Group and Inter-Individual Responses to the Effect of New Zealand Blackcurrant (*Ribes nigrum*) Supplementation on Recovery from Exercise Induced Muscle Damage

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Abstract

Exercise induced muscle damage (EIMD) causes reductions in neuromuscular function and muscle soreness with inter- and intra-individual variability. New Zealand blackcurrant (NZBC) is rich in polyphenols, namely anthocyanins, which improve blood flow and display anti-inflammatory properties that may improve recovery from EIMD. Therefore, the aim of this thesis is to examine whether NZBC extract supplementation improves recovery from EIMD. Study 1 showed that seven-days of NZBC extract supplementation had no effect on recovery from EIMD (Counter Movement Jump (CMJ), soreness, urinary interleukin-6 (IL-6)) in the 48 h following a half-marathon running race. Study 2 investigated the inter-individual variability in EIMD following the half-marathon and showed that by quantifying the Smallest Worthwhile Change (SWC), individuals within the NZBC group appeared to recover some CMJ variables quicker than placebo and the α-actinin-3 (*ACTN3)* and angiotensin-I converting enzyme (*ACE)* genotypes may partially explain recovery of muscle function. Study 3 showed that seven-days NZBC extract supplementation had no effect on EIMD following a 100-drop jump protocol (100-DJP) on markers of muscle function (CMJ and voluntary and electrically stimulated isometric contractions), muscle soreness and serum IL-6 and prostaglandin-E_2. Study 4 showed that following the 100-DJP, on the individual level (using the SWC), NZBC extract supplementation had no effect on recovery of muscle function but the *ACTN3* and *TTN* genotypes may influence recovery of muscle function. Study 5 quantified the time course and appearance of the phenolic acids vanillic acid (VA), gallic acid (GA) and protocatechuic acid (PCA) following a single dose of NZBC supplementation and it was observed that VA, GA and PCA were most abundant at 3, 4 and 1.5 h post-ingestion, respectively. Study 6 showed that seven-days of NZBC extract supplementation prior to a 100-DJP and three-days after (10 days total) increased plasma concentrations of PCA and GA but not VA, and there were no relationships with changes the EIMD. In summary, the research presented in this thesis shows that NZBC extract supplementation has no effect on recovery following EIMD on the group level but may be effective at an individual level. Acute and chronic supplementation of NZBC

extract increased plasma concentrations of key phenolic acids, which shows inter-individual variation but is not related to recovery from EIMD. Future research should investigate the appearance of a wider range of phenolic acids alongside exercise performance measures to help inform personalised use of polyphenol supplements to enhance individual's performance.

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List of Abbreviations

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

100-DJP	100-drop jump protocol
1-RM	One repetition maximum
AA	Arachidonic acid
ACE	Angiotensin-I converting enzyme
ACTN2	α-actinin-2
ACTN3	α-actinin-3
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
AOX	Antioxidant vitamins
ARE	Antioxidant response element
AST	Aspartate aminotransferase
AUC	Area under the curve
BC	Blackcurrant extract
BCN	Blackcurrant nectar
BP	Base pair
BPM	Beats per minute
BS	Blueberry smoothie
BTJ	Beetroot juice
Ca^{2+}	Calcium
CI	Confidence interval
CJ	Cherry juice
CK	Creatine kinase
*C*max	Concentration maximum

CMJ	Countermovement jump
COL5A1	Collagen type 1 alpha 1
COX	Cyclooxygenase
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CuZnSOD	Copper zinc superoxide dismutase
CV	Coefficient of variation
d	Cohen's *d*
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DOMS	Delayed onset muscle soreness
EC	Excitation-contraction coupling
ECM	Extracellular matrix
EGCG	Epigallocatechin galate
EIMD	Exercise induced muscle damage
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ES	Electrical stimulation
FFQ	Food frequency questionnaire
FMD	Flow mediated dilation
FORD	Free oxygen radical defence
FORT	Free oxygen radical test
FRAP	Ferric reducing ability of plasma
FVPD	Fruit and vegetable puree drink

G	Grams
GA	Gallic acid
GPx	Glutathione peroxidase
GSH	Glutathione
GSSG	Oxidised glutathione
H	Hours
HCL	Hydrochloric acid
HELENA	Healthy Lifestyle in Europe by Nutrition in Adolescence
HFF	High frequency fatigue
HO-1	Heme oxygenase-1
HPA	Hectopascal pressure unit
HPLC	High performance liquid chromatography
hsCRP	High sensitivity C-reactive protein
HUVECs	Human umbilical vein endothelial cells
Hz	Hertz
JH	Jump height
ICC	Intraclass correlation coefficient
IFN- γ	Interferon-gamma
IL-1 α	Interleukin-1-alpha
IL-1β	Interleukin-1-beta
IL-1ra	Interleukin-1 receptor antagonist
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
IL-8	Interleukin-8

IL-10	Interleukin-10
ISAK	International Society for the Advancement of Kinanthropometry
KEAP-1	Kelch-like ECH-associated protein 1
Kg	Kilograms
kJ	Kilojoules
LDL	Low density lipoprotein
LFF	Low frequency fatigue
LoA	Limits of agreement
LOD	Limits of detection
LOOH	Lipid hydroperoxides
LOQ	Limit of quantification
Ltd	Limited company
M	Meters
m^2	Meter squared
MAC	Mid-arm circumference
MBI	Magnitude based inference
MCJ	Montmorency tart cherry juice
MCP-1	Monocyte chemotactic protein-1
MDA	Malondialdehyde
MeOH	Methanol
Mg	Milligrams
Min	Minutes
mL	Millilitres
MPH	Miles per hour
mRNA	Messenger ribonucleic acid

MUAC	Mid-upper arm circumference
MuSCs	Muscle-specific stem cell
MVC	Maximal voluntary contraction
MVIC	Maximal voluntary isometric contraction
N	Newton
NAC	*N*-acetyl-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
Neu:Lym	Neutrophil to lymphocyte ratio
NGF	Nerve growth factor
NF-kB	Nuclear factor kappa light chain enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NIRS	Near-infrared spectroscopy
nM	Nanomolar
NO	Nitric oxide
Nrf-2	Nuclear factor erythroid 2-related factor 2
Nurr1	Nuclear receptor related-1 protein
ηp^2	Partial-eta square
NSAIDs	Non-steroidal anti-inflammatory drug
NQO-1	NAD(P)H:quinone oxidoreductase-1
NZBC	New Zealand blackcurrant
ORAC	Oxygen radical absorbance capacity
OS	Oxidative stress
PGE_2	Prostaglandin-E_2
PC	Protein carbonyls
PCA	Protocatechuic acid

PCR	Polymerase chain reaction
Pg	Picograms
PG	Propyl gallate
PGE	Prostaglandins
PLA	Placebo
POM	Pomegranate juice
PPT	Pressure pain threshold
PT-L2P	PrepIT
pRFD	Peak rate of force development
Q	Quercetin
qPCR	Real-time polymerase chain reaction
RAS	Renin-angiotensin system
RBE	Repeated bout effect
RNOS	Reactive nitrogen oxygen species
ROM	Range of motion
ROS	Reactive oxygen species
RSI	Reactive strength index
RSImod	Reactive strength index modified
S	Seconds
SD	Standard deviation
SEM	Standard error of the mean
SOD	Superoxide dismutase
SSC	Stretch shortening cycle
SNP	Single nucleotide polymorphism
SPSS	Statistical package for the Social Sciences

SWC	Smallest worthwhile change
TAC	Total antioxidant capacity
TBARS	Thiobarbituric acid
TE	Typical error
TFA	Trifluoroacetic acid
*T*max	Time to maximum
TMCJ	Tart Montmorency cherry juice
TNF-α	Tumour-necrosis-factor-alpha
TTN	Titin
TTT	Time to take off
UA	Uric acid
UK	United Kingdom
µL	Microliters
USA	United States of America
USDA	United States Department of Agriculture
UV	Ultraviolet light
VA	Vanillic acid
VAS	Visual analogue scale
$\dot{V}O_{2max}$	Maximal volume of oxygen uptake
$\dot{V}O_{2peak}$	Peak rate of oxygen uptake
W	Watt
X^2	Chi-square test

Declaration of Authorship

I, Rianne Costello,

declare that the thesis entitled

Group and Inter-Individual Responses to the effect of New Zealand blackcurrant (*Ribes nigrum*) Supplementation on Recovery from Exercise Induced Muscle Damage

and the work presented in the thesis are both my own and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- Parts of this work have been published as:

  Costello, R., Willems, M.E.T., Myers, S.D., Myers, F., Lewis, N.A., Lee, B.J., & Blacker, S.D. (2020). No effect of New Zealand blackcurrant extract on recovery of muscle damage following running a half-marathon. *International Journal of Sports Nutrition & Exercise Metabolism, 30(4),* 287-294. doi:10.1123/ijsnem.2019-3012

Signed: 

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Dedication

I dedicate my PhD and this thesis to my dad.

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Peer Reviewed Publications Arising from Studies Conducted Within and Alongside This Course of Investigation

Costello, R., Willems, M.E.T., Myers, S.D., Myers, F., Lewis, N.A., Lee, B.J., & Blacker, S.D. (2020). No effect of New Zealand blackcurrant extract on recovery of muscle damage following running a half-marathon. *International Journal of Sports Nutrition & Exercise Metabolism, 30(4),* 287-294. doi:10.1123/ijsnem.2019-3012.

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Conference Communications and Published Abstracts During Doctoral Studies

Costello, R., Myers, S.D., Willems, M.E.T., Myers, F., Lewis, N.A., & Blacker, S.D. (2019). Effect of New Zealand blackcurrant extract on recovery from exercise induced muscle damage following half marathon running: 355 Board #193 May 29. *Medicine & Science in Sports & Exercise, 51S,* 90. (*Thematic poster presentation at ACSM Annual Meeting 2019, 29th May 2019, Orlando, FL, USA*).

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induced by drop jumps. *International Journal of Sport Nutrition & Exercise Metabolism, 30(Suppl1),* S1-S14. doi:10.1123/ijsnem.2020-0065. (*Oral presentation at the International Sport & Exercise Nutrition Conference, December 2019, Newcastle, UK*).

1. General Introduction

1.1 Background

Strenuous exercise can result in exercise induced muscle damage (EIMD), which is characterised by ultrastructural muscular disruption, reductions in neuromuscular function, increased muscle soreness and an increase in circulatory intramuscular proteins in the days following exercise.

Neuromuscular function is most accurately quantified by the force producing capability of a muscle or muscle group (Warren et al. 1999) and can be quantified by using measures such as isometric and isokinetic dynamometry (Warren et al. 1999; Morton et al. 2005), electrical stimulation (Edwards et al. 1977; Blacker et al. 2013), interpolated stimulation during voluntary contraction (Paillard et al. 2005; Blacker et al. 2013) and through counter-movement jumps (Gathercole et al. 2015; Doma et al. 2017; Clifford et al. 2015). These measures have previously been applied to quantify the changes in neuromuscular function during activities such as running and skiing (Millet and Lepers, 2004), down-hill running (Webb and Willems, 2010) and drop-jumps (Jakeman et al. 2017; Clifford et al. 2015). Muscle soreness is defined as a sensation of discomfort and localised pain which is most evident in the 24–72 h following strenuous exercise and is therefore also commonly referred to as delayed onset muscle soreness (DOMS) (MacIntyre et al. 1995; Warren et al. 1999).

Exercise induced muscle damage (EIMD) is particularly evident following exercise that involves eccentric contractions, where a muscle is placed under tension to produce force (Paulsen et al. 2012). Eccentric contractions occur in a wide variety of actions such as running, jumping, sprinting, change of direction and decelerating, thus often resulting in some magnitude of muscle damage (Hydahl and Hubal, 2014; Byrne, Twist and Eston, 2004). These types of actions and the resultant reductions in neuromuscular function and increases in soreness are often experienced by sports performers, athletes and personnel engaged in physically demanding occupations (Montgomery et al. 2008; Wilkinson et al. 2011; Fallowfield et al. 2014). From a practical perspective, there is the need to identify interventions to enhance

the recovery from EIMD to reduce the risk of musculoskeletal injury and enhance physical performance (Blacker, 2017; Howatson and van Someren, 2008; Owens et al. 2018).

The precise origins of EIMD have still not been fully elucidated, despite the extensive body of research that has been carried out in this area for more than thirty years (Hydahl and Hubal, 2014; Peake et al. 2017). Exercise-induced muscle damage can be identified by histological markers such as ultrastructural disruptions and indirect markers such as decreases in muscle function and a transient increase in myofibrillar proteins in the blood (Hubal et al. 2007). Despite the lack of clarity about the origin of EIMD, it is generally accepted that there is a biphasic response following damaging exercise (Howatson and van Someren, 2008; Hubal, Chen, Thompson and Clarkson, 2008). The initial phase of EIMD or primary damage occurs during the exercise bout and are a direct cause of the eccentric contractions that occurred during the exercise (Howatson and van Someren, 2008). During this phase, physical damage to the muscle structures and/or impairments in excitation-contraction (EC) coupling are most likely the cause for initiating the muscle damage cascade. The subsequent process, secondary damage, is believed to then trigger a series of biochemical changes in the physiological system that may cause further harm to the muscle and surrounding connective tissues (Howatson and van Someren, 2008; Hubal et al. 2008). Key markers of secondary damage are inflammation and oxidative stress, which characteristically appear in the hours and days following the strenuous exercise event (Nikolaidis et al. 2008; Paulsen et al. 2012). Despite this biological response being fundamental to the repair and protect process to support damaged tissues, it may also have further negative consequences for muscle function recovery at least in the short term (McArdle et al. 1999). However, it has been suggested that an organism undergoes an adaptive response to repeated exposure of reactive oxygen species (ROS) via exercise bouts and sees a shift toward a more reducing environment and an induction of increased stress resistance (Mattson, 2008; Pingitore et al. 2015; Lewis et al. 2016). It has been suggested that it is only when the physiological system is pushed past it's hypothetical tolerable threshold and the equilibrium of hormesis is lost that the production of

ROS, inflammation and myofibrillar proteins leads to a greater magnitude of damage in the affected tissue and possibly maladaptation to training (Peake et al. 2015; Owens et al. 2018).

Quantifying the magnitude of EIMD experienced by an individual has been problematic for researchers and practitioners seeking to provide advice to individuals regarding the optimal recovery strategy following a strenuous exercise bout due to the large inter-individual variability of some of the aforementioned surrogate markers and the heterogeneity between them (i.e. neuromuscular function, systemic inflammation, ROS) (Damas et al. 2016; Hubal et al. 2007; Margaritelis et al. 2014). Therefore, it can be difficult to interpret the results of some EIMD markers as the responses from the different measurements do not always converge (Damas et al. 2016). Paulsen et al. (2012) and Damas et al. (2016) have published comprehensive reviews on the individual variation in the responses to EIMD. Possible factors for the variance in EIMD responses include: previous maximal or submaximal exercises (Chen et al. 2012), training status (Newton et al. 2008), use of muscles in daily activities (Chen et al. 2011), flexibility (McHugh et al. 1999), effort exerted during the exercise (Sayers et al. 2003; Chen et al. 2012), female contraceptive use (Hicks et al. 2017) and genotype (Baumert et al. 2016). Although still in its infancy, single nucleotide polymorphism (SNP) analyses have highlighted how some genetic allele variants are associated with an individual's susceptibility to markers of EIMD (Baumert et al. 2016; Goodlin et al. 2014; Del Coso et al. 2018).

Over the past two decades, there has been a plethora of research examining the role of 'functional foods' to support recovery from EIMD. So-called 'functional foods' are those that are claimed to exert positive physiological effects that are related to improved or preserved human health and disease prevention (Bell, McHugh, Stevenson and Howatson, 2014). Functional foods have grown in popularity over recent years due to their potential properties to mitigate the negative effects associated with strenuous exercise. In addition, these foods are often deemed 'natural' and have minimal interference from manufacturing processes and so are an attractive alternative for those are cautious of anti-doping violations (Maughan et al. 2018; Myburgh, 2014). Polyphenols are deemed functional food components and are found

in many fruits and vegetables that are consumed as part of a balanced diet and have purported health benefits (Seeram et al. 2008; Sousa et al. 2014; Kimble et al. 2018). Examples of foods containing polyphenols that have been the subject of investigation to enhance recovery following EIMD include: quercetin (O'Fallon et al. 2012), pomegranate (Trombold et al. 2011), tart Montmorency cherry (Connolly et al. 2006; Howatson et al. 2010), beetroot (Clifford et al. 2015; Clifford et al. 2016), bilberry (Lynn et al. 2018) and blueberry (McLeay et al. 2012). The evidence as to whether polyphenols can help improve recovery from EIMD following strenuous exercise is equivocal, with some showing favourable benefits (Trombold et al. 2011; Connolly et al. 2006; Howatson et al. 2010; Clifford et al. 2015) and some even claiming to have hindered recovery (Lynn et al. 2018). It is likely that the equivocal findings are related to the inherent inter-individual variability that exists with polyphenol metabolism and subsequent polyphenol-derived metabolite appearance when measured systemically (Kay et al. 2017; Koli et al. 2010). It has been suggested that divergent polyphenol metabolite appearance is partly driven by differences in individual's gut microbiome, which has been shown to be a target site for polyphenol metabolism and breakdown (Williamson and Clifford, 2010; Vendrame et al. 2011). The potential mechanisms for how polyphenols exert their effects following EIMD is not abundantly clear, but it is thought that polyphenols are unlikely to interact with the primary phase of the mechanical stress during the exercise bout (Bell et al. 2014; Howatson and van Someren, 2008). Instead, it is likely polyphenols interact with the secondary cascade, which presents in inflammation and in ROS being produced after the damaging exercise (Owen et al. 2018). In addition, some polyphenols possess the ability to attenuate the inflammatory arachidonic acid pathways by inhibiting cyclo-oxygenase (COX) 1 and 2 production (Seeram et al. 2001) to a similar magnitude to over-the-counter Non-Steroidal Anti-Inflammatory Drugs (NSAIDS) (Bondesen, Mills, Kegley and Pavlath, 2004).

Blackcurrant (*Ribes nigrum*) is a rich source of the bioactive compound, polyphenols and namely of the flavonoid variety, anthocyanins. Anthocyanins are water-soluble and act as natural pigments causing purple, blue, red and orange colouration to flowers, leaves, fruits

and vegetables (Mazza Cacace, and Kay, 2004). The specific make-up of anthocyanin found within blackcurrant is primarily delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside (Kähkönen, Heinämäki, Ollilainen and Heinonen, 2003). Interestingly, the parent anthocyanins have poor bioavailability and instead the degradation products, such as phenolic acids, appear in greater quantity when measured *in vivo* (Scalbert and Williamson, 2000). Given their increased presence *in vivo*, these anthocyanin-derived breakdown products have been suggested to be responsible for the observed health and physiological benefits (Keane et al. 2016). The phenolic acids, protocatechuic acid (PCA) and gallic acid (GA) are the most abundant degradation products of cyanidin and delphinidin, respectively, the two major parent anthocyanins detected in New Zealand blackcurrant (NZBC) whole berry (Slimestad and Solheim, 2002) and concentrate (Matsumoto et al. 2001). Whereas, vanillic acid (VA) is a reported breakdown phenolic acid of peonidin 3-*O*-rutinoside, which has been identified as a minor compound in blackcurrant (Matsumoto et al. 2001). The differences in the specific anthocyanin make up is what has been suggested to be responsible for the divergent purported health and physiological effects (Bowtell and Kelly, 2019; Cook and Willems, 2018). Blackcurrant extract supplements are commercially available and have recently been the subject of much research after showing promise for producing favourable physiological responses and performance benefits during rowing (Lyall et al. 2009), cycling (Willems et al. 2014; Willems et al. 2015; Cook et al. 2015; Murphy et al. 2017; Strauss et al. 2018), high intensity intermittent running (Perkins et al. 2015; Willems et al. 2016; Godwin et al. 2017), sustained isometric contractions (Cook et al. 2017) and at rest (Willems, Parktin, Widjaja and Ajjimaporn, 2018; Cook et al. 2017). However, the anthocyanin content of polyphenol foods is highly dependent upon many factors such as, cultivar, cultivation site, processing, storage and ripeness (Chalker-Scott, 1999; Del Rio et al. 2010; Mikulic-Petkovsek et al. 2017). Blackcurrant cultivars grown in European countries have been shown to contain less anthocyanin content (mg·100 mL) than those grown in New Zealand (Moyer et al. 2002; Schrage et al. 2010). This has led to the focus of exercise performance studies using NZBC as a dietary supplement (Braakhuis, Somerville and Hurst, 2020).

To date, limited research has explored the effect of NZBC extract supplementation on recovery following strenuous exercise that is likely to cause EIMD. Lyall et al (2009) demonstrated that consuming NZBC extract powder (240 mg·d^{-1} anthocyanins), immediately prior to and immediately post a 30-minute indoor row at 80% maximal volume of oxygen uptake ($\dot{V}O_{2max}$), reduced plasma creatine kinase (CK) activity 24 h post and attenuated the transient rise in plasma oxidative generating capability and protein carbonyls (PC) in recreationally active individuals. Lewis et al. (2017) observed that seven-days of NZBC capsule supplementation (315 mg·d^{-1} anthocyanins) during controlled performance tests similar to those of the pentathlete's regular training sessions, did not attenuate plasma CK or improve countermovement jump (CMJ) performance compared to baseline. However, similar to Lyall et al. (2009), Lewis et al. (2017) observed that NZBC extract supplementation reduced systemic inflammation and oxidative stress with no effect on physical performance. In a double-blind, placebo-controlled parallel design, Rowland (2018) provided active individuals with NZBC extract capsules (105 mg·d^{-1} anthocyanins) for 12 days, on the eighth day the participants performed a muscle damaging protocol in the form of repeated maximal concentric and eccentric elbow flexor contractions. Rowland (2018) observed that the NZBC group presented with significantly less muscle soreness immediately post- and a reduction in CK concentration at 96 h post the eccentric exercise bout, compared to the placebo group. However, NZBC extract supplementation was unable to attenuate maximal voluntary isometric contraction (MVIC) force losses, elbow range of motion (ROM) or localised inflammation as measured through mid-upper arm circumference (MUAC). Lyall et al. (2009), Lewis et al. (2017) and Rowland (2018) all used different exercise modalities to assess recovery with only the latter using a protocol reported to induce EIMD. It is difficult to draw firm conclusions regarding the effectiveness of NZBC as a recovery intervention when there are clear methodological differences between these three studies such as study design, participant characteristics (mixture of men and women with no menstrual cycle controls), physical training status, inter-individual variation in the response to polyphenol supplementation and the

markers used to assess the magnitude of recovery from EIMD. However, these observations suggest that NZBC extract supplementation is able to facilitate recovery from EIMD by attenuating increases in CK, oxidative stress and muscle soreness, so warrants further investigation to explore its efficacy further at both the group and individual level. In addition, none of the aforementioned studies assessed the time course or appearance of key anthocyanin-derived phenolic acids following NZBC extract supplementation. It has been suggested that future work in this field should ideally quantify exercise performance outcomes alongside measurement of plasma phenolic metabolites to enable identification of the bioactives metabolites and inform optimisation of the polyphenol blends consumed (Bowtell and Kelly, 2019). Thus, it is pertinent that this gap in the literature is addressed when assessing the efficacy of NZBC extract supplementation as a recovery aid following EIMD.

1.2 Thesis aims

Therefore, the overarching aim of this thesis is to investigate the effects of NZBC extract supplementation on recovery following EIMD. This will be addressed through six experimental chapters with the specific aims to examine the:

1. Effect of NZBC extract supplementation taken 7-days before and 2-days following running a half-marathon race on markers of EIMD (Chapter 3).
2. Inter-individual variability in response to EIMD induced by a half-marathon event with NZBC extract or placebo supplementation (Chapter 4).
3. Effect of NZBC extract supplementation taken 7-days before and 3-days following a 100 drop-jump protocol (100-DJP) on markers of EIMD (Chapter 5).
4. Intra- and inter-individual variability in response to EIMD induced by a 100-DJP with NZBC extract or placebo supplementation (Chapter 6).
5. Time course of the plasma concentration of the phenolic acids; vanillic acid (VA), gallic acid (GA) and protocatechuic acid (PCA), following acute ingestion of a single

dose of NZBC extract in individuals following a non-polyphenol restricted diet (Chapter 7).

6. Time course of plasma concentrations of the phenolic acids; VA, PCA and GA following 10-days of NZBC extract supplementation alongside a non-polyphenol restricted diet and the relationship between these phenolic acids and muscle function measures of MVIC and CMJ pre and following a 100-DJP (Chapter 8).

2. Review of the Literature: A Review of Dietary Polyphenols on Exercise Induced Muscle Damage

2.1 Polyphenols in food and as supplements

Polyphenols are a group of phytochemicals present in fruits and vegetables and their products, including berries, wine, green tea and chocolate (Somerville, Bringans and Braakhuis, 2017; Wisnuwardani et al. 2018). More than 8,000 distinct structures of polyphenols have been identified and their activity is based on functional groups capable of accepting a free radical's negative charge (Martin and Appel, 2010). Polyphenols are a very diverse and multi-functional group of active plant compounds with a pleiotropic array of potential health benefits in, but not limited to, areas such as cancer, hypertension, asthma, diabetes, and cardiovascular health (Martin and Appel, 2010; Somerville et al. 2010). Polyphenols exist primarily in a conjugated form with one or more sugar residues (glycosides) linked to one or more of the many hydroxyl groups. Polyphenols with direct links to the sugar ring, which is most commonly glucose, can also occur and polyphenols without sugar linkages are referred to as aglycones (Martin and Appel, 2010). When ingested, a large proportion of polyphenols are not absorbed within the small intestine and continue to the colon where they are acted upon by enzymes present within the microbiota to release aglycones that then undergo ring fission to produce bioavailable metabolites such as phenolic acids and hydroxycinnamates (Bowtell and Kelly, 2019).

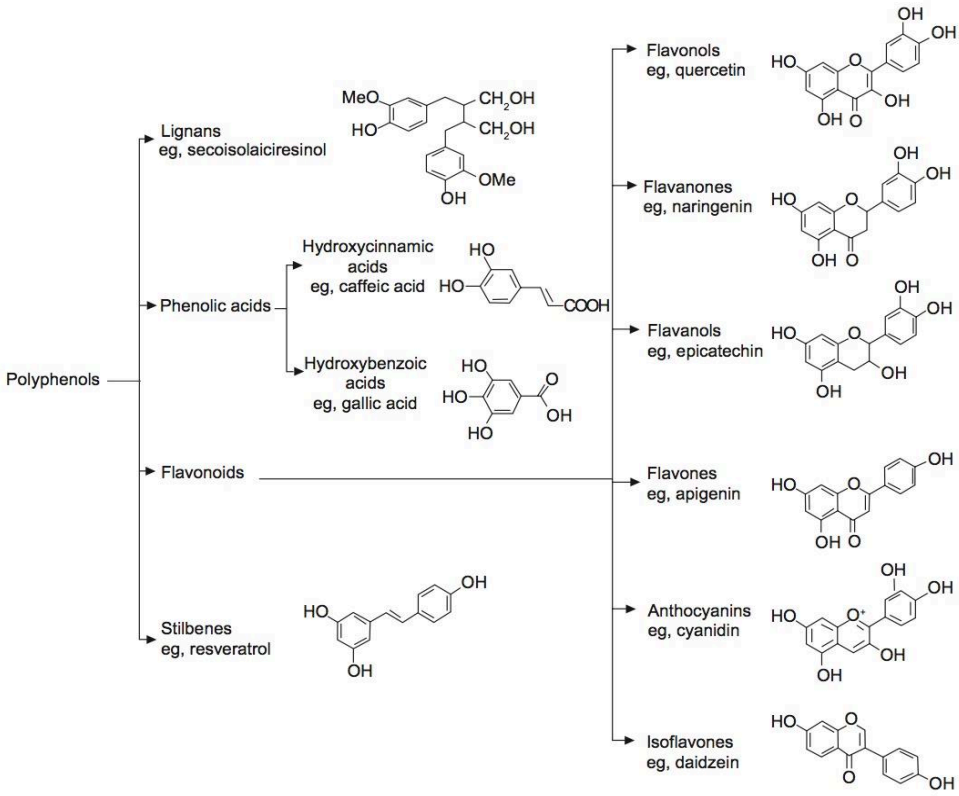


Figure 2.1 Dietary polyphenols classification and chemical structure. Adapted from Spencer et al. (2008).

Polyphenols can be organised into at least 10 different classifications based on the number of phenol rings that they contain and the basis of the structural elements that bind these rings to one another (Figure 2.1) (Pandey and Rizvi, 2009). The most common sub-groups of polyphenols are phenolic acids, flavonoids, stilbenes and lignans (Pandey and Rizvi, 2009). Due to flavonoids being the most abundant of the naturally occurring dietary polyphenols, they are one of the most commonly studied groups and to date, more than 5,000 varieties have been identified (Martin and Appel. 2010). Flavonoids can be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones. Quercetin, myricetin, catechins and anthocyanins are some of the most common flavonoids (Scalbert & Williamson, 2000). The chemical structure of flavonoids affects their bioavailability and absorption (McGhie and Walton, 2007; Scalbert and Williamson, 2000). Despite current evidence not showing any classical deficiencies due to low polyphenol intakes, recently they have been proposed as

'lifespan essentials' due to their associations with reducing the risk of chronic disease development (Wisnuwardani et al. 2018; Williamson and Holst, 2008).

Quantification of dietary polyphenol content and intake is problematic due to a variety of reasons including; incomplete food composition databases, polyphenol content between foods being highly variable, different cultivation methods, cooking methods and the myriad of chemical structures and the lack of commercially available standards to analyse them (Martin and Appel, 2010; Martin, Krueger, Rodriguez, Dreher and Reed, 2009; Amarowicz, Carle and Dongowski, 2009; Stracke, Rufer, Weibel, Bub and Watzl, 2009; Sánchez-Morenco, Plaza, de Ancos and Cano, 2003). Despite these difficulties, estimated quantification of the most commonly consumed polyphenols in plant foods, has been possible. The United States, the Department of Agriculture (USDA) has one of the most complete sources of information on the content of individual polyphenols in foods that includes composition data for 38 flavonoid aglycones in over 300 foods (Pérez-Jiménez, Neveu, Vos and Scalbert. 2010). The USDA database has been used to estimate dietary flavonoid intake among Americans using the 1992-2002 National Health and Nutrition Examination Survey (NHANES) (Chun, Chung and Song. 2007). Intake was estimated at 187±11 mg, with the major dietary sources being tea, citrus fruit juices, wine, and citrus fruits (Chun, Chung and Song. 2007). In Europe, the multicentre cross-sectional Healthy Lifestyle in Europe by Nutrition in Adolescence (HELENA) study conducted between 2006-2007 used the more recently developed Phenol-Explorer database (http://www.phenol-explorer.eu) (Neveu et al. 2010; Pérez-Jiménez, Neveu, Vos and Scalbert. 2010). The HELENA study showed that median, lower and upper quartiles of dietary polyphenol intake among adolescents were 326, 167 and 564 $mg \cdot d^{-1}$, respectively (Wisnuwardani et al. 2018). The main polyphenol contributors to the dietary intake in Europe were fruit (23%), chocolate products (19%) and fruit and vegetable juices (16%), with flavonoids being the main constituent of the daily dietary polyphenol intake (75-76%) (Wisnuwardani et al. 2018).

Various surveys, such as the NHANES, have highlighted that dietary supplements are used by a substantial amount of the population (68%) in the United States (Chun, Chung and Song. 2007). This is not surprising, when it has been reported that many of those living in the Western world do not consume sufficient micronutrients from their diet, thus making supplementation an attractive 'insurance' given the purported health benefits of eating sufficient fruit and vegetables (Hu, 2007). Polyphenol-rich dietary supplements in the form of isolated polyphenols or combinations are on the increase due to greater public knowledge and interest with them (Martin and Appel, 2010). The polyphenol-rich dietary supplements provide greater exposure level to polyphenols than would normally be found in a typical Western diet, with reported intakes ranging from 17.8 to 64.9 $mg \cdot day^{-1}$ (Zamora-Ros et al. 2011; Jennings et al. 2014) and ~300 $mg \cdot day^{-1}$ with polyphenol supplements (Martin and Appel, 2010). Mennen et al. (2005) reported that dosage recommendations of some of these polyphenol-rich supplements identified on the internet were ~100 times higher than the intake that is common of a Western diet. The risk of toxicity with these doses is low given that polyphenols and their downstream metabolites have relatively short half-lives. Studies of the absorption and elimination of polyphenols have demonstrated that maximum plasma levels generally occur at ~2 hours post-ingestion and the elimination half-life is ~5-7 hours for green and black tea catechins, respectively (Van het Hof, Kivits, Westtrate and Tijburg. 1998) and ≤48 h for most metabolites derived from both phenolic rings of the parent anthocyanin structure post labelled tracer ingestion (Czank et al. 2013). One exception was seen with ferulic acid, which remained elevated still at 48 h above baseline (Czank et al. 2013). The Phenol-Explorer database shows that the food group with the greatest content of polyphenols are spices and herbs, with cloves (15,188 mg mg·100 g), followed by peppermint (11,960 mg·100 g) and star anise (5460 mg·100 g), demonstrating the highest concentrations [all determined by chromatography and of proanthocyanin oligomers as determined by direct-phase High Performance Liquid Chromatography (HPLC)]. Furthermore, the berries richest in polyphenols, namely anthocyanin, are all darkly coloured: black chokeberry (878 mg·100 g), black elderberry (1316 mg·100 g) and blackcurrant (595 mg·100 g) (Pérez-Jiménez, Neveu,

Vos and Scalbert. 2010). What must be considered when interpreting and using these data is that content values for every food and drink are based on the currently available literature and as more data are published, the Phenol Explorer database will be revised and updated.

2.2 Effect of polyphenols on exercise

Polyphenol use in exercise testing – a brief history

The number of peer reviewed publications investigating the effects of polyphenol supplementation on exercise performance has steadily grown over the past 30 years. Figure 2.2 shows the number of PubMed hits returned when searching 'polyphenol and exercise' in the title and/or abstract has risen from one in 1987 to 65 in 2019. The 1989 study by Lupandin was the first study conducted to investigate possible applications of polyphenols, where it was observed that dietary polyphenols were able to inhibit the degradation of catechol-O-methyltransferase, a key enzyme which degrades mood regulation hormones such as dopamine or norepinephrine *in vitro* (Lupandin, 1989). However, the first study to specifically focus on investigating the effects of dietary polyphenol supplementation and exercise performance was conducted in 1996 by Bar-Or and Wilk utilising grape-flavoured water supplementation to assess its efficacy as a hydration strategy in children exercising in the heat. Prior to this study, the effect of dietary polyphenol supplementation on exercise performance was preceded by a focus on the effect of antioxidant vitamins (AOX) for performance tests, redox status and recovery (vitamin C, vitamin E or the combination of vitamins C and E). A number of studies have combined AOX and polyphenols such as that by MacRae and Mefferd (2006), who investigated the use a supplement containing vitamin C and E, green tea extract and quercetin compared to one identical, minus any quercetin, on 30 km time trial performance. MacRae and Mefferd (2006) observed that the AOX supplementation improved time trial performance by 3.1% compared to baseline the time trial. However, recent reviews have suggested that the current evidence in favour of recommending AOX supplements to enhance exercise performance, balance redox status or aid recovery are

lacking (Nikolaidis, Kerksick, Lamprecht and McAnulty, 2012; Owens et al. 2018). The disparity in findings to date is due to the possibility that AOX, when taken in supra-physiological quantities, may interfere with specific exercise adaptations such as suppressing mitochondrial biogenesis (Paulsen et al. 2014; Gomez-Cabrera, Salvador-Pascual, Cabo and Viña. 2015; Draeger et al. 2014). Therefore, it would be negligent to recommend chronic AOX supplementation to athletes, especially in the supra-physiological dosing ranges (e.g. 1000 mg·day^{-1} vitamin C). The present review of the literature will be restricted to those studies that have specifically focused on providing a dietary polyphenol supplement for the actions that the polyphenol has on exercise performance.

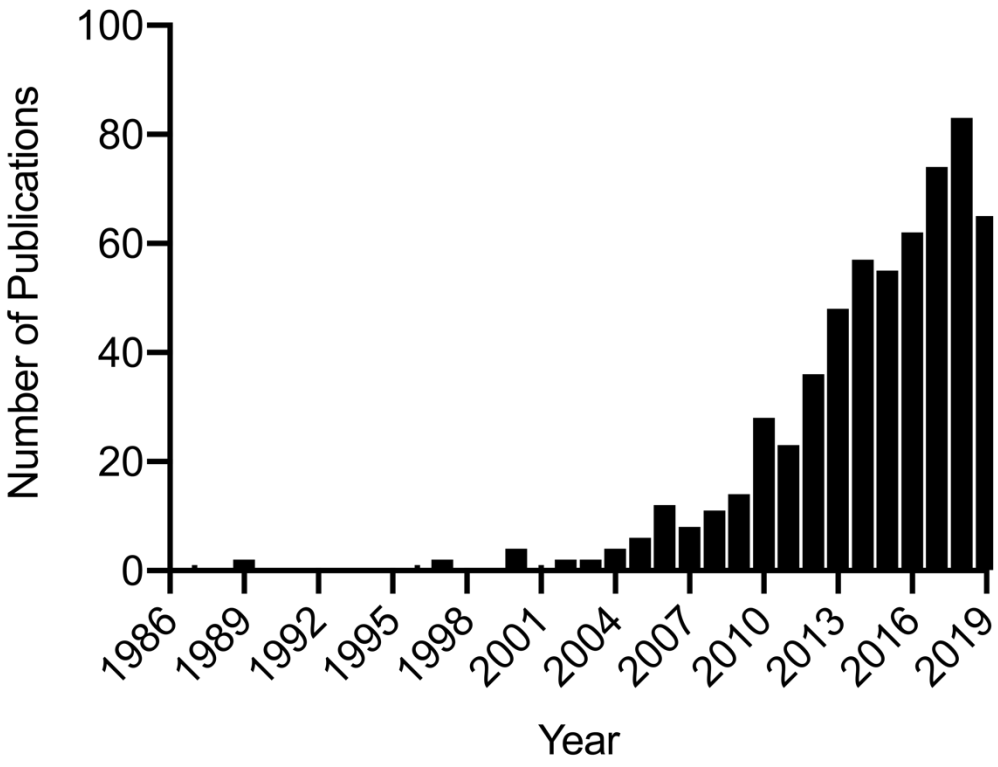


Figure 2.2. Publication metrics for number of publications per year since 1987 when search terms 'polyphenol and exercise' are used in PubMed. Search performed 13/06/2020.

The exercise models used to examine the effects of dietary polyphenol supplementation can be broadly categorised in to three areas:

1. Performance tests (focus on exercise performance, fat oxidation, muscular strength, sleep)
2. Redox status (focus on biomarkers of reactive oxygen and nitrogen species)
3. Recovery (focus on exercise-induced muscle damage)

Figure 2.3 illustrates the number of studies and polyphenol supplements that have been investigated in each of the exercise models. Furthermore, Figure 2.3 highlights that the majority of previous polyphenols studies have focused on the use of performance test and redox status models and a minority have used exercise recovery models with a focus on exercise-induced muscle damage.

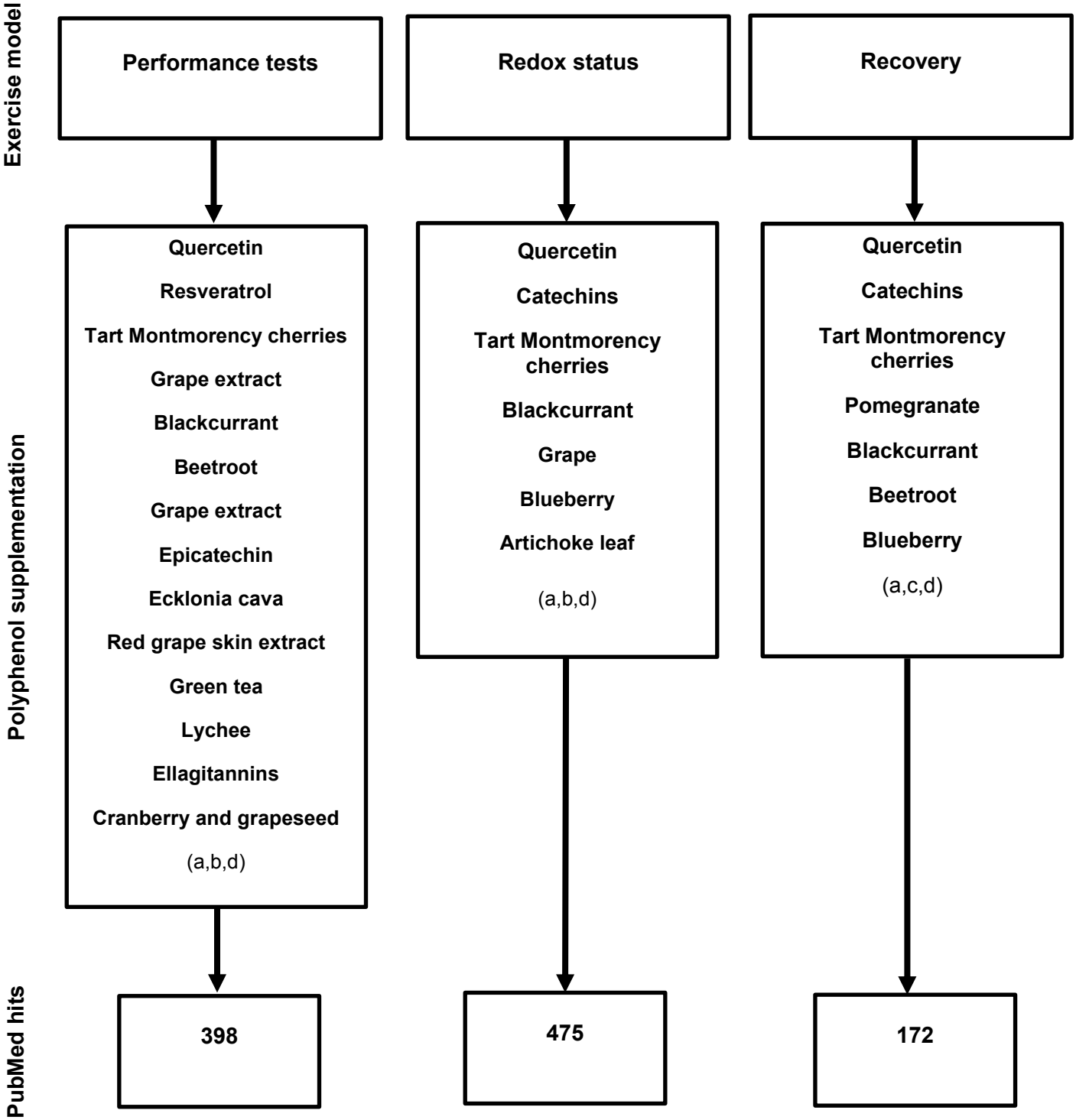


Figure 2.3. High-level overview of cited polyphenol supplementation strategies used in different exercise models according to most up-to-date reviews with the number of PubMed hits when searching the term 'polyphenol and exercise performance tests/redox status or exercise recovery'. Search performed 13/06/2020. a Myburgh (2014), b Braakhuis and Hopkins (2015), c Owens et al. (2018), d Bowtell and Kelly (2019).

The main focus of this review is on the effect of polyphenol intake and recovery. Therefore, the effects of polyphenol supplementation on performance tests and redox status are summarised below to provide context and an overview of the current knowledge on the performance and physiological effects of polyphenol supplementation during exercise. This is followed by a more comprehensive review of the effects of polyphenol supplementation on recovery with a focus on EIMD.

For reader clarity, the definitions below describe the different polyphenol intake methods that will be used as terms in this thesis as the terms are often used interchangeably in the scientific literature:

1. Dietary intake – through foods (i.e., whole berries)
2. Supplementation – drinks, extract in capsules, extract powder (i.e., tart Montmorency cherry juice)
3. Intake – covering both supplementation and dietary intake

Performance Tests

Somerville and colleagues (2017) recently published a systematic review and meta-analysis, which highlighted how polyphenol supplementation (anthocyanins, epigallocatechin gallate and *trans*-resveratrol) used for at least seven-days or more, increased exercise performance by 1.90% (95% confidence interval (CI): 0.40-3.39). The authors also carried out a sub-analysis of seven studies using quercetin and identified a performance increase of 2.82% (95%CI: 2.05-3.58). It should be noted that for the meta-analyses, the authors created a common performance metric to pool the data together to express the performance change as a percentage based on the original publication test results, which covered exercise time to fatigue, distance covered in a pre-selected time period, time to complete a set distance, maximum power output and anaerobic threshold. Furthermore, purple grape juice, beetroot juice and NZBC extract supplementation have all been shown to increase time-to-exhaustion in healthy, recreationally active individuals (Toscano et al. 2015; Vanhatalo et al. 2010;

Perkins et al. 2015). Acute ingestion of tart Montmorency cherry juice (TMCJ) has been shown to improve total work by 10% during a 60 s all-out sprint following a time-to-exhaustion trial and reduce systolic blood pressure (SBP) by 5±2 mmHg in 10 trained male cyclists compared to placebo (Keane, Bailey, Vanhatalo, Jones and Howatson, 2018). Similarly, seven-days supplementation of NZBC extract supplementation increased cardiac output and stroke volume and decreased total peripheral resistance in a dose-dependent manner in endurance trained male cyclists during supine rest (Cook, Myers, Gault, Edwards and Willems, 2017). This observation is in line with the observation of a recent review by Braakhuis, Somerville and Hurst (2020), which suggested that NZBC has a small, but significant, effect on sport performance when taken for seven-days at a dose of 105-210 mg anthocyanins, with a final dose 1-2 h prior to exercise. The mechanisms by which polyphenols exert their effects to enhance exercise performance have been suggested to occur in one of three ways, via mitochondrial biogenesis, improvements in vascular function and/or by increasing fat oxidation (Somerville et al. 2017; Braakhuis, Somerville and Hurst, 2020).

Previously, catechins and NZBC extract supplementation have been reported to increase fat oxidation at rest and/or during exercise in both healthy men and endurance trained men and women, respectively (Dulloo et al. 1999; Kim, Quon and Kim, 2014; Cook, Myers, Gault, Edwards and Willems, 2017; Strauss, Willems, and Shepherd, 2018). However, the findings are not always consistent in favour of beneficial performance effects for these various polyphenols. A TMCJ supplement did not improve time-trial performance in moderately trained cyclists and triathletes (Clifford, Mitchell and Scott, 2013) nor did it improve marathon finishing time compared to placebo in recreational marathon runners (Howatson et al. 2010). Furthermore, six weeks of quercetin supplementation did not improve various military simulated tasks in moderately trained cadets conducting military physical training (Bigelman et al. 2010). Interestingly, Randell and colleagues (2013), demonstrated that neither one-day or seven-days ingestion of green tea extract (catechins) improved fat oxidation in recreationally active individuals. Clearly, whether polyphenols are able to improve various

performance measures is dependent on many factors including but not limited to, the dose, composition of supplement/extract, timing and duration of intake, physical characteristics of the participants and exercise intensity and modality. For a more in-depth review of polyphenols and exercise performance, the reader is referred to recent reviews by Braakhuis et al. (2015), Braakhuis et al. (2020) and Myburgh (2014).

In summary, although the evidence for supplementation with TMCJ, quercetin or green tea extract and exercise performance improvements is less clear, it is plausible based on the aforementioned studies from Cook et al. (2017), Perkins et al. (2015) and the review from Braakhuis, Somerville and Hurst (2020) that supplementing with NZBC for at least seven-days at a dose of 105-210 $mg \cdot d^{-1}$ anthocyanins, with a final dose 1-2 h prior to exercise, can improve markers of exercise performance such as cardiac output, stroke volume, decreased total peripheral resistance and time-to-exhaustion. Therefore, although the overall aim of this thesis is on NZBC's efficacy for enhancing recovery from EIMD and not exercise performance *per se,* this prior evidence with NZBC supplementation helped inform the choice of the EIMD exercise modality with the half-marathon in Chapter 3 and the 100-DJP in Chapter 5 as well as the supplementation strategy (7-days prior and 2- and 3-days post, respectively) and final dose prior to exercise (~2 h).

Redox Status

Redox status can be defined as a deviation from the steady state arising as a result of an imbalance between the production and metabolism of reactive species, such as lipid, protein and deoxyribonucleic acid (DNA) oxidation (Cobley, Close, Bailey and Davison, 2017). One of the first studies to examine the effects of polyphenols on redox status in well-trained athletes was conducted by Skarpanska-Stejnborn et al. (2006). In that study, 19 male rowers supplemented with 250 $mg \cdot d^{-1}$ of blackcurrant extract for six weeks and performed a 2000 m rowing ergometer test pre- and post-the supplementation period. Measures of oxidative stress were collected both pre- and post-intervention and it was observed that those who were in the

blackcurrant condition, showed an increase of their total antioxidative defence capacity and decrease in levels of oxidative stress [as determined by total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPx) and thiobarbituric acid (TBARS)]. Furthermore, Gonçlaves et al. (2011) showed that 300 $mL \cdot d^{-1}$ of organic grape juice supplementation for 20 days with male national triathletes, decreased red blood cell SOD suggesting a reduction in oxidative stress levels. Similarly, NZBC extract supplementation has also been found to be able to modulate markers of oxidative stress, free oxygen radical test (FORT) and free oxygen radical defence (FORD), in elite Olympic level modern pentathletes during a key training phase prior to world championship preparations (Lewis et al. 2017). In recreational marathon runners, TMCJ supplementation has previously been shown to increase TAC when supplemented for five-days prior to running a marathon event, on the day of and for 48 h post (Howatson et al. 2010). Furthermore, blueberry supplementation was found to increase plasma antioxidant capacity and decrease oxidative stress 36 h post an EIMD protocol in 10 healthy females (McLeay et al. 2012). However, previous research has demonstrated no effect of chronic TMCJ supplementation on oxidative stress (lipid hydroperoxides) in trained cyclists after completing a 109-minute cycling race simulation trial (Bell, Walshe, Davison, Stevenson and Howatson, 2014). Furthermore, competitive rowers who supplemented with artichoke-leaf extract for 5 weeks showed no protective effect with regards to oxidative damage to erythrocytes following a 2000 m maximal rowing ergometer test (Skarpañska-Stejnborn, Pilaczynska-Szczesniak, Basta, Deskur-Smielecka and Horoszkiewicz-Hassan, 2008).

Most often, oxidative stress is measured in blood samples rather than other biological tissues due to the comparative ease of both collecting the sample and performing the measurement. Furthermore, it has been reported that the most commonly used redox biomarkers measured in blood do adequately reflect tissue redox status (Margaritelis et al. 2015). However, a recent review by Cobley et al. (2017) highlighted that many of the assays typically used to assess oxidative stress and TAC, namely, TBARS and TAC, are flawed and do not adequately reflect

what is occurring within the tissues of interest, such as skeletal muscle. Furthermore, redox biology is extremely complex, and analysis is often difficult due to the short half-lives of most reactive species, typically a few milliseconds, with even more stable reactive species such as nitric oxide being consumed within two seconds (Halliwell and Whiteman, 2004; Thomas, 2015; Thomas, Liu, Kantrow and Lancaster, 2001). It is generally well accepted that a 'gold standard' method or single biomarker that accurately reflects the oxidative stress or redox balance in human tissues does not exist and as such, the most recent reviews suggest assessing redox status by using a range of biomarkers that focus on different cellular targets to assess redox signalling and/or oxidative stress (Cobley et al. 2017; Powers, Smuder, Kavazis and Hudson (2010). For in-depth review of the complexities in assessing redox status the reader is referred to reviews by Cobley et al. (2017) and Powers et al. (2010).

In summary, whilst the evidence is still equivocal as to whether or not polyphenol supplementation can consistently mitigate oxidative stress following exercise, the two studies that have assessed NZBC's efficacy for dampening the oxidative stress response and upregulating markers associated with endogenous antioxidant defence following exercise (Lewis et al. 2017; Skarpanska-Stejnborn et al. 2006), show promising results. Thus, although there is still a lack of well-conducted human trials, which precludes any definitive conclusions as to its potential benefits, there is enough preliminary evidence to postulate that NZBC is capable of protecting skeletal muscle tissues from oxidative stress damage. This makes the expectation reasonable that supplementation with NZBC could be used to mitigate the secondary cascade in EIMD (reviewed in detail in Section 2.3.3) following strenuous exercise and, as a result, serve as an effective means of enhancing exercise recovery.

2.3 Effect of polyphenols on exercise induced muscle damage

Many studies have investigated the effects of polyphenols on recovery following exercise, specifically, their use to mitigate symptoms associated with EIMD. Exercise induced muscle damage is a common model used to assess a nutrition interventions efficacy on recovery, although it is not certain that this eccentric exercise model is an appropriate way to evaluate soft tissue injuries in individuals (Tipton, 2015). However, given the need to apply various experimental controls in order to assess proof of concept with a given intervention (i.e., polyphenol supplementation), EIMD is the most widely used model to date (Owens et al. 2018). As Figure 2.3 highlights, there has been an abundance of research focusing on various dietary polyphenol supplementation strategies to enhance performance tests and maintain redox balance, with somewhat less focusing specifically on recovery and EIMD. Despite the growing interest in the area and the various dietary polyphenol supplements used, no clear consensus exists regarding which of them would be best placed to help attenuate varying EIMD symptoms (i.e., DOMs, muscle force loss, impaired proprioception), whether there is an optimal dose for polyphenols and adequate knowledge regarding the mechanistic pathways of how polyphenols exert their potential beneficial effects (Owens et al. 2018; Sousa et al. 2014; Sureda et al. 2014).

Exercise induced muscle damage is often characterised by symptoms that present both immediately and for up to ~14 days after the initial bout (Owens et al. 2018). The implications for an individual are the direct impact on functional capacity through a reduction of a muscles force producing capability as a result of the acute mechanical damage to the skeletal muscle (Proske and Morgan, 2001; Howatson and van Someren, 2008). This could expose an individual to be at a greater risk of developing a musculoskeletal injury through the muscle not being able to attenuate the impact forces to the same extent as before the muscle damaging exercise, thus increasing the load burden on the skeleton and stress fracture risk (Blacker, 2017). A nutritional intervention, such as polyphenols, that could mitigate the reduced force

reducing capability and accelerate recovery may have the potential to mitigate musculoskeletal injury risk (Sousa et al. 2014; Blacker, 2017).

It is important to consider the aetiology of EIMD when reviewing the potential effects of polyphenols on the recovery from EIMD. High force eccentric muscle contractions typically produce ultrastructural muscular disruption (i.e. Z-line streaming and fibre degradation), DOMS, increases in specific intramuscular proteins in circulation, swelling of the affected limb, decreased range of motion and impaired muscle force producing capability (Byrne et al. 2004; Hydahl and Hubal, 2014; Mackey and Kjaer, 2017; Howatson and van Someren, 2008), especially when the individual is unaccustomed to the exercise (Newton et al. 2008). The process of EIMD can be classified into a primary and secondary phase, where the primary phase occurs as a consequence of the mechanical work performed and the secondary damage that propagates that damage through processes associated with the inflammatory cascade (Howatson and van Someren, 2008). With regards to the primary phase of EIMD, it is beyond the scope of this thesis to review all the potential mechanisms of how eccentric contractions might cause EIMD, instead the reader is referred to previous reviews of the area (Hyldahl and Hubal, 2014; Proske and Morgan, 2001; Warren et al. 2002). However, it is pertinent to address the two main theories that have featured heavily within the literature to provide context for the primary cascade believed to initiate EIMD along with the secondary cascade, which propagates the initial damage.

2.3.1 Primary muscle damage

The 'popping sarcomere hypothesis' was the first theory put forwards by Morgan (1990), which proposes that EIMD occurs as a result of non-uniform lengthening of half-sarcomeres when a muscle is stretched beyond an optimum length for force generation, especially on the descending limb of the length-tension curve (Yeung et al. 2002). Sarcomeres extended beyond the point of optimal length become longer and weaker, and consequently, less able to maintain tension. These sarcomeres will then lengthen rapidly until a point of passive tension

is reached with the surrounding structures of the muscle preventing further lengthening (Proske and Morgan, 2001; Morgan and Proske, 2004; Proske et al. 2004). The failure of sarcomeres to maintain tension following an eccentric action increases the relative tension on remaining functional sarcomeres, with the process of lengthening, weakening and failure repeating itself on the next weakest sarcomere. This process continues for the duration of loading caused by eccentric action (Morgan, 1990; Proske et al. 2004). Upon completion of a contraction, the majority of sarcomeres re-interdigitate, however, some sarcomeres become damaged to a point where re-interdigitation cannot occur, leading to myofilament disruption. Inconsistencies in sarcomere strength are heterogenous along each myofibril, leading to non-uniform lengthening, which can expose cell membranes to large deformation (Morgan and Proske, 2004). Yeung et al. (2002) have suggested that repeated non-uniform lengthening of sarcomeres following eccentric action can cause further physical damage in the extracellular matrix (ECM) and propose that this may in turn lead to disrupted calcium (Ca^{2+}) and sodium homeostasis, abnormal pH regulation and subsequent inflammation (Morgan, 1990; Morgan and Proske, 2004). The loss of Ca^{2+} homeostasis is proposed to be largely responsible for the facilitation of secondary muscle damage (Beaton et al. 2002; Howatson and van Someren, 2008). Further, the disruption to the sarcomere can be observed in the form of Z-line streaming, broadening and total disruption through histological analyses (Fridén, Sjöström and Ekblom, 1983).

Warren et al. (2002) proposed an alternative theory, which suggests that failure of the EC coupling process is the primary event that propagates EIMD. The EC coupling process refers to the sequence of events responsible for muscular contraction, culminating in the release of Ca^{2+} from the sarcoplasmic reticulum (Warren et al. 2001). Yeung et al. (2002) demonstrated that eccentric stretching could cause damage to the sarcoplasmic reticulum and T-tubular network in mouse muscle leading to disruptions in the diffusion of molecules into and out of the T-system. Increases in intracellular Ca^{2+} and sodium resulting from damage to T-tubules were also considered as contributing to the mechanisms of EIMD. The effect of disrupted Ca^{2+}

release following interruption of the EC coupling process was highlighted by Balnave and Allen (1995) who concluded that both reduced Ca^{2+} release and reduced Ca^{2+} sensitivity contribute to reductions in muscle force generating capacity. Failures in the EC coupling process can cause losses in force production to an extent of as much as 75% in the days after muscle injury (Warren et al. 2001). However, research concerning the contribution of EC coupling failure to force loss is equivocal. Though Warren et al. (2001) indicated that a large amount of force loss could be associated with EC coupling interruptions, Balnave and Allen (1995) suggested that for a similar loss in force production (approximately 70%), a much lower proportion could be attributed to EC coupling failure, with a larger contribution to force loss stemming from disruption of force-bearing elements within the muscle fibre. Martin et al. (2004) have suggested that following an eccentric exercise protocol, alterations to the EC coupling process may be predominately involved in strength loss in the first two days after muscle insult, with damage to the force-generating structures accounting for strength losses 2-4 days following EIMD.

The disruption of the EC coupling process is an important factor in EIMD (Jones et al. 1997; Allen, 2001; Warren et al. 2001), and is likely to be one of the major causes of reduced force production following EIMD (Balnave and Allen, 1995). However, the evidence concerning the stage at which the process is affected, the subsequent recovery pattern, and the potential for further calcium induced damage is unclear (Balnave and Allen, 1995; Warren et al. 2001). Irrespective of the exact mechanisms and their relative contributions, the latest consensus suggests that both sarcomere inhomogeneity and failure of the EC coupling process are likely to have a prominent role in the manifestation of EIMD (Hydahl and Hubal, 2014).

2.3.2 Metabolic damage

In support of metabolic damage, as opposed to mechanical, initiating muscle damage during exercise, Tee, Bosch and Lambert (2007) suggested that the initial events in EIMD are caused by metabolic deficiencies within the working muscle, or that these deficiencies increase the

vulnerability of the muscle fibre to mechanical stress. This theory is based on observations from studies where markers of muscle damage, such as increased CK and muscle force loss, following exercise tasks, which do not require significant eccentric muscle contractions (e.g., cycling) (Bell et al. 2014; Bell et al. 2015; Lyall et al. 2009). For example, Lyall et al. (2009) observed modest increases in plasma CK, markers of inflammation (TNF-α and IL-6) and oxidative stress (protein carbonyls) following a 30-min ergometer row at 80% $\dot{V}O_{2max}$ in ten healthy male and females in the placebo group but not the blackcurrant supplemented group. Thus, the presence of muscle damage as indicated by the increases in plasma CK concentrations, despite the absence of any significant eccentric muscle contractions, supports the concept that metabolic factors seem to have at least a partial role in provoking EIMD.

The specific mechanisms by which exercise-induced metabolic stress causes EIMD, in the absence of significant mechanical stress, are poorly understood. However, it is beyond the scope of this thesis to review them in detail and instead the reader is referred to the theoretical model outlined by Tee et al. (2007). Nonetheless, what can be suggested, is in sporting scenarios such as team sports or distance running where both mechanical and metabolic stress are likely involved, although the principal cause of EIMD is still likely due to mechanical factors due to the repeated number of eccentric muscle contractions, both metabolic and mechanical stress are likely contributing or interacting with one another in provoking EIMD.

2.3.3 Secondary muscle damage

Dietary polyphenol interventions are unlikely to interact with the primary phase of the mechanical damage during the exercise bout (Bell et al. 2014; Howatson and van Someren, 2008). It is more probable, that polyphenols will affect the secondary cascade associated with EIMD, where inflammation and the production of reactive oxygen species (ROS) are present, given that polyphenols are known to possess both anti-inflammatory and anti-oxidative properties *in vitro* (Seeram et al. 2008; Seeram et al. 2001; Wang et al. 1999) and *in vivo* (Lyall et al. 2009). Therefore, dietary polyphenols may be able to attenuate further damage by

modulating this response and aid the subsequent recovery process. However, in order to fully contextualise the role that polyphenols may play in mitigating the secondary cascade in EIMD, the aetiology of inflammation and oxidative stress will be discussed in further detail in this section before the previous EIMD and polyphenol supplementation literature is presented and discussed.

Inflammation

The mechanical and/or metabolic damage described in the previous sections has been purported to orchestrate a series of biochemical alterations that have the capacity to exacerbate the magnitude of damage in a cell, which has been termed secondary muscle damage or the secondary cascade (Howatson and van Someren, 2008). It is believed that this secondary damage is initiated by a loss of Ca^{2+} homeostasis resulting from sarcolemma damage and opening of stretch activated channels (Armstrong, Warren and Warren, 1991; Ebbeling and Clarkson, 1989). The loss of Ca^{2+} homeostasis then triggers an acute phase inflammatory response, primarily from the cytokine family, to orchestrate the degradation and subsequent regeneration of muscle cells (Armstrong et al. 1991; Toumi, F'Guyer and Best, 2006). The subsequent inflammatory cascade is a vital process that clears damaged tissue, and initiates tissue repair and adaptation to the exercise stimulus (Chazaud, 2016). Damaged muscle cells synthesise a large number of inflammatory cytokines that are either deemed pro-inflammatory (e.g., tumour-necrosis-factor-alpha; TNF-α, interleukin-1-beta; IL-1β, interleukin-6; IL-6, interleukin-8; IL-8 and interferon-gamma; IFN- γ) or anti-inflammatory (interleukin-1-alpha; IL-1α; interleukin-4; IL-4 and interleukin-10; IL-10) depending on their primary biological functions in skeletal muscle and other cells (Cannon and St Pierre 1998; Smith, Kruger, Smith and Myburgh, 2008). The pro-inflammatory cytokines TNF-α and IL-1β play a key role in attracting immune cells to damage muscle (Butterfield et al. 2006). The main leukocytes recruited by damaged muscle tissues are neutrophils, which infiltrate muscle tissue immediately following the initiation of muscle damage and may remain elevated for up to five-days post, reflective of the magnitude and intensity of the exercise task (Cannon and St Pierre

1998; Tidball, 2005; Toumi et al. 2006). Further, these immune cell types infiltrate the damaged tissue, which also include mast cells, neutrophils, T regulatory lymphocytes, eosinophils and CD8 T lymphocytes (Burzyn et al. 2013; Castiglioni et al. 2015; Cote, Tremblay, Duchesne and Lapoite, 2008; Heredia et al. 2013; Zhang et al. 2014) to carry out specific roles in a highly organised, temporal manner. Neutrophils are likely the first group of immune cells to infiltrate muscle at the site of injury, activated by Ca^{2+} stimulated proteolysis and increased intracellular Ca^{2+} signalling pro-inflammatory cytokine release (Butterfield, Best and Merrick, 2006; Hyldahl and Hubal, 2014). Neutrophils phagocytose necrotic myofibers and cellular debris (Pizza et al. 2005), however, in doing so, they produce high concentrations of cytolytic and cytotoxic molecules through NADPH oxidase derived superoxide anion dependent mechanisms that can aggravate existing damage (Nguyen and Tidball, 2003). Thus, it has been suggested that while neutrophil mediated phagocytosis is necessary step in the repair process, it may also exacerbate the existing damage and hinder the rate of recovery (Lapointe et al. 2002; Pizza et al. 2005; Tidball, 2005; Toumi et al. 2006). Pizza (2005) suggested that neutrophils are the main molecules involved in the secondary muscle damage cascade and primarily cause further damage by inhibiting the growth of new myofibrils or degrading undamaged ones, due to the collective activities of RONS, proteases and pro-inflammatory cytokines. Further, it is probable that, depending on the magnitude and intensity of the muscle damage sustained, the aforementioned activities of neutrophils may result in further or a biphasic loss of muscle function in the proceeding days (Howatson and van Someren, 2008; Toumi and Best, 2003). However, this observation is lacking in many studies (Warren, Call, Farthing and Baadom-Piaro, 2017). Nevertheless, it has been suggested that regardless of whether or not 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 following days (Toumi and Best, 2003; Warren et al. 2017).

Oxidative stress

The term oxidative stress was first defined in the 1980s as “a disturbance in the pro-oxidant-antioxidant balance in favour of the former” (Sies, 1985). Although this meaning has been widely accepted for over two decades, the definition of oxidative stress has been challenged many times. Due to the complexity associated with the assessment of cellular redox balance, it has been argued that the term oxidative stress defines a simple prooxidant versus antioxidant definition (Azzi, Davies and Kelly, 2004; Jones, 2006). However, Powers et al. (2010) defined oxidative stress as “a disturbance in the redox balance in cells in favour of oxidants, with this imbalance resulting in oxidative damage to cellular components”. Although the area of redox biology is complex and ever evolving, for reader clarity, the term oxidative stress will be used throughout this thesis in line with the definition put forward by Powers et al. (2010). Reactive oxygen species (ROS) produced during the respiratory burst may be activated by increases in intracellular Ca^{2+} concentration, TNF-α and/or neutrophils. The respiratory burst involves an increase in non-mitochondrial oxidative metabolism, which results in the production of superoxide anion (O_2^-) and associated ROS (Halliwell and Gutteridge, 1986). This respiratory burst has been shown to occur three days post eccentric muscle contractions directly using rodent models (Zerba, Komorowski and Faulkner, 1990; McArdle et al. 1999). Specifically, superoxide anion has been shown to be generated during 2 h of downhill running in rats (Liao et al. 2010). Superoxide is converted to hydrogen peroxide, which can peroxidise lipids and damage cell membranes (Hampton, Kettle and Winterbourn, 1998). Liao et al. (2010) also demonstrated an increase in hydrogen peroxide 24 h post downhill running compared to rest. Hydrogen peroxide can also form hypochloride acid, which can damage cell membranes (Tidball, 2005). Thus, it is feasible that ROS may cause lipid oxidation and exacerbate damage in the days following eccentric exercise (Close et al. 2005). In humans, there is evidence of lipid oxidation where serum malondialdehyde (MDA) has been shown to be increased immediately (Sacheck et al. 2003), 48 h (Goldfarb, Bloomer and McKenziem 2005), 72 h (Close et al. 2004; Close et al. 2006) and 96 h (Close et al. 2006)

post muscle damaging exercise. Furthermore, $F_{2\alpha}$-isoprostanes are increased up to 72 h following muscle damaging exercise (Sacheck et al. 2003), and serum TBARS are significantly increased from 48 to 96 h (Nikolaidis et al. 2007).

Proteins can also be oxidised, which is indicated by the presence of carbonyl groups. Following isokinetic lengthening muscle actions, plasma protein carbonyls are increased from 48 to 96 h post in females (Nikolaidis et al. 2007), and at 24 and 48 h in males (Goldfarb, Bloomer and McKenzie, 2005). Differences in timelines may be attributed to gender and the muscle group utilised during the damaging protocol. However, it is difficult to identify which proteins are being oxidised as different proteins may vary greatly in their susceptibility to oxidative damage (Nikolaidis et al. 2008). In summary, the activation of ROS may lead to lipid and protein oxidation (e.g., oxidative stress), further exacerbating the magnitude of damage to the muscle cells, which may overwhelm the endogenous antioxidant defence network and lead to functional impairments. Subsequently, these functional impairments could have implications for muscular fatigue and muscle damage, which may augment exercise recovery and performance (Powers and Jackson, 2008). However, one should be aware that the majority of studies which implicate a direct relationship between systemic oxidative stress and muscle function loss after exercise stem from animal models and therefore, may not hold true in humans. Nikolaidis et al. (2008) highlights that in human trials, the time course of muscle function loss and systemic oxidative stress do not appear to follow one another with regards to exercise recovery. Lastly, the ambiguity surrounding oxidative stress's role within EIMD is further confounded by the complexity of accurately measuring redox biology *in vivo* (see Section 2.14). Thus, it is clear that much more work is required to shed further light on the exact role of oxidative stress in EIMD.

In addition to the aforementioned inflammatory and oxidative stress responses in secondary muscle damage, other degradative pathways such as the caplain or ubiquitin-proteasome pathway are also significantly upregulated following EIMD (Raastad et al. 2010). It is likely that they operate in parallel or in conjunction with the aforementioned inflammatory and

oxidative stress responses in the secondary muscle damage cascade. However, it is beyond the scope of this thesis to investigate these pathways and instead the reader is referred to previous reviews on the area (Belcastro, Shewchuk and Raj, 1998; Goll, Thompson, Li, Wei and Cong, 2003).

Previous literature on polyphenol supplementation and EIMD

To date, seven different polyphenol rich foods have been the focus of research to modulate symptoms associated with EIMD and facilitate recovery (Table 2.1). Varying EIMD protocols have been previously used to assess polyphenol supplementation efficacy with recovery such as drop-jumps (Clifford et al. 2015), repetitive eccentric knee (McLeay et al. 2012; Trombold et al. 2011; Bowtell et al. 2011) and elbow flexor contractions (Connolly et al. 2006; O'Fallon et al. 2012; Rowland, 2018; Trombold et al. 2010; Trombold et al. 2011), half-marathon (Lynn et al 2018; Levers et al. 2016), marathon (Howatson et al. 2010; Clifford et al. 2016), 160 km endurance run (Nieman et al. 2007a), intensified repeated cycling bouts (Nieman et al. 2007b; Bell et al. 2014), simulated cycling road race (Bell et al. 2015) and a metabolically-demanding intensive ergometer row (Lyall et al. 2009), with equivocal results. To draw comparisons between each polyphenol supplementation study, each study will be briefly discussed in turn with the EIMD protocol as the grouping variable.

Table 2.1. Polyphenol supplementation and recovery focused studies as identified by Pubmed (search performed on 10/07/2020).

Author (year)	Polyphenol supplement and dose	Study design	Exercise (EIMD) model	Participants	Biomarker response	Performance outcome	Key finding
Connolly et al. (2006)	TMCJ, 355 ml twice a day for 8 days or placebo (P)	Randomized, repeated crossover (blinding not clear)	40 (20 x 2) maximal eccentric contractions of elbow flexors	14 male college students	N/A	No difference between TMCJ or P for muscle tenderness or elbow range of motion (⟷). ↓ in pain and strength loss with TMCJ vs P.	TMCJ group strength loss averaged only 4% vs. 22% in P over 4-days following eccentric exercise
Howatson et al. (2010)	TMCJ, 237 ml a.m and p.m or placebo (P) for 5 days pre Marathon, and 8 days in total	Independent, matched groups (based on predicted finish time)	2008 London Marathon (two participants ran marathon distance 14-days later)	20 recreational Marathon runners (13 males, 7 females)	TMCJ↓ IL-6, CRP, UA, TAS, PC vs. P. No effect for TMCJ on CK, LDH, TBARS (⟷)	TMCJ↑ MVIC strength vs. P No effect for TMCJ on DOMS	TMCJ may enhance recovery by increasing TAC, ↓ inflammation and OS

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Kuehl et al. (2010)	TMCJ, 355 ml at a.m and p.m or placebo (P) for 7-days and on race day	Double blind, randomized, placebo-controlled trial	Hood to Coast relay Total distance 26.3±2.5 km	54 healthy runners (36 males, 18 females)	N/A	↓ Smaller increase in pain in TMCJ vs. P	TMCJ for 7-days prior to and during a strenuous running event can minimize post-run muscle pain
Bell et al. (2014)	TMCJ, 30 ml at a.m and p.m or placebo (P) for 7-days with restriction of dietary AOX for 10 days (3-days pre-trial and 7-days during)	Double blind trial (7-days)	3 consecutive days of simulated lab based cycling road race: 109 mins, with 66 sprints, 5, 10, 15 secs duration	16 well trained male cyclists	TMCJ ↓ LOOH vs. P TMCJ ↓ IL-6 and HS-CRP vs. P. No effect for TMCJ on IL-1 β, IL-8, CK, TNF-α (↔)	No difference between TMCJ or P (↔)	TMCJ may enhance recovery from consecutive days of high intensity exercise through ↓ oxidative stress and inflammation

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Bell et al. (2015)	TMCJ, 30 ml a.m and p.m or placebo (P) for 4-days with restriction of dietary AOX 4-days pre, 3-days post test	Double blind trial (4-days)	Laboratory test of simulated cycling road race 109 min total with 66 sprints of 5, 10, 15 sec, with additional 9 min time trial	16 well trained male cyclists	TMCJ ↓ IL-6 and hs-CRP vs. P. No effect for TMCJ on IL-1 β, IL-8, CK or TNF-α (↔)	DOMS – no effect for TMCJ vs. P. MVIC maintained in TMCJ, whereas Post-test with P ↓ at 24, 48, 72 h. Cycling efficiency maintained in TMCJ, ↓ in P post 24 h. No effects for total work performed or 6 sec peak power for TMCJ vs. P	TMCJ may maintain performance (MVIC and cycling efficiency) following a bout of high intensity exercise through ↓ inflammation.

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Bowtell et al. (2011)	TMCJ, 30 ml a.m and p.m or PLA for 10 days, EIMD trial on day 8	Double blind, repeated crossover, placebo-controlled trial	Two trials of 10x10 single leg knee extension @ 80% 1RM, opposite leg for repeat trial	10 well trained males (resistance backgrounds)	TMCJ ↓ PC vs. PLA. No effect of MTCJ on CK, nitrotyrosine, hsCRP or TAC	TMCJ ↑ MVIC recovery vs. PLA	TMCJ improved the recovery of isometric muscle strength after intensive exercise
Clifford et al. (2015)	Beetroot juice (BTJ), 3 x serving immediately post of High-BTJ, low-BTJ or PLA then 2x250 ml, 2x125 ml or 2x250 ml, respectively for	Double blind, independent groups design, place-controlled trial	100-drop jump exercise	30 recreationally active males	CK, IL-6, TNF-α and IL-8 were unaffected by BTJ (↔)	CMJ recovered ↑ in hight-BTJ vs PLA at 48 h and 72 h. Pressure pain threshold higher in both low- and high-BTJ vs. PLA at 24, 48 and 72 h. MVIC unaffected by BTJ.	Acute BTJ attenuated muscle soreness and decrements in CMJ performance following 100-drop jumps

	2-days (24, 48 h post)						
Author (year)	**Polyphenol supplement and dose**	**Study design**	**Exercise model**	**Participants**	**Biomarker response**	**Performance outcome**	**Key finding**
Clifford et al. (2016)	Beetroot juice (BTJ), 3x250 ml or PLA for 3-days (event day, 24 and 48 h post)	Double blind, independent groups design	2016 Druridge Bay Marathon	34 runners (~16 previous marathons) (21 males, 13 females)	No effect of BTJ on CK, IL-6, AST or hsCRP	No effect of BTJ on CMJ, DOMS or MVIC.	BTJ did not attenuate inflammation or muscle damage ↓ following a marathon
Clifford et al. (2016)	Beetroot juice (BTJ), 2x250 ml or PLA for 3-days (24, 48 and 72 h) post initial bout	Double blind, independent groups design	Repeated sprint test (20x30 m) and second repeated sprint at 72 h post initial bout	20 male team-sport players	No effect for BTJ on hsCRP, CK, LOOH, PC or ascorbyl free radical (↔)	BTJ ↑ CMJ and RSI vs PLA. PPT, mean and fastest sprint times or fatigue index not different between groups (↔)	BTJ ↓ the decrement in CMJ and RSI following repeated sprint tests but had no effect on sprint performance or OS

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Nieman et al. (2007a)	Quercetin (Q) (1000 mg·day^{-1}) or PLA for 3 weeks pre-test and 3-days during ergometer cycle test days	Double blind, independent groups design	Ergometer cycle for 3 h/day at ~57% maximal work rate	40 trained male cyclists	Q ↓ leukocyte IL-8 and IL-10 mRNA vs PLA. No effect for Q on muscle NF-kB, COX-2 mRNA, plasma IL-6, IL-10, IL-1ra, IL-8, TNF-α (↔)	N/A	Q diminished post-exercise expression of leukocyte IL-8 and IL-10 mRNA. However, Q did not influence any of the muscle measures.
Nieman et al. (2007b)	Quercetin (Q) (1000 mg·day^{-1}) or PLA for 3 weeks	Double blind, independent groups design	160 km Western States endurance run	39 ultramarathon athletes (32 males, 7 females)	Q ↑ plasma quercetin levels vs PLA. No effect for Q on IL-6, IL-8, IL-1Ra, IL-10 mRNA, CK, CRP and cortisol (↔)	N/A	Chronic Q ingestion significantly ↑ plasma Q levels but failed to attenuate muscle damage, inflammation, in plasma cytokine and hormone levels, and alterations in leukocyte mRNA expression ↑

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Kastello et al. (2014)	Cherry Juice (CJ, CherryFlex®), or PLA, 1 capsule, twice daily for 16 days prior to eccentric exercise and 3 days post	Double blind, repeated crossover design	5 sets x 10 reps, maximal eccentric arm extensions, collateral arm used in repeat	14 healthy participants (4 males, 10 females)	CJ↓ CRP vs. PLA. No effect of CJ on PC, CK, TBARS or myoglobin	CJ↓ perceived pain from VAS scale No effect of CJ on limb volume, limb girth, peak torque or peak work (⬌)	Chronic CJ supplementation may have a protective effect on inflammation and perceived pain
Trombold et al. (2010)	Pomegranate juice (POM) or PLA, 500 ml 2xday for 9 days. EIMD on day 5.	Double blind, repeated crossover design	2 sets x 20 reps maximal eccentric elbow flexor contractions	16 recreationally active males	No effect of POM on CK, myoglobin, IL-6 or CRP (⬌)	POM↓ strength loss at 48 and 72 h post vs PLA.	POM significantly improves recovery of isometric strength 2-3 d after a damaging eccentric exercise bout

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Trombold et al. (2011)	Pomegranate juice (POM) or PLA, 250 ml twice daily for 15 days. EIMD on day 8.	Double blind, repeated crossover design	3 sets x 20 reps unilateral eccentric elbow flexions and 6 sets of 10 reps unilateral knee extensions	17 resistance trained males	N/A	POM significantly ↑ elbow flexion strength and elbow flexor muscle soreness vs PLA.No effect for POM on isometric strength and DOMS in knee extensors(↔)	POM attenuates weakness and reduces soreness of the elbow flexor but not of the knee extensor muscles in resistance trained males
Kersick et al. (2010)	*N*-acetyl-cysteine (NAC), epigallocatechin gallate (EGCG) or PLA, for 14 days	Double blind, parallel design	10 sets x 10 reps at 30º/s of dominant knee extensor	30 healthy, non-resistance trained males	Neu:Lym ratio in EGCG ↓ than NAC at 48 h. EGCG↓ less vs. PLA at 48 h. No effect for NAC or EGCG on SOD,	NAC and EGCG ↓ DOMS vs. PLA.	NAC and EGCG appeared to invoke little change over the secondary muscle damage cascade

					8-isoprostane, cortisol, neutrophil counts		
Author (year)	**Polyphenol supplement and dose**	**Study design**	**Exercise model**	**Participants**	**Biomarker response**	**Performance outcome**	**Key finding**
Lyall et al. (2009)	NZBC capsules (NZBC) or PLA, 2x capsules before exercise, 2x post exercise	Double blind, repeated crossover design	30-minute row on ergometer @ 80%V_{O_2}max	10 healthy recreationally active participants (5 males, 5 females)	NZBC ⬇ PC, IL-6 and TNF-α vs. PLA	N/A	NZBC can alleviate OS and possibly enhance immune responsiveness to potential pathogens
Coelho et al. (2017)	New Zealand blackcurrant extract capsules (NZBC) or PLA, for 12-days, EIMD on day 8	Double blind, parallel design	60 maximal eccentric contractions of biceps brachii	13 recreationally active healthy females	No effect of NZBC on CK, IL-6 (⬌)	NZBC ⬇ DOMS at 24 and 48 h vs PLA No effect of NZBC on MVIC, mid-arm circumference or range of motion	Chronic NZBC supplementation can reduce DOMS post strenuous eccentric exercise

						(⬌)	
Author (year)	**Polyphenol supplement and dose**	**Study design**	**Exercise model**	**Participants**	**Biomarker response**	**Performance outcome**	**Key finding**
Hutchinson et al. (2014)	Blackcurrant nectar (BCN) or PLA, 455 ml 2x day for 8-days, EIMD on day 4.	Double blind, placebo-controlled design	3 sets of 10 reps of eccentric knee extensions (leg squats) @ 115% of 1RM	16 recreationally active college students (3 males, 13 females)	BCN⬇ CK at 48 and 96 h vs PLA. IL-6 change⬆ at 24 h vs. PLA. ORAC levels⬆ in BCN vs. PLA at 48 h	No effect for BCN on DOMS (⬌)	BCN supplementation prior to and post a bout of eccentric exercise attenuates muscle damage and inflammation
Skarpańska-Stejnborn et al. (2006)	Blackcurrant extract (BC) (250 mgx3 per day) vs PLA	Single blind controlled trial (6 weeks)	2000 m rowing ergometer test	19 male rowers from Polish national team	GPx and SOD TBARS 24 h post-test in BC vs P,⬆ TAC in BC 24 h post-test vs. PLA	No difference between B vs PLA	6-weeks BC extract⬇ lipid peroxidation and raises TAC post exhaustive exercise (⬇ oxidative stress)
McLeay et al. (2012)	Blueberry smoothie (BS) or PLA, for 3-	Randomized, repeated	3 sets x 100 reps eccentric	10 recreationally	BS⬇PC at 36 h post and ⬆	BS⬆ recovery of peak isometric tension vs. PLA.	BS accelerates recovery of muscle peak isometric strength

	days. EIMD on day 1	crossover design	quadricep contractions	active healthy females	FRAP (oxidative capacity) No effect of BS on IL-6 (⟷)	No effect of BS on concentric or eccentric strength (⟷)	
Author (year)	**Polyphenol supplement and dose**	**Study design**	**Exercise model**	**Participants**	**Biomarker response**	**Performance outcome**	**Key finding**
Levers et al. (2016)	Powdered tart cherries (PTC) or PLA, one capsule (480 mg·day^{-1} for 10-days, including race day and up to 48 h post race	Randomised, double-blind and placebo-controlled trial	Half-marathon race	27 endurance trained runners or triathletes (18 males, 9 females)	Attentuations in PTC group for creatinine, urea/blood nitrogen, total protein, cortisol, IL-2, IL6, IL-13 and increased TAC vs. PLA	PTC group averaged 13% faster half-marathon finish times compared to PLA. VAS score↓ in PLA vs. PTC	PTC attenuated markers of muscle catabolism, reduced immune and inflammatory stress, better maintained redox balance and increased performance surrounding a half-marathon race

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Brown et al. (2019)	TMCJ or PLA for 8-days (2x30 mL·day^{-1}), 4-days prior to and day of and 3-days post exercise	Randomised, double-blind, placebo-controlled trial	Repeated sprint protocol (15x30 m maximal sprints, rapid 10 m deceleration phase, 60 s rest)	20 active females	CK and hsCRP remained unaffected by TMCJ or PLA.	TMCJ accelereated recovery of CMJ height vs. PLA. Trend for lower DOMS and higher pressure pain threshold in TMCJ vs. PLA. MVC, 30 m sprint time, reactive strength index, flexibility, claf gurth, thigh girth remained unaffacted by TMCJ.	TMCJ may be a practical nutritional intervention to help attenuate symptoms of muscle damage and improve recovery in females

Author (year)	Polyphenol supplement and dose	Study design	Exercise model	Participants	Biomarker response	Performance outcome	Key finding
Lamb et al. (2019)	TMCJ, POM or PLA 2x250mL·day^{-1} for 9-days (4-days prior, day of exercise and 4-days post)	Randomised, double-blind, placebo-controlled, parallel trial	Elbow flexor of non-dominant arm (5x10 sets)	36 non-resistance trained males	CK remained unaffected by TMCJ or PLA vs. PLA	MVIC, DOMS and range of motion remained unaffected by TMCJ and POM vs. PLA	TMCJ and POM were unable to accelerate recovery from high-force eccentric exercise of the elbow flexors

CK = creatine kinase; UA = uric acid; DOMS = delayed onset muscle soreness; FRAP = Ferric reducing ability of plasma; IL-2 = interleukin-2; IL-6 = interleukin-6; IL-8 = interleukin-8; IL-1 β = interleukin-1 beta; IL-13 = interleukin-13; LDL = Low density lipoprotein; LOOH = lipid hydroperoxides; MCP-1 = Monocyte chemotactic protein-1; MVIC = maximum voluntary isometric contraction; AST = aspartate aminotransferase; GSH = Glutathione; GPx = Glutathione peroxidase; GSSG; Oxidised glutathione; ORAC = Oxygen radical absorbance capacity; PC = Protein carbonyls; CMJ = Countermovement jumps; RSI = reactive strength index; TAC = Total antioxidant capacity; TBARS = Thiobarbituric acid reactive substances; TAS = Total antioxidant status; TMCJ = tart Montmorency cherry juice; TNF- α = tumour necrosis factor alpha; SOD = Superoxide dismutase; hsCRP = highly sensitive C-reactive protein; CRP = C-reactive protein; OS = Oxidative stress; 1RM = One rep maximum; PPT = pain pressure threshold; NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells; Neu:Lym = neutrophil:lymphocyte ratio; V_{O_2}max = Maximal volume of oxygen uptake; VAS = visual analogue scale; PTC = powdered tart cherries.

2.4 Drop jumps

Clifford et al. (2015) observed that when recreationally active males supplemented with high- or low-dose beetroot juice (BTJ) immediately post, 24 and 48 h after completing a 100-DJP, countermovement jump (CMJ) performance recovered quicker at 48 and 72 h post-exercise under the BTJ condition compared to the placebo (PLA) condition. In addition, participant's pain pressure threshold (PPT), a measure of muscle soreness, was greater in both high and low BTJ groups at 24, 48 and 72 h post-exercise, returning to baseline by 72 h post-exercise, whilst remaining elevated in the PLA group. However, BTJ was unable to attenuate declines in maximal voluntary isometric contraction (MVIC) force compared to PLA, suppress elevations in systemic inflammation or intramuscular protein secretion as measured by plasma creatine kinase (CK) activity. Due to the paucity of evidence on whether a polyphenol supplement is able to accelerate recovery following a plyometric exercise, such as 100-DJP, Chapter 5 will examine whether intake of NZBC extract 7-days before and 3-days following 100-DJP affect the recovery of markers of EIMD.

2.5 Repetitive knee-extensor contractions

McLeay et al. (2012) found that when recreationally active females supplemented with a blueberry smoothie or PLA, five and 10 h prior to and then immediately post, 12 and 36 h after performing 300 strenuous eccentric contractions of the quadriceps, isometric peak torque was greater in the blueberry smoothie group compared to PLA at 60 h suggesting a faster rate of recovery in the former. Oxidative stress decreased at a faster rate in the blueberry smoothie group, although it was not statistically significant until 36 h post-exercise. However, no group differences were observed for muscle soreness, scored on a VAS, or for systemic inflammatory markers. Bowtell et al. (2011) investigated whether 9-days of tart Montmorency cherry juice (TMCJ) (specifically CherryActive®) supplementation could attenuate declines in muscle function and oxidative damage post

repeated unilateral knee extensions in well-trained males. Participants performed the muscle damaging exercise on day seven and measures of quadricep maximal voluntary contractions (MVC), CK, inflammation, oxidative stress and total antioxidant capacity were performed immediately post, 24 and 48 h after the damaging exercise. Bowtell et al. (2011) found that TMCJ was able to accelerate the recovery of quadricep MVC force at 24 and 48 h compared to PLA and attributed this to TMCJ possibly attenuating the oxidative damage induced by the damaging exercise. Similarly, Trombold et al. (2011), assessed the efficacy of pomegranate juice supplementation on the recovery of skeletal muscle strength in resistance trained men after repeated unilateral eccentric elbow flexion and knee extension exercises. Maximal isometric elbow flexion and knee extension strength and elbow and knee muscle soreness (VAS) measurements were made at baseline and 2, 24, 48, 72, 96 and 168 h post-exercise. It was observed that elbow flexion strength was significantly higher during the 2- to 168 h period post-exercise and elbow muscle soreness was attenuated with pomegranate juice compared to PLA. However, knee isometric strength and muscle soreness were not statistically different with pomegranate juice compared to PLA. Both McLeay et al. (2012) and Bowtell et al. (2011) found that a blueberry smoothie and TMCJ were able to attenuate the decline in force producing capability of the quadriceps as defined by isometric peak torque and MVIC, respectively, following repeated knee extensor damaging exercise. In contrast, Trombold et al. (2011), observed no difference when supplementing with pomegranate juice on knee isometric strength.

2.6 Repetitive elbow-flexor contractions

Connolly et al. (2006) was one of the first studies to assess the efficacy of polyphenol supplement to accelerate recovery following EIMD. The authors utilised a repeated crossover design with a TMCJ blend or PLA in recreationally active males consuming the beverage twice a day for eight consecutive days. On the fourth day, the participants performed a bout of eccentric elbow flexor contractions, with isometric elbow flexion

strength, pain, muscle tenderness and relaxed elbow angle being recorded immediately before and for four days following the eccentric exercise. The opposite arm was then used in the repeat condition to avoid the repeated bout effect (RBE). It was observed that TMCJ was able to attenuate strength losses and increases in pain compared to the placebo group. However, relaxed elbow angle and muscle tenderness were not different between trials. Quercetin has good bioavailability in plasma after consumption (Egert et al. 2008) and could therefore have potential benefits *in vivo* for recovery from strenuous exercise. As such, O'Fallon et al. (2012) examined whether a of 1000 $mg \cdot day^{-1}$ quercetin containing bar seven-days before the first and five days after the second exercise session could attenuate EIMD symptoms in recreationally active males and females. Participants were randomized to quercetin or PLA and performed two separate sessions of 24 eccentric elbow flexor contractions. Muscle strength, muscle soreness, resting arm angle, upper arm swelling, serum CK, plasma quercetin and systemic inflammatory markers were assessed before and for five days after exercise. Plasma quercetin levels reached 202±52 $ng \cdot mL^{-1}$ after seven-days of supplementation and remained elevated during the five-day post-exercise recovery period. Surprisingly, no differences were observed between quercetin and PLA groups for any of the aforementioned markers. Similar to Connolly et al. (2006), Rowland (2018) observed that NZBC extract supplementation was unable to attenuate losses in elbow range of motion (ROM) following maximal concentric and eccentric elbow flexor contractions in recreationally active males and females. Additionally, NZBC extract was also unable to attenuate the force decline in MVIC, despite being able to significantly reduce the serum CK response at 96 h post-exercise compared to PLA. In contrast, pomegranate extract supplementation has been shown to mitigate isometric strength losses compared to PLA at 48 h following maximal eccentric elbow flexion contractions in recreationally active males (Trombold et al. 2010). However, the authors reported that serum markers of systemic inflammation and muscle damage were not different compared to PLA, therefore not providing any insight regarding potential mechanisms for how the

pomegranate extract may have exerted its effects. Similarly, in a follow-up study by the same group, pomegranate juice was also able to mitigate elbow flexion strength losses and increases in elbow muscle soreness compared to placebo during the 2- to 168 h period following repeated unilateral eccentric elbow flexion contractions (Trombold et al. 2011).

2.7 Half-marathon

Levers et al. (2016) assessed whether supplementing with Montmorency powdered tart cherries in a capsule form (CherryPURE®) for 10-days before a half-marathon race and up to 48 h post-race could affect markers of muscle damage in endurance-trained male and female runners or triathletes. It was observed that supplementation of Montmorency powdered tart cherries was able to attenuate markers of muscle catabolism, reduce immune and inflammatory stress, better maintain redox balance and increase performance (as measured by a 13% faster half-marathon performance finish time compared to PLA). In contrast, bilberry juice supplementation for five-days before a half-marathon, on race day, and for up to 48 h post-race, did not accelerate recovery according to the markers assessed (Lynn et al. 2018). In fact, the authors concluded that bilberry juice evoked small to moderate increases in exercise induced DOMS and systemic inflammation. However, these conclusions should be interpreted with caution due to methodological concerns with the study design. Which are that, firstly the authors did not report restricting participants from the use of any other recovery aids such as NSAIDS, which have been shown to attenuate strength losses, blood CK, inflammation and increases in muscle soreness following strenuous exercise (Morelli et al. 2017; Trappe et al. 2001). Furthermore, the authors used magnitude-based inferences (MBI) to analyse the effect of bilberry juice on EIMD responses. Whilst MBI has been suggested to be a useful statistical tool to support studies with suboptimal sample sizes (Hopkins and Batterham, 2016; Batterham and Hopkins, 2006), it has also been shown to inflate the chance of creating a Type 1 error (Sainani, 2018). Thus, as it remains unclear

whether a polyphenol supplement is able to facilitate recovery following a half-marathon event, Chapter 3 will address this issue by examining the effect of NZBC extract supplementation taken before and following running a half-marathon race on markers of EIMD.

2.8 Marathon

Similar to the findings of Levers et al. (2016), Howatson et al. (2010) found that supplementing with TMCJ for five-days before, on the day and for up to 48 h post a marathon, helped accelerate recovery possibly through systemic inflammation modulation and by increasing total antioxidant status in recreational male and female runners. In addition, isometric strength (MVIC) recovered significantly faster in the TMCJ group compared to the PLA. However, no differences were observed between groups for muscle soreness as assessed by a VAS scale. On the other hand, Clifford et al. (2016) showed that BTJ supplementation on the three-days following a marathon race did not attenuate losses in isometric strength or CMJ performance when compared to PLA in recreational male and female runners. Furthermore, in the same study, markers of systemic inflammation, CK and muscle soreness were all elevated following the marathon, but no group differences were observed between BTJ and PLA.

2.9 160-km endurance run

Nieman et al. (2007b) assessed whether 1000 $mg \cdot d^{-1}$ quercetin supplementation for three-weeks before a 160-km endurance run could influence systemic markers of inflammation and leukocyte cytokine messenger ribonucleic acid (mRNA) expression in male and female ultramarathoners. Similar to the findings of O'Fallon et al. (2012), although quercetin supplementation significantly increased plasma quercetin levels, it failed to attenuate muscle damage, increases in plasma inflammation and hormone levels and alterations in leukocyte cytokine mRNA expression.

2.10 Intensified repeated cycling bouts

Nieman et al. (2007a) assessed whether 1000 mg·d^{-1} quercetin supplementation three-weeks prior to and during a three-day period of intensified cycling training could attenuate cytokine mRNA expression in muscle and blood leukocytes and lower plasma cytokine levels in endurance trained male cyclists. The quercetin supplementation diminished post-exercise expression of leukocyte IL-8 and IL-10 mRNA, indicating that elevated plasma quercetin levels exerted some effects within the blood compartment. However, quercetin supplementation did not influence any of the muscle measures, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) content, cytokine mRNA, or cyclooxygenase-2 (COX-2) mRNA expression across a three-day intensified exercise period. Bell et al. (2014) investigated the impact of seven-days TMCJ supplementation on indices of EIMD such as oxidative stress, inflammation and muscle damage (inferred through CK measures) following a simulated, high-intensity, stochastic cycling trial in trained male cyclists. Measures of oxidative stress, inflammation and CK were collected at baseline, immediately pre- and post-exercise on days five, six and seven. It was observed that TMCJ was able to attenuate the oxidative markers and inflammatory response over the repeated cycling bouts compared to PLA. However, CK response was not different between groups, indicating that TMCJ was unable to mitigate the muscle damage response to the exercise compared to PLA.

2.11 Simulated cycling road race

Bell et al. (2015) examined whether eight-days of TMCJ supplementation could facilitate recovery following a simulated cycling road race in trained male cyclists. Functional performance (MVIC, cycling efficiency and 6 s peak cycling power) and muscle soreness were assessed at baseline, 24, 48 and 72 h post-exercise, whilst inflammation, oxidative stress and CK were assessed at 1, 3, 5, 24, 48 and 72 h post-exercise. There was no post-exercise decline in MVIC performance with TMCJ supplementation compared to PLA indicating a protective effect on muscle function. In addition, TMCJ did improve

cycling efficiency at 24 h and some systemic inflammatory markers were attenuated with TMCJ compared to PLA.

2.12 Metabolically demanding intensive ergometer row

Lyall et al. (2009) demonstrated that supplementing with a NZBC extract powder immediately prior to and immediately post a 30-minute indoor row at 80% $\dot{V}O_{2max}$, reduced plasma CK activity 24 h post-exercise and attenuated the transient rise in plasma oxidative generating capability and protein carbonyls in recreationally active males and females. However, ratings of perceived muscle soreness post-exercise were not attenuated by NZBC extract supplementation.

2.13 Summary of effects of polyphenols on EIMD

After reviewing the available literature on polyphenols and EIMD, it is clear that there are a range of exercise environments where polyphenols may serve to help facilitate exercise recovery and also some where there were no observable benefits of polyphenol supplementation. However, what can be suggested when reviewing the aforementioned experimental studies in sections 2.4 – 2.12, is that of the 16 studies which implemented a longer polyphenol supplementation strategy (e.g., more than on one single day), 12 demonstrated favourable recovery benefits following muscle damaging exercise (Bell et al. 2014; Bell et al. 2015; Bowtell et al. 2011; Clifford et al. 2015; Connolly et al. 2006; Howatson et al. 2010; Levers et al. 2016; McLeay et al. 2012; Nieman et al. 2007a; Rowland, 2018; Trombold et al. 2010; Trombold et al. 2011). Thus, highlighting that longer supplementation periods may prove beneficial in terms of improving exercise recovery. However, it is important to note that acute polyphenol supplementation strategies have also shown favourable benefits such as that of Lyall et al. (2009) with NZBC supplementation where plasma CK concentration was mitigated following a 30-min ergometer row at 80% $\dot{V}O_{2max}$. As the majority of studies utilise a longer supplementation period, further work is warranted to establish whether an acute

polyphenol supplementation strategy is the most optimal or one which is implemented over several days, before any definitive conclusions can be drawn. Nevertheless, in light of the available evidence to date, the NZBC supplementation strategies that will be utilised within this thesis will focus on longer supplementation periods (e.g., a total of 9- or 10-days for Chapters 3 and 5, respectively).

Furthermore, in terms of participant sex, it would appear that polyphenol supplementation efficacy in exercise recovery is most effective in males. However, this is likely due to the paucity of research evidence in females, which is a known problem within the domain of sport and exercise medicine (Costello, Bieuzen and Bleakley, 2014), where only nine out of 17 studies included in this review included female participants. Nonetheless, of those nine, five demonstrated favourable recovery benefits in the female participants following muscle damaging exercise compared to the placebo group (Howatson et al. 2010; Levers et al. 2016; Lyall et al. 2009; McLeay et al. 2012; Rowland, 2018). It is beyond the scope of this review to explore the discrepancies between the sexes in terms of polyphenol supplementation and recovery from EIMD and instead, the reader is refereed to previous reviews in the area (Köhne, Ormsbee and McKune, 2016; Tiidus, 2000). Interestingly, both of the NZBC extract supplementation studies highlighted within Table 2.1 (Lyall et al. 2009; Rowland, 2018) utilised both male and female participants and positive recovery benefits were observed following strenuous exercise. Thus, both male and female participants will be included within Chapters 3, 4 and 7 of this thesis, to build on the available evidence to date with NZBC extract supplementation as an efficacious recovery strategy for females as well as males.

Lastly, out of all of the aforementioned studies discussed in Table 2.1, the polyphenol supplement, which has been observed to provide the most consistent benefit on exercise recovery from strenuous and/or muscle damaging exercise is TMCJ (Bell et al. 2014; Bell et al. 2015; Bowtell et al. 2011; Connolly et al. 2006; Howatson et al. 2010; Levers et al. 2006) where at least one of the markers of exercise recovery has been improved

compared to the placebo group. Second to TMCJ supplementation is pomegranate (Trombold et al. 2010; Trombold et al. 2011) and NZBC (Lyall et al. 2009; Rowland, 2018) supