exercise period for detecting potential differences between the

groups. Likewise, if supplements had been consumed in the days before the marathon, as in the study by Howatson et al. (2010), group differences might have been apparent at the post-marathon time point, or have at least been easier to detect, given the majority of biomarkers were significantly elevated at this point (Table 13 and Table 14).

In contrast to many of the cytokines, the chemokine, MCP-1, and the growth factor, EGF, were still significantly elevated at 2 days' post-marathon, suggesting that they have more prominent roles in muscle damage after exercise. Indeed, MCP-1, also known as CCL2, is thought to play a particularly important role in resolving muscle damage after exercise, via its effects on facilitating macrophage infiltration and activating resident satellite cells (Hubal et al., 2008; Urso et al., 2013; Warren et al., 2004). Nonetheless, as with the other inflammatory markers measured in this study, MCP-1 was unaffected by BTJ supplementation.

As seen in previous marathon studies, serum CK activity was markedly increased, peaking 1 day after the race (Howatson et al., 2010; Hill et al., 2015). The magnitude of CK release did not differ between the groups however, which is consistent with previous Chapters (5 and 7) and the work of others who report no benefit of functional foods akin to BTJ on CK efflux after muscle damaging exercise (Bell et al., 2014; Howatson et al., 2010; Trombold et al., 2010).

In conclusion, this study reports that consuming BTJ for 3 days after a marathon does not attenuate muscle soreness, enhance the recovery of muscle function or attenuate biochemical markers of inflammation and muscle damage in experienced marathon runners. Because muscle fucntion recovery was largely complete, and muscle soreness absent by 2 days after the marathon, the experienced runners in this study might have already been sufficiently well-trained to protect against high levels of muscle damage; thus, BTJ provided no additional benefit. Notwithstanding, future studies

might benefit from including a loading phase in the days leading up to the marathon.

8.6 Perspectives

The overall aim of this thesis is to determine whether BTJ is more effective than a PLA for attenuating muscle damage and enhancing recovery following strenuous exercise. In previous Chapters (5 and 7), BTJ was associated with an improved recovery of muscle function and reduced feelings of muscle pain after high volume plyometric exercise and high-intensity RSE. It was therefore anticipated that BTJ might also help to protect against muscle damage following a marathon, which involves a high volume of eccentric muscle contractions and, thus, typically results in a prolonged muscle damage response. However, the results presented in this Chapter suggest that BTJ is not an effective recovery aid following marathon running. Muscle function, as measured by MIVC and CMJ performance, and muscle soreness, as measured with a VAS, did not differ in the BTJ and PLA groups in the 2 days after the marathon. Furthermore, a host of inflammatory markers were unaffected by BTJ supplementation.

There are several factors that could explain the discrepant results between this study and the previous studies in this thesis. These include but are not limited to differences in exercise model, participant training status, timing of measures and measurement tools used. Perhaps the biggest difference in the present study is that the magnitude of muscle damage was much lower than that reported in the two previous studies. This could be because the marathon runners in the present study were experienced (16 previous marathons completed on average per group) and, therefore, possibly sufficiently well accustomed to protect against muscle soreness and prolonged deficits in muscle function. Irrespective of the exact reasoning, this study does not support the use of BTJ to alleviate muscle damage and

accelerate recovery in athletes already accustomed to the EIMD associated with long distance running events.

Furthermore, although the original hypothesis was that BTJ might enhance recovery by reducing post-exercise inflammation, there was no evidence of this in the present study. Perhaps this could explain why BTJ was ineffective at enhancing functional recovery. Nonetheless, the fact that inflammation did not differ between the groups would suggest that any benefits of BTJ on exercise recovery could be unrelated to an anti-inflammatory effect *per se*. In order to gain a deeper understanding of the potential mechanisms by which BTJ has benefitted exercise recovery, it would be useful to know which compounds in BTJ are most likely to exert positive effects for recovery. Thus, the final Chapter in this thesis attempted to isolate the effects of the nitrate in BTJ from the other phytonutrients (i.e., phenolic and betalainic compounds) on EIMD.

9 Comparison of the effects of beetroot juice and sodium nitrate on exercise-induced muscle damage

9.1 Introduction

The data presented in this thesis so far has demonstrated that BTJ might be beneficial for attenuating indices of EIMD after some (plyometric exercise and RSE; Chapters 5 and 7, respectively) but not all (marathon running; Chapter 8) forms of exercise. Notwithstanding these equivocal findings, an intriguing question that still remains is what are the compounds in BTJ primarily responsible for exerting some of these positive effects? Indeed, it is unclear whether some of the beneficial effects of BTJ seen in previous Chapters is attributable to the phenolic and betalainic compounds in BTJ, as is proposed for other functional foods (Bell et al., 2014; Bell et al., 2015; Bowtell et al., 2011; Howatson et al., 2010; Trombold et al., 2010; Trombold et al., 2011), or, the nitrate in BTJ, as has been suggested by other work (Lomonosova et al., 2014; Rigamonti et al., 2013). Thus, the aim of the final investigation in this thesis was to gain a more in-depth understanding of the role the nitrate component of BTJ has in exerting protection against EIMD.

As demonstrated in Chapter 4, BTJ is rich in a variety of phenolic and betalainic compounds. Many of these compounds are endowed with interesting biological activities that include, but also extend beyond anti-inflammatory and AOX and, thus, at least theoretically, they could be of benefit for exercise recovery (see Chapter 2.6). In support, several studies have demonstrated that EIMD is attenuated after consuming functional food supplements rich in AOX phenolic compounds (Bell et al., 2014; Bell et al., 2015; Bowtell et al., 2011; McLeay et al., 2012; Howatson et al., 2010; Trombold et al., 2010; Trombold et al., 2011), evidencing that this class of nutrients appears to have a positive role in recovery after exercise. Consequently, it would be reasonable to assume that any beneficial effects of BTJ on exercise recovery would be mediated, in large part, by the high number of phenolic and betalainic compounds it contains.

However, unlike other functional foods shown to attenuate EIMD, BTJ is unique in that it also contains high amounts of nitrate, a precursor for endogenous NOx production (Lundberg et al., 2008; Kapil et al., 2014). It has become apparent that NOx has a regulatory influence on several of the biological processes often impaired (microvascular blood flow, Ca^{2+} handling) (Ferguson et al., 2013; Ferguson et al., 2014; Hernandez et al., 2013; Hoon et al., 2015) or upregulated (phagocytosis, calpain activity and myogenesis) (Jädert et al., 2012; Lomonosova et al., 2014; Rigamonti et al., 2013) after muscle-damaging exercise. Due to the potential involvement of NOx in the damage and repair processes in skeletal muscle, it has been suggested that NOx donors, such as nitrate for example, could offer a therapeutic approach to enhance recovery after muscle injury (Lomonosova et al., 2014; Rigamonti et al., 2013). However, the effects of nitrate on muscle recovery have not been tested in humans. A greater understanding of the role of nitrate in EIMD could help to elucidate the mechanisms behind some of the benefits seen with BTJ on recovery in this thesis so far. It would also help to answer the question of whether these effects are exclusive to BTJ and its somewhat unique mixture of phytonutrients, or whether similar effects could be expected with foods that are just simply rich in nitrate. Thus, the aim of this study was to investigate the effects of BTJ versus a nitrate only containing drink (SN) on muscle damage following a bout of eccentric-heavy exercise. It was hypothesized that BTJ and SN would be similarly effective for attenuating indices of muscle damage compared to a PLA.

9.2 Methods

9.2.1 Participants

According to the findings from Chapter 5, a sample size of n = 10 per group was sufficient to detect an 8% (ES = 1.25) between group difference (SD: 6%) in CMJ at a power of 0.80 and α level of 0.05. Consequently, 30 healthy male participants were recruited to participate in this study

Table 15). All participants were recreationally active but none had completed intense plyometric exercise for ≥12 months.

Table 15 - Physical characteristics, nitrate content of the supplements, and macronutrient content of participant's dietary intake throughout the study.

Characteristic	Group		
	BTJ	**SN**	**PLA**
Age (years)	23±3	22±3	21±1
Mass (kg)	76.7±12.1	76.0±7.5	72.8±10.8
Height (cm)	178.9±7.5	179.2±6.9	176.1±5.0
Daily energy intake (kcal)	2460±494	2491±706	2108±387
CHO (%)	42	44	43
PRO (%)	18	22	20
FAT (%)	40	34	37
Nitrate (mg per serving)	~210	~210	N/A

Values are means ± SD; *n*=10 per group. Groups did not differ for any variable ($P > 0.05$).

9.2.2 Experimental design

The study employed a double blind, randomized, independent groups design. Prior to data collection, participants were required to attend a familiarisation session in which height, mass and MIVC were established. Using MIVC scores as a blocking factor, participants were then randomized to 1 of 3 experimental treatment groups: SN; *n* =10, BTJ; *n* =10 or PLA; *n* =10. At least 1 week after familiarisation participants attended the laboratory on 4 consecutive mornings. As in Chapter 5, on day 1, participants performed a strenuous plyometric exercise protocol to induce muscle damage. A venous blood draw, muscle soreness and measures of muscle function were taken pre and immediately post muscle-damaging exercise on day 1 and on the following 3 mornings (24, 48 and 72 h post-exercise). After

the muscle-damaging exercise on day 1, participants consumed 1 serving of their allocated supplement alongside a standardized breakfast meal. Breakfast consisted of cereal (Rice Krispies, Kellogs, UK) and milk (semi-skimmed, Tesco Ltd, UK) and provided 10% of daily energy requirements calculated from age and body mass (kg) (Schofield, 1984). Following a 2.5 h absorption period a final blood draw was taken. Participants could consume water *ad libitum* during the absorption period but were required to avoid consuming any other foods until the final blood draw.

9.2.3 Muscle-damaging protocol

The muscle damaging protocol consisted of 100 drop jumps from a 0.6 m high box. See Chapter 3.5 for further information.

9.2.4 Muscle soreness

Muscle specific soreness was assessed as PPT with a handheld algometer. See Chapter 3.6.3 for further information.

9.2.5 Maximal isometric voluntary contraction

Please refer to Chapter 3.6.1 for details on MIVC measurement.

9.2.6 Counter movement jump

Please refer to Chapter 3.6.2 for details on CMJ measurement.

9.2.7 Blood sampling and analysis

See Chapter 3.7. for blood sampling procedures. CK, hs-CRP and NOx were measured according to the methods described in section 3.7.1, 3.7.2 and 3.7.3, respectively.

9.2.8 Supplementation

Participants received BTJ, SN or an isocaloric PLA for 3 days' post muscle-damaging exercise. Similar to a previous study in this thesis (Chapter 5), supplements were consumed on three occasions on day 1; one 30 min post-exercise alongside a breakfast meal, one 2.5 h post after an additional blood sample, and a third with their evening meal. Participants consumed 2 more servings at 24 and 48 h post (the first within 30 min of completing all dependent variables and the second with their evening meal). Details of the BTJ and PLA treatments used in this study are provided in Chapter 3.4. SN was purchased in powder form (BASF, Ludwigshafen, Germany) and mixed with water into bottles for participants to consume as a drink. Both maltodextrin (Myprotein, Manchester, UK) and flavourless protein powder (Arla Foods, Amba, Denmark) were added to each serving of SN to match the BTJ for macronutrient composition. The SN and BTJ were matched as closely as possible for nitrate content. The nitrate content of this particular batch of BTJ was approximately 210 mg (~3.4 mmol/L; see Table 15) per 250 ml serving (data from the manufacturer). This amount of nitrate was equivalent to 287 mg of SN after adjusting for differences in molecular weight; thus, 287 mg (~3.4 mmol/L) of SN powder was weighed and added to each 250 ml serving.

9.2.9 Dietary control

Participants were asked to record their dietary intake throughout the trial (see Chapter 3.2 for further information and Appendix 5 for a food diary example). In addition, prior to each study visit, participants were provided with a meal (Beef Lasagne, 450 g; Tesco Ltd, UK) and a snack bar (Honey and Oat Cunch Bar, 42 g; Natures Valley, UK) to consume as a replacement for their usual evening meal. Participants were instructed to consume both the foods together at least 12 h prior to their study visit the following morning and to avoid consuming any other food or drink (other than water) until all measures

had been completed that day. The meal and snack bar provided 836 kcal, of which 34.6% was carbohydrates, 18.9% protein and 43.7% fat.

9.2.10 Data analysis

All data were analysed using IBM SPSS Statistics 22 for Windows (Surrey, UK) and are presented as mean ± SD. Multiple one-way ANOVA's were used to test for group differences between participant's physical characteristics and dietary intake. CMJ, MIVC and PPT were measured using a mixed design ANOVA; 3 group levels (BTJ vs. SN vs. PLA) by 5 time levels (pre-exercise, post-exercise, 24, 48 and 72 h post-exercise). Biochemical markers were analysed with a 3 (group) × 6 (time; pre-exercise, post-exercise, 2.5 h post-breakfast, 24, 48 and 72 h post-exercise) mixed design ANOVA. Significance was read from the Greenhouse-Geisser adjustment if Mauchly's test of Sphericity had been violated. In the event of a significant interaction effect (drink*time) Fisher LSD *post hoc* analysis was performed to locate where the differences occurred. Where relevant, Cohen's *d* ES were calculated with the magnitude of effects considered small (0.2-0.49), medium (0.5-0.79) and large (≥0.8). Statistical significance was set at $P < 0.05$ prior to analyses.

9.3 Results

There were no significant differences between groups for their physical characteristics or macronutrient intake throughout the testing period (Table 15*; P* > 0.05). PPT showed a main effect for both time ($F_{(3, 249)} = 26.89$; $P = 0.001$) and group ($F_{(2, 87)} = 3.25$; $P = 0.043$) whereby PPT was reduced in all groups as a result of exercise but consistently higher in BTJ compared to the SN and PLA in the 72 h post-exercise period (Figure 21). PPT had recovered to baseline values in the BTJ group by 72 h (104.3 ± 25.9%) but remained depressed in both the SN (94.1 ± 16.0%) and PLA groups (91.2 ± 19.0%) (ES = 0.69 vs PLA and 0.53 vs. SN).

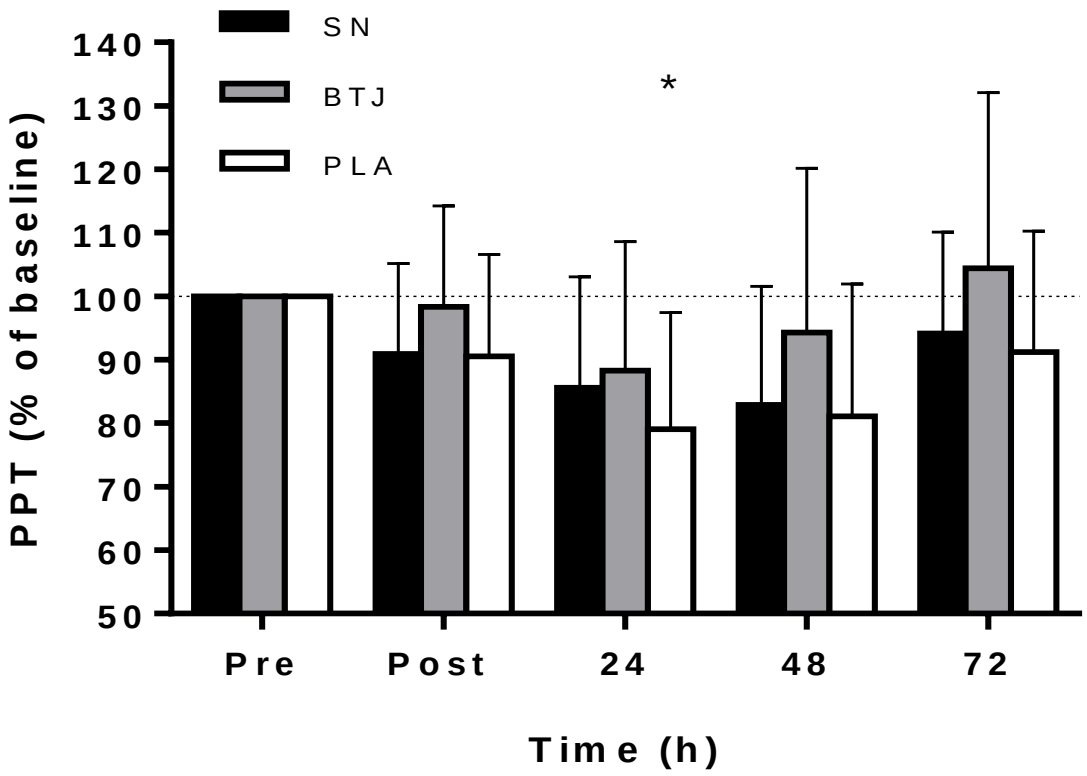

Figure 21 - Pressure pain threshold (PPT) before and 72 h after exercise (% of baseline). Values presented are average of the three sites measured (calf, CF; rectus femoris, RF; vastus laterialis, VL). *Denotes group effect; $P < 0.05$, beetroot juice (BTJ) higher than sodium nitrate (SN) and placebo (PLA). Values are mean ± SD; $n = 10$ per group.

Serum NOx concentrations showed group ($F_{(2, 25)}$ 6.11; $P = 0.007$) and group*time interaction effects ($F_{(7, 83)} = 3.38$; $P = 0.004$), increasing after BTJ and SN but not PLA. As shown in Figure 22, 2 h after consuming BTJ and SN serum NOx was markedly higher ($P < 0.001$; 135.5 ± 78.7 and 189.2 ± 78.8 µmol/L, respectively) than baseline and PLA concentrations. Serum NOx remained elevated above baseline for the rest of the trial in the BTJ and SN groups ($P < 0.05$).

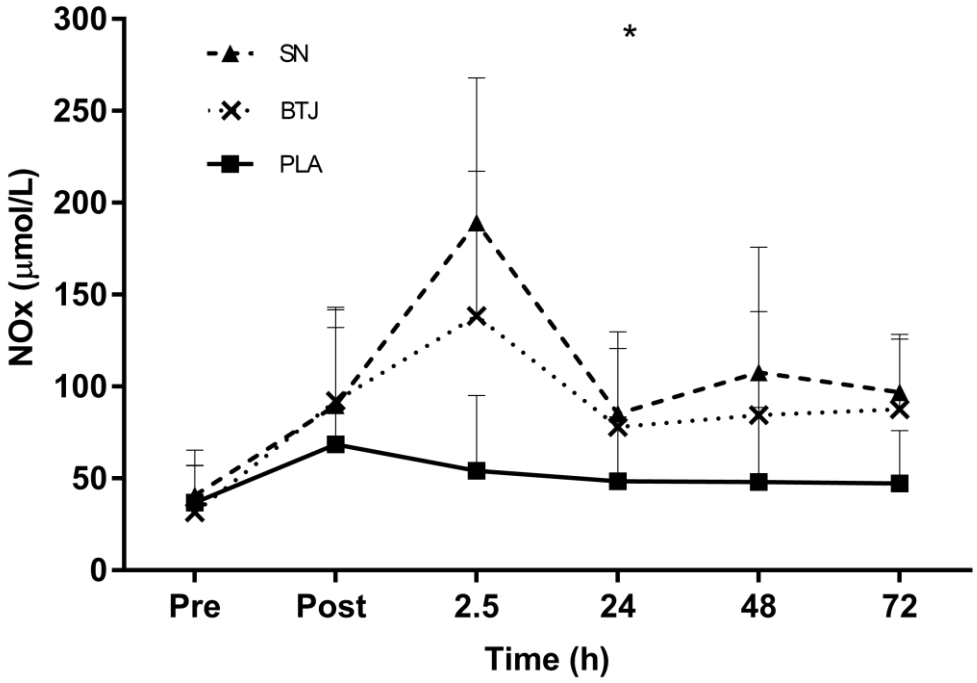

Figure 22 - Serum nitric oxide (NOx) concentrations before and 72 h after exercise. *Interaction effect; beetroot juice (BTJ) and sodium nitrate (SN) higher than placebo (PLA); $P < 0.05$. Data are mean ± SD, $n = 10$ per group.

There was an immediate decline in MIVC and CMJ following the exercise bout (time effect; $P < 0.05$) with neither variable recovering to baseline by 72 h post-exercise (Table 16). There were no differences between the three groups at any time point for MIVC and CMJ ($P > 0.05$). Plasma CK increased in response to the exercise bout (time effect; $F_{(1, 31)} = 6.43$; $P = 0.011$), peaking at 24 h post in all groups (Table 16); however, no group or interaction effects were present ($P > 0.05$). hs-CRP was unaltered following exercise and showed no time, group or interaction effects ($P > 0.05$).

Table 16 - MIVC, CMJ, CK and hs-CRP values pre and post muscle damaging exercise.

Variable	Pre exercise	Post exercise	2.5 h post exercise	24 h post exercise	48 h post exercise	72 h post exercise
MIVC (N)*#						
BTJ	602(100) ± 109(0)	479(80) ± 144(16)		487(81) ± 159(19)	510(87) ± 162(10)	556(95) ± 164(8)
SN	577(100) ± 100(0)	503(87) ± 136(9)		510(88) ± 126(10)	501(85) ± 151(19)	546(92) ± 149(17)
PLA	597(100) ± 117(0)	504(84) ± 111(8)		505(84) ± 153(15)	521(87) ± 136(13)	536(89) ± 131(10)
CMJ (cm)*#						
BTJ	31.8(100) ± 7.0(0)	29.1(90) ± 8.8(11)		29.2(91) ± 8.4(15)	29.4(91) ± 8.9(15)	30.9(97) ± 8.0(10)
SN	33.9(100) ± 6.2(0)	30.1(88) ± 7.0(9)		30.5(90) ± 5.5(7)	29.7(89) ± 5.2(11)	32.8(96) ± 5.3(7)
PLA	36.5(100) ± 4.8(0)	32.0(90) ± 5.2(11)		32.1(87) ± 6.7(14)	31.6(86) ± 6.9(15)	33.7(92) ± 5.6(10)
CK ($IU \cdot ^{-1}$)*						
BTJ	224 ± 104	242 ± 129	358 ± 142	714 ± 638	564 ± 615	351 ± 396
SN	198 ± 108	229 ± 121	264 ± 98	312 ± 122	192 ± 43	148 ± 31
PLA	224 ± 88	266 ± 97	298 ± 122	395 ± 245	274 ± 194	261 ± 126
hs-CRP ($mg \cdot L^{-1}$)						
BTJ	0.41 ± 0.28	0.43 ± 0.33	0.40 ± 0.29	0.35 ± 0.14	0.44 ± 0.12	0.58 ± 0.82
SN	0.42 ± 0.30	0.45 ± 0.30	0.42 ± 0.27	0.50 ± 0.28	0.46 ± 0.26	0.39 ± 0.18
PLA	0.44 ± 0.32	0.48 ± 0.34	0.42 ± 0.31	0.42 ± 0.29	0.34 ± 0.34	0.32 ± 0.13

#Values in brackets represents data normalised to percentage change from baseline. *Time effect*; $P < 0.05$. MIVC, maximal isometric voluntary contraction; CMJ, counter movement jump; CK, creatine kinase; hs-CRP, high sensitivity C-reactive protein.

9.4 Discussion

The aim of this study was to compare the effects of a nitrate matched BTJ and SN drink on muscle force loss and muscle pain after eccentric exercise, in an attempt to gain a better understanding of the potential effects of nitrate on EIMD. While ingestion of BTJ and SN increased serum NOx levels, neither drink protected against muscle force deficits (MIVC and CMJ) in the 72 h after the exercise bout. However, BTJ was more efficacious for attenuating muscle pain than SN and a PLA. A biochemical marker of muscle damage (CK) and a general marker of inflammation (hs-CRP) did not differ between supplement groups at any time point.

As expected, provision of BTJ and SN after muscle-damaging exercise evoked large increases in serum NOx concentrations compared to PLA (Figure 22). These results are in agreement with the findings of Chapter 4, and others who report large increases in NOx bioavailability after SN and BTJ ingestion (Cristensen, Nyberg, & Bangsbo, 2012; Joris & Menesik, 2013). Importantly, the rise in serum NOx was similar between SN and BTJ, indicating that the drinks were well-matched for nitrate content.

Recent data have shown that NOx is integral for normal muscle regeneration, and that administering NOx donors enhances the recovery of strength after eccentric exercise (Lomonsova et al., 2014; Rigamonti et al., 2013). Nevertheless, the present findings are in contrast to the above data, instead suggesting that SN supplementation is ineffective for attenuating force loss or any other parameter of EIMD after an eccentric-heavy exercise bout. The most obvious explanation for the discrepant findings between the present and aforementioned studies is the species difference; the present study was in humans, whereas most previous studies suggesting a role for NOx in EIMD were in animals (Corona & Ingalls, 2013; Lomonsova et al., 2014; Rigamonti et al., 2013; Sakurai et al., 2013). However, it is also possible that other methodological differences, such as exercise model, NOx

donor and dosage contributed to the discrepancies. Notwithstanding, the fact that SN had no influence on any of the indices of muscle damage measured in this study, would seem to indicate that nitrate, at least at this dose, might not exert any favourable effects on exercise recovery. These findings also suggest that nitrate might not be the main constituent in BTJ responsible for attenuating losses in muscle function, as seen in Chapters 5 and 7.

The inability of BTJ to attenuate the post-exercise loss in CMJ height is in direct contrast to a previous investigation in this thesis (Chapter 5), in which 48 and 72 h after the same bout of plyometric exercise, BTJ was found to attenuate the deficit in CMJ. Because in both studies muscle damage was induced with an identical protocol, and the participants were similar in terms of training status, this discrepancy is difficult to account for. With that said, it is important to not overlook the fact that the magnitude of EIMD in response to the same exercise stimulus, even amongst individuals of similar training status, can vary widely (Hubal, Rubenstein, & Scott, 2007; Sayers & Clarkson, 2001). Intuitively, this could also impact how an individual might respond to a recovery intervention. Thus, the inherent heterogeneity in susceptibility to EIMD makes it difficult to compare the findings between any two studies, irrespective of protocol and participant similarity.

With that said, there are subtle differences between these two studies that could account for the different effects of BTJ on CMJ recovery. One potential difference between these studies (and all the studies in this thesis) that could provide at least a partial explanation for these equivocal findings is the different batch of BTJ supplements used. It was not possible to elucidate the AOX and phenolic content of each batch of BTJ used in the studies in this thesis; however, it is reasonable to assume that the phytochemical content of each batch varied slightly due to seasonal variations in crop production. Speculatively, the batch of BTJ used in the present study could have contained less phenolic/betalainic compounds and had a lower AOX capacity than the batch used in Chapter 5. In this scenario, it would be reasonable to

assume that the BTJ with a higher AOX and phytochemical content would be more beneficial for enhancing CMJ recovery than one with a lower content (as suggested by Chapter 5). However, without data to compare the phytonutrient content of the two batches of BTJ used in these studies, this remains a very speculative attempt to explain these discrepant findings. A more plausible explanation could be related to the differences in dietary control between the two investigations. In the previous study (Chapter 5), a strict low-phytonutrient diet was imposed 48 h prior to and throughout the course of the trial (5 days in total) (see Chapter 3.3 for details and Appendix 4 for an example), whereas in the present study, participants were encouraged to not deviate from their usual eating patterns. This change in design was to ensure the findings of the present study were more ecologically valid and applicable to real-world scenarios, in which individuals, particularly athletes, do not restrict their phytonutrient intake. Nonetheless, it has been demonstrated that restricting the intake of AOX and phytonutrient rich foods in the diet can leave individuals more vulnerable to oxidative stress (Watson et al., 2005) and inflammation (Plunkett, Callister, Watson & Garg, 2009) after stressful exercise. This could have important implications for secondary muscle damage after strenuous exercise, and overall muscle function (Paschalis et al., 2016), and makes the expectation tenable that dietary intake of AOX and/or phytonutrient rich foods could influence an individual's susceptibility to EIMD, and their ability to recover from muscle-damaging exercise. Interestingly, the CMJ loss in response to the drop jumps in Chapter 5 was clearly greater than in the present study, which would lend some support to the idea that a low phytonutrient intake might impact an individual's rate of recovery. Indeed, the recovery of CMJ performance was more prolonged in the 72 h following the drop jumps in that study, and there was a clear secondary loss in muscle function without BTJ supplementation in the 24-72 h after exercise (see Figure 9), which was also less pronounced in the present study. These results provide tentative support for the idea that restricting phytonutrients through the diet might leave an individual more

vulnerable to muscle damage, at least after eccentric-heavy exercise, in which secondary damage is presumed to be of a higher magnitude and more prolonged (Howatson & van Someren, 2008). If this is the case, it would be reasonable to assume that BTJ would be more beneficial for functional recovery and performance in individuals with lower AOX intakes, as has been recently suggested with vitamin C supplementation (Paschalis et al., 2016). This might help to explain, at least in part, why BTJ attenuated the loss in CMJ in Chapter 5, when phytonutrient intake was restricted (and participants might have been more susceptible to EIMD) but not in present study, when phytonutrient intake was unrestricted (thus participants were possibly less vulnerable to EIMD). Clearly, without measuring phytonutrient intake and inflammation/oxidative stress (and other aspects that might influence secondary muscle damage) in these studies the above postulate is speculative, and needs to be clarified in future work.

As in Chapters 5, 6, 7 and 8, serum CK efflux increased after exercise, irrespective of supplementation. These data support the conclusions of others who have also found that functional foods do not seem to reduce CK efflux to a greater extent than a PLA (Bell et al., 2014; Bell et al., 2015; Goldfarb et al., 2011; Howatson et al., 2010; Peschek, Pritchett, Bergman, & Pritchett, 2013). It is important to note that CK is not considered a valid enough measure for assessing the extent of EIMD (Paulsen et al., 2012; Warren et al., 1999) and, thus, it might not be a sensitive enough marker to detect changes associated with an intervention. Hence, throughout this thesis, the primary outcome measures were changes in muscle function, which are proposed at the most sensitive and valid markers of EIMD (see Chapter 2.4.1).

Unlike the muscle function measures, muscle pain, as measured by changes in PPT, was alleviated by BTJ supplementation (Figure 21). This pattern for improved recovery of PPT is consistent with those from Chapter 5 and 7, where PPT recovered quicker with BTJ versus a PLA after eccentric-heavy

plyometric exercise and RSE, respectively. An explanation as to how BTJ might reduce muscle pain is still unclear. Part of the difficulty in determining the potential mechanisms involved stems from the fact that the precise causes of exercise-induced muscle pain are still uncertain (Yu, Liu, Carlsson, Thornell, & Stål, 2013). Nonetheless, as described in Chapters 5 and 7, a possibility is that BTJ might suppress the release of stimuli thought to sensitize nociceptive neurons in the muscle and ECM (Murase et al., 2013), the latter of which, to the best of the current knowledge, is proposed as the main site where muscle pain originates (Crameri et al., 2007; Malm et al., 2004). However, because muscle tissue samples could not be obtained as part of this thesis, this posit is somewhat speculative until confirmed by future studies.

Perhaps the most pertinent new finding of this study is that muscle pain was attenuated with BTJ but not SN. These results suggest that phytonutrients other than nitrate, such as betalains and phenolics, or interactions between them (or with nitrate), are likely responsible for its analgesic effects. Although there have been no previous attempts to directly compare the effects of nitrate and BTJ on muscle pain, reports that nitrate free but betalain-rich treatments can alleviate muscle pain appear to support this concept. In two studies (Pietrzkowski et al., 2010 & Pietrzkowski et al., 2014) muscle and joint pain, as measured with the McGill pain questionnaire, was significantly lower after 10 days of taking specially formulated betalain capsules derived from beetroot extracts. The supplements used in these studies did not contain any of the nitrate or phenolic compounds inherently found in BTJ, therefore suggesting that betalains, independent of any interactions with other biological compounds, were responsible for alleviating muscle and joint pain. Based on these findings, the improved PPT seen in the present study with BTJ might have been due to the high amount of betalains it contains (see Chapter 4). However, because the BTJ used in this study contained many other potentially bioactive compounds (see Chapter 4) it cannot be ruled out that these compounds worked synergistically and this had additive

effects. Regardless of the underpinning mechanisms, the present findings, coupled with the previous findings in this thesis, suggests there is scope for further research to examine the potential role of BTJ in muscle pain, perhaps not just in relation to EIMD but also in clinical settings.

In conclusion, this study found that acute supplementation with BTJ and SN did not influence the recovery of muscle function or attenuate CK efflux or hs-CRP after exercise. BTJ was more beneficial than SN for attenuating PPT though, advocating the use of BTJ over nitrate only containing drinks for attenuating exercise-induced muscle pain. These findings also suggest that the phenolics and betalains could be the compounds in BTJ most likely to exhibit analgesic effects after exercise.

9.5 Perspectives

The overall aim of this thesis was to establish whether BTJ could be an effective recovery aid after muscle-damaging exercise. The present investigation addressed the final aim of this thesis, which was to gain a deeper understanding of the phytonutrients in BTJ that might exert beneficial effects for recovery; specifically, the contributory effects of nitrate. The main finding from this study was that BTJ was more effective than SN for attenuating muscle pain after exercise and, therefore, suggests that the analgesic effects afforded by BTJ are likely due to phytonutrients in BTJ other than nitrate, such as the betalains and/or phenolics, or interactions between them.

The reduction in muscle pain with BTJ compared to a PLA is consistent with the findings from Chapters 5 and 7, and provides further evidence that BTJ could be a useful analgesic therapy. The beneficial effects of BTJ on reducing muscle pain might have important implications for athletes in a variety of sports, especially as many athletes frequently report suffering from muscle pain after exercise. These findings also raise the possibility that BTJ

would be of use for clinical populations who suffer from disorders associated with chronic muscle pain (i.e., fibromyalgia and osteoarthritis).

Interestingly, in contrast to the findings of Chapter 5, BTJ supplementation was not associated with an improved recovery of CMJ after eccentric-heavy exercise. The reason for the divergent findings between these two studies is not overtly clear, but it could be related to the fact that the magnitude of muscle damage in the present study was smaller, irrespective of supplement. Speculatively, muscle damage might have been lower in this study because dietary intake of phytonutrients was not restricted as in Chapter 5. Alternatively, the lower muscle damage response could just be an artefact of the inherently large variability in individual recovery rates after strenuous exercise. Notwithstanding, because neither SN nor BTJ were able to influence the recovery of muscle function, the independent effects of nitrate on this parameter remains elusive.

10 General discussion

10.1 Aims and summary

Recent years have seen a growing interest in the potential for plant based food supplements to attenuate EIMD and enhance exercise recovery. The interest in these foods stems from the fact that they contain large amounts of bioactive compounds. In turn, these compounds that might confer protection against cellular and physiological processes associated with muscle damage, most notably, inflammation and oxidative stress. Several of the compounds in the root vegetable, beetroot, demonstrate interesting biological effects that fall into this category. This led to the hypothesis that consuming a BTJ drink after exercise might attenuate some of the deleterious symptoms that can impair functional performance after muscle-damaging exercise, such as muscle soreness and deficits in force generating capacity. Consequently, the purpose of this thesis was, firstly, to establish whether a BTJ drink had the potential to be an effective recovery aid, based on its phytochemical profile; and, secondly, whether BTJ would be beneficial for attenuating muscle damage, principally the decrements in functional performance indices after strenuous exercise.

The series of investigations in this thesis have led to a number of new contributions to the body of knowledge. The opening investigation (Chapter 4) was the first to study the bioavailability of betanin in a commercially available BTJ. It was also the first to demonstrate that this BTJ is a rich source of phytonutrients (specifically, phenolic and betalainic compounds) and possesses a good level AOX capacity and, therefore, seemed to hold potential as a recovery aid following exercise. In further investigations, there was some evidence that consuming BTJ after strenuous exercise accelerated the recovery of dynamic muscle function and reduced muscle pain. These findings were not consistent across all studies; however, a pattern seemed to emerge, in which BTJ was only beneficial (at least in terms of muscle function recovery) when the magnitude of force loss was more pronounced and/or there was clear evidence of a secondary drop in

muscle function. Intriguingly, there was no benefit of BTJ on circulatory markers of inflammation, oxidative stress or muscle damage (CK) in any of the investigations in this thesis. Collectively, the results presented in this thesis add to the current body of literature by suggesting that application of BTJ in the exercise domain may extend beyond just enhancement of acute athletic performance to include exercise recovery.

10.2 Effects of beetroot juice on exercise-induced muscle pain

The most consistent finding in this thesis was that BTJ reduced muscle pain. Other than Chapter 8, which suggested that BTJ did not attenuate muscle soreness after a marathon race, all other Chapters found some evidence that BTJ reduced muscle pain more effectively than a PLA. In line with these findings, supplementation with other antioxidant rich foods, such as cherries (Connolly et al., 2006), curcumin (Drobnic et al., 2014) and ginger (Black, Herring, Hurley, & O'Connor, 2010; Wilson, Fitzgerald, Rhodes, Lundstrom, & Ingraham, 2015) have also been shown to attenuate exercise-induced muscle pain. As alluded to in Chapter 8, an explanation as to why BTJ attenuated muscle pain in Chapters 5, 6 and 9, but not Chapter 8, could be due to a number of factors, including differences in exercise protocol, training status of the participants, and methods used to measure muscle soreness/pain between these studies (PPT vs. VAS). With regards to the latter, a previous study reported inconsistent responses to PPT and VAS measures of muscle soreness following muscle-damaging exercise, suggesting that they might not correlate (Connolly et al., 2006). Nonetheless, another explanation for these equivocal findings could be entirely unrelated to the different methods used, and design of the studies, but instead due to the fact that muscle soreness after the marathon was modest, particularly in comparison to the muscle soreness after the drop jumps and the RST in Chapters 5, 9 and 7, respectively. Thus, the lack of an analgesic effect of BTJ in Chapter 8 could be mostly due to the fact that the magnitude of muscle soreness was not severe enough to warrant an intervention. This

seems the most plausible explanation, as unlike in all the other studies assessing BTJ as a recovery intervention in this thesis, none of the indices of EIMD were modulated by BTJ in this study.

It was beyond the scope of this thesis to examine, at least in great detail, the mechanisms by which BTJ might reduce muscle pain, largely because muscle tissue samples could not be obtained. Although the exact causes of muscle pain are still not fully understood, the most up to date understanding is that it stems from the sensitization of muscle fibre afferents in the muscle and ECM (Mizmuara & Murase, 2015; Mizumura & Tauchi, 2015). It was therefore felt that without obtaining muscle tissue, it would be almost impossible to identify, at least with any degree of certainty, the potential mechanisms involved.

While a mechanistic explanation as to how BTJ reduced muscle pain remains elusive, this thesis did shed some light on the constituents in BTJ that might be exerting pain-relieving effects. Indeed, Chapter 9 showed that BTJ was more effective than a nitrate-matched SN drink for attenuating muscle pain after exercise, which would suggest that the betalainic and/or phenolic compounds in BTJ contributed to the analgesic effects observed in this thesis. These results concur with others, who reported that muscle and knee joint pain was reduced by betalain-rich but nitrate free oral beetroot supplements (Pietrzkowski et al., 2010; Pietrzkowski et al., 2014). Based on these findings, and knowledge of betalains biological effects from previous work (Reddy et al., 2005; Vidal et al., 2014), a potential explanation for how BTJ might attenuate muscle pain can be offered. As alluded to in section 2.6.4, betalains can blunt the *in vitro* activation of COX-2, an enzyme that helps to orchestrate the transient inflammatory response in EIMD via the formation of prostanoids (Warden, 2010; Weinheimer et al., 2007; Vella et al., 2016). In relation to muscle pain, COX-2 has been shown to upregulate neurotrophins that sensitize muscle afferents, particularly glial cell-line derived neurotrophic factor (GDNF) (Mizumura & Taguchi, 2015; Murase et

al., 2013). Thus, one mechanism by which BTJ could have exerted pain-reliving effects is by suppressing COX-2, thus acting in a similar fashion to pharmacological COX-2 inhibitor drugs designed for pain-relief, i.e., NSAIDS. A similar mechanism of action has also been proposed as a partial explanation for ginger's analgesic effects in EIMD (Black et al., 2010; Wilson, 2015). One potential flaw with this theory however, is that reducing muscle pain via this pathway was shown to be more beneficial when NSAID treatment was given prophylactically (Murase et al., 2013), which was not the case in the studies in this thesis. However, this might only be specific to selective COX-2 inhibitor NSAIDS and not other chemical compounds. Another possibility is that the constituents in beetroot reduced local concentrations of pro-inflammatory cytokines, such as TNF-α, that are known activators of other neurotropic factors associated with muscle pain, such as NGF (Schafers, Sorkin, & Sommers, 2003). As described in section 2.6.4, BTJ and betalain compounds have been shown attenuate local inflammatory responses in several animal studies, which lends some support to this suggestion (El Gamal et al., 2014; Krajka-Kuźniak et al., 2012; Lu et al., 2009). It is important to stress that the mechanisms described above are merely speculative attempts to explain how BTJ might, at least in theory, reduce exercise-induced muscle pain. Obtaining and analysing muscle samples in future studies will be integral for establishing how BTJ exerted analgesic effects. Nonetheless, the findings in this thesis suggest that BTJ might help to relieve muscle pain, and could be more beneficial than pharmacological pain treatments such as NSAIDS, which might adversely affect anabolic signalling (Markworth et al., 2014) and/or cause gastro-intestinal issues (Jädert et al., 2012).

10.3 Effects of beetroot juice on muscle function recovery

There was some evidence that BTJ might enhance the rate of recovery for measures of dynamic but not isometric muscle function after muscle-damaging exercise. This was first demonstrated in Chapter 5, in which the

recovery of CMJ performance was significantly accelerated with BTJ versus a PLA at 48 and 72 h following a bout of high intensity plyometric exercise (100 drop jumps). A similar pattern was noted in Chapter 7, as BTJ was shown to be more effective than a PLA for enhancing the recovery of CMJ and RSI performance following high intensity RSE in team-sports players. The findings in these two studies agree with others who have observed a similar pattern of recovery with functional food supplementation after single bouts of muscle damaging exercise (Bell et al., 2015; Bowtell et al., 2011; Howatson et al., 2010; Trombold et al., 2010; Trombold et al., 2011). In contrast to these studies, however, in Chapter 8 BTJ was no more effective than a PLA for attenuating CMJ or other markers of muscle function in the days after a marathon race, and, in Chapter 9, BTJ was not associated with improved functional recovery following the drop jump protocol used in Chapter 5. The reason for the equivocal findings between the studies in this thesis could be attributable to several factors, including the differences in exercise models used to initiate EIMD (excluding Chapter 5 & 9), training status of the participants, and, as discussed in Chapter 9, differences in dietary control throughout the testing periods. Nonetheless, perhaps the most convincing explanation is related to the variation in the magnitude of EIMD in each study. With regards to Chapter 8, in which BTJ did not enhance dynamic muscle function after a marathon, the magnitude of muscle damage was much smaller, and the rate of recovery much quicker than in the studies where BTJ did enhance the recovery of function (Chapter 5 and 7). In addition, a secondary loss in muscle function was observed in these studies; as shown in Figures 9 (Chapter 5), and 15 and 16 (Chapter 7), muscle function actually deteriorated in the 24-72 h period post-exercise without BTJ supplementation, where as in Chapter 8, when BTJ was ineffective, muscle function steadily recovered from 24 h onwards (Figure 19). The variation in muscle damage and rate of recovery could also help to explain, at least in part, why BTJ enhanced the recovery of CMJ in Chapter 5 but not in Chapter 9, despite the almost identical design of the studies. As

explained in section 9.4, the overall loss in dynamic muscle function was far less in Chapter 9 than in Chapter 5, and this could have rendered BTJ less effective for accelerating the recovery of this parameter. In view of these findings, it could be speculated that BTJ was ineffective in the aforementioned studies because either; 1) the muscle function loss was smaller and, thus, group differences were more difficult to detect, and/or; 2) there was no further loss in muscle function and, therefore, the secondary muscle damage response was minimal and not of a large enough magnitude for BTJ to be of benefit. These findings would suggest that the magnitude of force loss in the days after the initiating event could be a key determinant of the effectiveness of BTJ as a recovery aid and subsequent research should consider if the exercise stimulus is sufficiently strenuous to actually warrant an intervention. Nonetheless, the inconsistent findings mean that a definitive conclusion cannot be reached as to the efficacy of BTJ for enhancing muscle function recovery on these studies alone.

While it was not the primary aim to elucidate the potential mechanism(s) by which BTJ might attenuate decrements in force loss after exercise, where possible, markers of inflammation and oxidative stress were measured. As suggested with other functional foods, it was initially hypothesized that BTJ would attenuate EIMD by dampening the inflammatory and/or oxidative stress response associated with muscle-damaging exercise; however, contrary to this hypothesis, biomarkers of inflammation and oxidative stress were not different after BTJ or a PLA in any of the studies in this thesis. It is important to note however, that these markers were all taken systemically and, thus, it is possible that BTJ mitigated oxidative stress and or inflammation at the local level, but was not detectable in blood. Others have also reported that functional foods improved recovery of muscle function after exercise in the absence of any changes in systemic levels of inflammation and oxidative stress (Bowtell et al., 2011; Trombold et al., 2010).

Nonetheless, the lack of systemic changes in these markers with BTJ raises the possibility that mechanisms other than AOX and anti-inflammatory could have been responsible for the improved muscle function recovery in Chapters 5 and 7 with BTJ. In support of this notion, nitrate (via its conversion to NOx) and phenolic compounds, might influence other biological processes that could reduce the secondary muscle damage response and accelerate recovery, such as increasing satellite cell activity (Kruger & Smith, 2012; Rigamonti et al., 2013; Skaurai et al., 2013) and inhibiting proteolysis (Lambert et al., 2014; Louis et al., 2014). NOx, in particular, is involved in several of these processes, and, unlike most phytonutrients, is known to be highly bioavailable (see Chapter 4). An attractive mechanism by which NOx might help attenuate muscle damage is by blunting the activation of calpain isoforms that are known to promote the secondary degradation of cytoskeletal proteins after eccentric-heavy exercise (Koh & Tidball, 2000; Lomonosova et al., 2014). Although evidence for such effects has not been investigated in humans, increasing *in vivo* NOx availability has been shown to simultaneously inhibit calpain activation, reduce EIMD, and preserve muscle function in rats (Lomonosova et al., 2014), which lends some support to this notion. Alternatively, the enhanced dynamic function (CMJ and RSI) in these studies might not be entirely related to a dampening of the biochemical processes thought to provoke secondary cell damage. Instead, they could merely be an artefact of NOx mediated improvements in muscle contractile function, as demonstrated by animal (Hernandez et al., 2012) and some recent human studies (Coggan et al., 2014; Hoon et al., 2015; Flanagan et al., 2016). Although most studies have not found BTJ to improve voluntary isometric strength compared to a depleted nitrate control (Haider & Folland et al., 2014; Hoon et al., 2015), Coggan et al. (2014) reported that muscle strength and power at higher angular velocities were enhanced with an acute pre-exercise dose of BTJ. Thus, another possible mechanism by which BTJ could have enhanced dynamic function after exercise (independent of AOX or anti-inflammatory

effects) is related to NOx-mediated increases in muscle power potential. Such effects would be possible if NOx availability was significantly increased in the muscle when function was assessed; unfortunately, this was not measured in Chapters 5 and 7 to endorse this speculation.

Nonetheless, it is clear from the above discussion that there are a number of potential mechanisms that could explain why BTJ was able to enhance the recovery of muscle function in Chapters 5 and 7. As such, it is also important to consider that these effects are unlikely to be explained by just one biochemical mechanism; rather, several might converge to produce these effects. In other words, BTJ might act to decrease inflammation, oxidative stress (in muscle tissue only) and proteolysis, but also simultaneously increase satellite cell activity and contractile function. However, if these effects are involved, they are likely to be largely dependent on the extent of muscle damage present, as highlighted above. The possible role of these mechanisms and their relative contributory effects requires further exploration.

When discussing the potential mechanisms by which BTJ might have enhanced recovery of both muscle function and PPT in these studies, it is important to not rule out the influence of psychosocial factors that are unrelated to the content of BTJ and its potential for biochemical effects, i.e., the placebo effect. Although the studies in this thesis were placebo-controlled, insofar as an isocaloric drink with negligible nitrate and phenolic content was examined alongside BTJ, the two drinks could not be matched for taste and texture. As outlined in Chapter 3.4, taste matching was not possible for several reasons and therefore additional controls were relied upon to try and limit the psychological influence that knowingly consuming different tasting drinks might have on the results. Specifically, in each experimental study: 1) double blinding was employed, 2) the PLA was made with a fruit squash to give it the appearance of an AOX-rich fruit drink, 3) participants were split into independent groups (so participants were

unaware of the other treatment(s) being tested) and, 4) the studies specific aim was concealed (to ensure participants were unaware if the supplement they had was actually the treatment under investigation). Notwithstanding these controls, it is conceivable that at least some of the participants were aware of BTJs proposed ergogenic effects from performance studies and, irrespective of the other supplement(s) under investigation, this was enough to fill them with the expectation that it would also be of benefit for recovery. This is based on the notion that the placebo effect is dependent on an individual's pre-conceived belief about how beneficial an intervention might be in a given situation or for a specific ailment and, ultimately, this conclusion dictates the expectation of a positive or negative effect (Beedie & Foad, 2009; McClung & Collins, 2007; Pollo, Carlino, & Benedetti, 2008). This makes the expectation tenable that perhaps just believing BTJ can positively influence recovery is enough to alter an individual's perception of muscle pain and/or an individual's sense of muscle fatigue. Such effects were recently suggested with a sham water immersion intervention (Broatch, Peterson, & Bishop 2014).

The potential for a beneficial effect of BTJ, independent of any actual physiological or biochemical effects stemming from its contents, is an intriguing idea and is lent some credence by the fact that none of the biochemical markers associated with improved functional recovery (i.e., inflammation and oxidative stress) was influenced by BTJ in this thesis. Thus, a potential role for the placebo effect to, at least partially, explain the findings in this thesis warrants further attention. Future studies could consider adding a no-placebo arm to intervention trials with BTJ (or indeed any recovery intervention) to try and help elucidate the influence, if any, the placebo effect might be having on the outcome measures (Beedie & Foad, 2009). A brief questionnaire asking participants whether they believed they were on an experimental treatment or placebo could also serve as an added layer of control to rule out the potential of influence of a placebo effect.

10.4 Beetroot juice and acute adaptation

Given that many of the cellular processes BTJ aims to blunt after muscle damaging exercise (i.e., oxidative stress and inflammation), are, paradoxically, critical mediators of the acute and chronic adaptive response to exercise (Gomez et al., 2015), it could be argued that BTJ supplementation might interfere with the normal adaptations to eccentric-heavy exercise and actually have negative effects. Indeed, it has been mooted that attempts to diminish these responses might supress cell signalling responses that promote physiological adaptations following a bout of exercise (Gomez et al., 2015).

It was not within the scope of this thesis to investigate the long term implications of BTJ supplementation on exercise adaptations; however, Chapter 6 did address the issue of short-term BTJ provision on the acute adaptive response to muscle damaging exercise, typically referred to as the RBE. In this study there was no evidence that BTJ negatively affected the RBE, despite attenuating the magnitude of EIMD after the first bout of exercise (Chapter 5). Although there are no similar studies with BTJ, these findings are consistent with those of He et al. (2015) who found that vitamin C and E did not affect the RBE between two bouts of downhill running. Interestingly, this study, and the findings from Chapter 6, are in contrast to the findings from two longer-term studies, whereby vitamin C and E blunted strength gains following 12 weeks of resistance training (Bjørnsen et al., 2015; Paulsen et al., 2014b). In view of the disparate findings in the acute and longer-term studies, it could be speculated that vitamin C and E (or other AOX supplements) might only supress exercise-induced adaptive responses when taken over an extended period. Nonetheless, it is also important to stress that the dose of vitamin C and E used in the above studies is ≥10 × the RDA and, therefore, with such high doses perhaps these deleterious effects should be unsurprising. The effects of lower doses of AOX compounds over shorter periods of time (as in this thesis) might be more

appropriate. Similarly, this recommendation might also be extended to include other recovery strategies that attempt to limit post-exercise oxidative stress and inflammation, such as cold water immersion, which was recently shown to blunt strength adaptations after long-term exposure (12 weeks) (Roberts et al., 2015).

It is also noteworthy that in later Chapters' oxidative stress and inflammation were unaffected by BTJ, so perhaps it is unsurprising that BTJ did not appear to interfere with the acute adaptive response. As discussed above, the mechanisms by which BTJ might attenuate EIMD could differ to vitamin C and E AOXs and, thus, the influence of each supplement on exercise-induced adaptations might differ. Nonetheless, until the implications of more chronic BTJ doses have been examined, it would seem prudent to not recommend BTJ as a long-term recovery strategy. Rather, in scenarios where BTJ has shown some promise as a recovery aid, such as after high intensity plyometric exercise and RSE, BTJ could serve as a useful short term strategy to provide relief from EIMD, particularly muscle pain. For example, BTJ could be of benefit after training or events/matches when the need to minimise the deleterious effects of EIMD and optimise recovery is of far greater importance than physiological adaptation. From an applied perspective, there are several examples when such situations might arise in sport, including during tournament scenarios, congested fixture schedules and in preparation for important events/matches, such as cup finals and Olympic qualifying.

10.5 Limitations and future research directions

While the series of investigations in this thesis have produced a number of novel and interesting findings, they have also raised several questions for future research. In addition, it is important to acknowledge that there are several limitations in the interpretation of the findings presented in this thesis.

As such, the following section will discuss both potential future research directions and the limitations of the findings in this thesis.

A pertinent question raised by the first study in this thesis (Chapter 4), is what is the fate of betanin after ingesting BTJ and, therefore, its potential for biological effects associated with EIMD *in vivo*? Interestingly, in previous studies, betanin ingestion has decreased inflammation and oxidative stress, which, in contrast to the findings in Chapter 4, would suggest, albeit indirectly, that betanin is bioavailable in humans (Pietrzkowski et al., 2010; Pietrzkowski et al., 2014; Tesoriere et al., 2004b). One potential explanation for these seemingly contrasting findings is related to the fact that the plasma bioavailability of betanin is not necessarily reflective of total bioavailability, as betanin might have been present in urine or faeces (Frank et al., 2005). It is also possible that betanin was bioavailable in this study but just not detectable in the circulation at the specific time points measured due to being rapidly distributed to cells. Thus, an important limitation of this work is that betanin bioavailability was possibly underestimated by failing to measure additional time points and other excretory pathways. Nonetheless, the findings from the aforementioned studies and Chapter 4 could also be interpreted to suggest that it is not betanin *per se* that exerts biological activity *in vivo*, but perhaps secondary metabolites of betanin that are produced from biochemical interactions in the large intestine. The argument has been made by others (Frank et al., 2005; Netzel et al., 2005) and was acknowledged in Chapter 4, but the chemical standards for unambiguously identifying betanin metabolites were not commercially available and, thus, they could not be measured in this study. As such, in the series of investigations that followed, it was unclear as to what extent betanin and its metabolites contributed to some of the beneficial effects on recovery. Additionally, other betalains proposed to exert biological effects such as indicaxanthin (Allegra et al., 2005; Tesoriere et al., 2008), or individual phenolic compounds were not measured in Chapter 4 and, thus, the precise role they might have in the muscle damage process can only be speculated.

Given the above discussion, the results of this study are clearly limited in that the bioavailability of other potentially bioactive compounds in BTJ were not considered. Consequently, there is a need for future research to characterise the bioavailability of betalain compounds and their metabolites before the biological significance of these compounds *in vivo* can be firmly established and subsequently their role, if any, in EIMD. This has become particularly relevant in light of the findings of Chapter 9 that suggest the betalain and phenolic compounds are the main compounds in BTJ with analgesic potential after muscle-damaging exercise.

Another important question for future studies is what are the mechanisms by which BTJ might attenuate muscle-damage and enhance recovery? An acknowledged limitation of this work is the inability to obtain muscle tissue samples to investigate the underpinning mechanisms in detail. Intuitively, in order to truly understand the effect of any intervention on EIMD, at least from a mechanistic perspective, the affected muscle tissue needs to be analysed. Nonetheless, the fact BTJ did not attenuate systemic markers of inflammation or oxidative stress after exercise in any of the studies in this thesis, provides rationale for future studies to instead focus on changes in these biomarkers at the local level, or look to investigate some of the other potential mechanisms suggested in section 10.3 above, including the potential influence of a placebo effect.

Future research examining the effects of BTJ as a recovery aid should also aim to focus on; 1) its application in real world sporting settings i.e., after races or actual training sessions, and; 2) in higher calibre athletes. With regards to the former, apart from Chapter 8 (marathon race), muscle damage was assessed after exercise bouts performed in well controlled laboratory conditions and, in Chapters 5 and 9, bouts that did not reflect the physiological demands or movement patterns of a real world sporting activity. It could therefore be argued that the findings from these studies lack ecological validity and as such their generalizability to real-world settings is

limited. Although the RST in Chapter 7 did attempt to reproduce some of the mechanical (i.e., acceleration and deceleration) and metabolic (i.e., high intensity sprints) stress imposed by team-sport activity, the protocol could not account for the other factors that likely contribute to EIMD in competitive settings, such as clashes with opponents, kicking/hitting, multi directional changes and jumping actions. As such, this test cannot be considered a valid representation of an actual team sports match and, therefore, the beneficial recovery effects of BTJ might not be directly transferable to real-world scenarios. Given these limitations, an important direction for future research will be to investigate whether BTJ is a useful recovery intervention in real world sporting situations, specifically after competitive matches or high intensity resistance or plyometric training sessions.

In line with the above suggestion, it is critically important for future studies to examine the effects of BTJ in better trained athletic populations, especially if BTJ is to gain any recognition as a recovery intervention in elite sporting settings. Indeed, participants in Chapters 5 and 9 were only recreationally active and unfamiliar with the exercise task. Given that better trained populations are more likely to be accustomed to bouts of high volume eccentric exercise, the muscle damage and pattern of recovery observed in these studies is probably not reflective of that observed in higher calibre athletes, especially those at the elite level (Barnett, 2006). Thus, a key limitation of these findings is that they cannot be readily applied to more elite athlete populations. Another limitation of this work is the lack of investigations in female participants. Indeed, apart from Chapter 8, in which a mixed gender cohort was recruited, all studies were performed with males. Given the suggestion that males and females might recover at different rates after EIMD (Minahan, Joyce, Bulmer, Cronin, & Sabapathy, 2015; Sayers & Clarkson, 2001), and even respond differently to functional food supplements (Rankin, Stevenson, Cockburn, 2015), future research should also look to compare the effects of BTJ on recovery in male and female athletes.

Another important direction for future research is to establish the most effective supplementation strategy of BTJ for minimising EIMD and enhancing recovery. It was beyond the scope of this thesis to address this question in detail, but Chapter 5 showed that several servings of a 250 ml dose of BTJ was more effective than the equivalent number of servings with a 125 ml dose for enhancing the recovery of CMJ. Intuitively, the 250 ml serving was more beneficial because it supplied a higher dose of phytonutrients than the 125 ml serving; however, this needs to be clarified with further research, especially as muscle pain was attenuated to a similar extent with the 125 and 250 ml dose of BTJ. This latter finding suggests that perhaps the dose of BTJ needed to attenuate decrements in muscle function might be greater than the dose needed to attenuate muscle pain. In view of this, future studies might benefit from providing higher doses of BTJ when attempting to accelerate the recovery of muscle function after strenuous exercise.

Other important aspects relating to dosage strategy that require further investigation are the frequency and timing of BTJ ingestion after exercise. Because this thesis was the first to investigate BTJ supplementation in terms of its effects of muscle damage and recovery, there was no previous literature on which to base a dosage strategy on. As such, the dose, frequency and timing of supplementation in each study was based on knowledge regarding; 1) the time course of secondary muscle damage and likely pattern of recovery (i.e., post-exercise); 2) other food supplements demonstrating favourable effects on EIMD, and; 3) what would be most easily applied to a real-world scenario. Notwithstanding this rationale, it is unclear whether this approach was the most effective with BTJ and, therefore, future studies should look to compare this strategy to more acute or chronic strategies, including the pre-load strategy used by others (Bell et al., 2014; Bell et al., 2015; Howatson et al., 2010; Trombold et al., 2010; Trombold et al., 2011). Moreover, in developing an appropriate dosing strategy for BTJ, the practical feasibility from an athlete's perspective needs

to be factored in. Therefore, elements such as taste preferences, cost, availability and safety/risks associated with BTJ supplementation all need to be carefully considered.

10.6 Practical recommendations

Based on the findings from the studies in this thesis, some practical recommendations for BTJs use as an acute recovery intervention following muscle-damaging exercise can be offered:

1 – In general, BTJ might be of more benefit after novel or unaccustomed bouts of eccentric-heavy exercise and/or in situations when fruit and vegetable intake might be compromised. With regards to the first point, the potential recovery benefits of BTJ might become more apperant after exercise that typically results in moderate to severe EIMD (defined as muscle pain and/or pronounced losses in muscle function lasting for >48 h; Paulsen et al., 2012), and which might cause further deterioration in muscle function before recovering. Thus, the beginning of a new exercise regime/training technique, returning to exercise after injury, or pre-season training, are all situations in which BTJ might help to provide short term relief from EIMD symptoms. Improved functional capacity could be beneficial for mitigating injury risk in the days after muscle-damaging exercise and for improving adherence at the outset of a new exercise regimen. With regards to the second point, BTJ might be a more effective recovery aid while traveling and competing in foreign countries, where access to phytochemical rich foods (i.e., fruits and vegetables) might be more limited.

2 – BTJ might help to preserve dynamic muscle power after exercise that incorporates high intensity repeated sprints, enabling athletes to perform closer to their optimum in the ensuing days. Thus, from an applied perspective, team-sport players (at least at the amateur level) might benefit from consuming BTJ during congested fixture periods, tournaments, or in

other scenarios where recovery time might be insufficient such as intensified training periods.

3 – In terms of dosage strategy, for muscle function recovery, a 250 ml serving of BTJ seems more efficacious; however, for relief from muscle pain, a 125 ml serving might be equally as effective. Two to three servings should be taken at intervals throughout the day (AM and PM) for 3 days after the exercise bout or until symptoms subside.

In conclusion, the series of investigations in this thesis have provided the first evidence that a commercially available BTJ is rich in phytonutrients and has potential as a recovery intervention, at least after exercise that results in marked and prolonged symptoms of EIMD. With that said, it is critically important that future work corroborates these findings in real world sporting scenarios, and in better trained athletes. If some of the findings in this thesis can be replicated in this population, BTJ could serve as an easy to implement and efficacious intervention for attenuating the some of the undesirable effects of EIMD and, thus, might help to facilitate functional recovery.

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12 Appendices

12.1 Appendix 1 – Example informed consent document.

Faculty of Health & Life Sciences

INFORMED CONSENT FORM

Project title:

	please tick or initial where applicable
I have carefully read and understood the Participant Information Sheet.	☐
I have had an opportunity to ask questions and discuss this study and I have received satisfactory answers.	☐
I understand I am free to withdraw from the study at any time, without having to give a reason for withdrawing, and without prejudice.	☐
I agree to take part in this study.	☐
I would like to receive feedback on the overall results of the study at the email address given below. Email address……	☐

Signature of participant..... Date.....…….. (NAME IN BLOCK LETTERS).....…….
Signature of Parent / Guardian in the case of a minor .....
Signature of researcher..... Date.....…….. (NAME IN BLOCK LETTERS).....…….

INFORMED CONSENT FOR REMOVAL AND STORAGE OF TISSUE

Principal Investigator: Tom Clifford

I agree that the following tissue or other bodily material may be taken and used for the study:

Tissue/Bodily material	Purpose	Removal Method
Blood	For analysis of oxidative stress and inflammation.	Via venepuncture

I understand that if the material is required for use in any other way than that explained to me, then my consent to this will be specifically sought. I understand that I will not receive specific feedback from any assessment conducted on my samples, but should any kind of abnormality be discovered then the investigator will contact me.

I understand that the University may store this tissue in a Licensed Tissue Bank only for the duration of the study, it will then be destroyed.

I consent to the University distributing this tissue to partners in this research study, outside of the University, for further testing (please tick the box if you agree). ☐

Signature of participant..... Date.....……..

Signature of Parent / Guardian in the case of a minor

.....
Date.....……..

Signature of researcher..... Date.....……..

12.2 Appendix 2 – Example of institutional ethical approval

Mic Wilkinson <mic.wilkinson@northumbria.ac.uk>

26/01/2016

Hi Tom,

Thanks for your responses. As they were very minor, the reviewer was happy for me to sign off once they had been made. The project listed below now has ethical approval. Please keep the message for your records.

Regards,

Mick

HLSTC120116

The influence of recovery drinks on muscle damage following a marathon race

Mick Wilkinson, PhD

Senior Lecturer

Sport, Exercise and Rehabilitation

Northumbria University

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England

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12.3 Appendix 3 – General health history questionnaire

HEALTH HISOTRY QUESTIONNAIRE

D.O.B:……..

As you are participating in exercise within this laboratory, please can you complete the following questionnaire. Your co-operation is greatly appreciated.
All information within this questionnaire is considered confidential.
Where appropriate please circle your selected answer.

1. How would you describe your current level of activity?
   Sedentary / Moderately Active / Highly Active
2. How would you describe your current level of fitness?
   Very Unfit / Moderately Fit / Trained / Highly Trained
3. How would you describe your current body weight?
   Underweight / Ideal / Slightly Overweight / Very Overweight
4. Smoking Habit: -

Currently a non-smoker	Yes / No	
Previous smoker	Yes / No	
If previous smoker, how long since you stopped? ……Years		
Regular smoker	Yes / No	of …… per day
Occasional smoker	Yes / No	of …… per day

5. Alcohol Consumption: -

Do you drink alcohol?	Yes / No	
If yes then do you -	have an occasional drink	Yes / No
	have a drink every day?	Yes / No
	have more than one drink per day?	Yes / No

6. Have you consulted your doctor within the last 6 months?
   Yes / No
   If yes, please give details …….
7. Are you currently taking any medication (including anti-inflammatory drugs)?
   Yes / No
   If yes, please give details …….
8. Do you, or have you ever suffered from:-

Diabetes	Yes / No
Asthma	Yes / No
Epilepsy	Yes / No
Bronchitis	Yes / No
Elevated cholesterol	Yes / No
High Blood Pressure	Yes / No

9. Do you suffer from, or have you ever suffered from any heart complaint or pains in your chest, either associated with exercise or otherwise?

Yes / No

10. Is there a history of heart disease in your family?
    Yes / No
11. Do you feel faint or have spells of severe dizziness when undertaking exercise or otherwise?
    Yes / No
12. Do you currently have any form of muscle joint injury?
    Yes / No
    If yes, please give details ……
13. Have you ever suffered from any knee joint injury or thigh injury?

    Yes / No
    If yes, please give details ……
14. Do you currently take any form of nutritional supplement (e.g. creatine, whey and casein protein, HMB, etc)?
    Yes / No
    If yes, please give details ……
15. Do you have any food allergies?
    Yes/No
    If yes, please give details ……
16. Are you currently on any special diet or have dieted in the past? (e.g. weight loss/ high protein)
    Yes/No
    If yes, please give details
……
    ……
….

17. Is there anything to your knowledge that may prevent you from successfully completing the test that has been explained to you?
    Yes / No
    If yes, please give details ……..

Please provide any further information concerning any condition/complaint that you suffer from and any medication that you may be taking by prescription or otherwise.
……
……

Signature of participant: …… Date:
……..
Signature of test supervisor: ……

12.4 Appendix 4 – Low phenolic diet food diary example (Chapters 4, 5 and 6).

CONFIDENTIAL

Participant ID:_____

FOOD RECORD DIARY

Please record everything you eat and drink for **2 days prior to your trial and throughout the trial (72 hours).** You will then be asked to consume similar food and drink prior to and during the second trial. **Please avoid consuming the foods listed at the back of this document throughout the 2 days prior to and during the trial periods. This is vitally important to the results of the study so please adhere to this strictly.** Instructions and examples are given inside.

Information about your diet will be treated in confidence.

If you have a problem, please contact: Tom 07508200021
tom.clifford@northumbria.ac.uk

Department of Sports, Exercise and Rehabilitation

School of Life Sciences

Northumbria University

Newcastle Upon Tyne

NE1 8ST

INSTRUCTIONS FOR USING THE FOOD DIARY

Everything that you eat and drink over the course of the day should be recorded.

Do not forget to record second helpings and between meal snacks.

Eating Out – Most people eat foods away from home each day, please do not forget to record these. Take your diary with you where ever it is possible. If this is too inconvenient just record the type of food eaten.

Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

e.g. Cheese – is insufficient information.
Cheese, cheddar (Shape reduced fat) – is sufficient information.

Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line.
e.g. 10:30 am McVities Digestive Biscuits (2) 50g

The space provided at the foot of each page for general comments is for you to give any further information about your diet and your training/activity for that day.

e.g. Steady run, morning 1 hour.
Missed lunch due to stomach pains

DAY 1 (48 hours pre-trial 1)
Date: / /

Please use a separate line for each item eaten; write in weight of plate; leave a line between different 'plate' entries.

A	B		C	D	E	F	
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away		-cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	(gms)	(gms)	(gms)

GENERAL COMMENTS and ACTIVITY UNDERTAKEN:

Dietary Restrictions

Please see below for foods that you can/cannot eat during the period 2 days prior to and during each trial. It is vitally important that you strictly follow these restrictions in order to make sure any changes we see in your samples (bloods) are directly as a result of the beverage you are provided with. The foods on the 'should NOT' eat list are those are high in polyphenols (AOXs) and nitrate/nitrite, if you come across foods not listed there but you know are high in AOXs, please avoid these also.

Foods that should NOT be eaten during the diet

- Tea, coffee, drinking chocolate, alcohol (specially red wine and apple cider), fruit juice
- Fruits and Vegetables
- Chocolate and chocolate products
- Cereals / wholemeal bread / grains
- Spices (such as curry) and herbs
- Cured meats (Bacon, sausages, salami, ham, chorizo and hot dogs).

Foods that you may eat

- White bread
- Butter, vegetable oil (avoid olive oil)
- Pasta, rice
- Meat, eggs, fish
- Peeled potatoes (mash, crisps, french fries)
- Mushrooms
- Digestive biscuits
- Milk and milk products (plain yogurts, cheese)

EXAMPLES OF DIET FOR THE STUDY

Here below is an example of what you can eat during the 2 days preceding your visit and the 4 days of testing:

Breakfast:

- Toast (white bread) with butter (**NO** jam)
- Glass of milk or plain yogurt (or Greek yogurt but without Honey)
- Scrambled eggs
- Croissant, pastries (**NO** jam or chocolate coated)
- Waffles, pancakes
- Rice Krispies with milk and sugar

Lunch:

- Sandwich (white bread, white bagels, white pitta bread, or panini) with any of the following fillings (**NO** lettuce, or tomatoes):
    - Chicken
    - Cheese
    - Tuna
    - Prawns
    - Butter
    - Mayonnaise
- Pack of crisps (Ready salted)
- Burger and chips

Dinner:

- Fish and chips with salt and vinegar (**NO** tomato sauce)
- Chicken with mushrooms and mash potatoes or chips (**NO** vegetable or peas)
- Chicken and fried rice / fried noodles with eggs, and oyster sauce (**NO** peas and **NO** soya sauce)
- Macaroni and cheese (cream cheese sauce)
- Tuna, salmon, cod, with rice or pasta (**NO** tomato-based or vegetable-based sauce)
- Steak, pork chops
- Spaghetti carbonara (cream cheese sauce, bacon)

Snacks:

- Digestive biscuits (**NO** chocolate coated)
- Short breads
- Custard rice pudding
- Crisps (Ready salted)
- Ice cream (vanilla flavour)
- Vanilla flavour milk-shake
- Plain scones with butter, clotted cream

You can drink water at all times.

WHAT YOU MUST NOT EAT OR DRINK

- Coffee

- Tea

- Alcohol (especially red/white wine, apple cider, beer)

- Fruits (especially apples, pears, apricots, prunes, plums, cherries, peaches, blueberries, grapes)

- Vegetables (especially tomatoes, spinach, asparagus, cabbage, carrots, peppers, onions, broccoli, cauliflower)

- Fruits juices (especially Ribena), soft drinks (coca cola, irn bru…)

- Chocolate / chocolate products (biscuits, cakes, chocolate bars) / drinking chocolate

- Wholemeal bread, granary bread, brown bread

- Wholegrain cereals (such as Weetabix, bran flakes, Corn flakes, oat cakes, porridge, Muesli)

- Wholemeal food (such as wholemeal pasta, wholemeal rice…)

- Beans (such as baked beans, green beans), lentils, peas

- Curry dishes, curry spice, chilli, paprika

- Herbs (such as basil, parsley, coriander, chives…)

- Tomato sauce (such as Ketchup, ready-made pasta sauce in jar…)

- Cured meats (Bacon, sausages, salami, ham, chorizo and hot dogs).

We are aware that this may be difficult for you to follow such a diet, as it may be really different from your usual diet. The reason we ask you to change your normal diet is that the foods listed above contain similar components that we want to be testing in the AOX drinks. Therefore, if you do eat the foods above, this may interfere with the study.

Thank you for your co-operation.

12.5 Appendix 5 – Food diary (unrestricted; Chapters 7,8 and 9).

CONFIDENTIAL

Participant ID:_____

FOOD RECORD DIARY

Please record everything you eat and drink **1 day prior to the trial, the day of the trial, and for the remainder of the testing period. Please do not deviate from your usual eating patterns.** Instructions and examples are given inside.

Information about your diet will be treated in confidence.

If you have a problem, please contact: Tom 07508200021
tom.clifford@northumbria.ac.uk

Department of Sports Science

School of Life Sciences

Northumbria University

Newcastle Upon Tyne

NE1 8ST

INSTRUCTIONS FOR USING THE FOOD DIARY

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Do not forget to record second helpings and between meal snacks.

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Names and descriptions of foods should be as detailed as possible, including the brand name and any other information available.

e.g. Cheese – is insufficient information.
Cheese, cheddar (Shape reduced fat) – is sufficient information.

Start a new page in your diary for each day, and record each item on a separate line. Record the time of day in the first column of each line.
e.g. 10:30 am McVities Digestive Biscuits (2) 50g

The space provided at the foot of each page for general comments is for you to give any further information about your diet and your training/activity for that day.

e.g. Steady run, morning 1 hour.
Missed lunch due to stomach pains

DAY 1 (48 hours pre-trial 1)
Date: / /

Please use a separate line for each item eaten; write in weight of plate; leave a line between different 'plate' entries.

A	B		C	D	E	F	
Time	Food eaten		Brand name of each item (except fresh food)	Full description of each item including: -whether fresh, frozen, dried, canned -cooked: boiled, grilled, fried, roasted. -what type of fat food fried in	Weight Served	Weight of Leftovers	Actual Weight
am/pm	home	away			(gms)	(gms)	(gms)

GENERAL COMMENTS and ACTIVITY UNDERTAKEN: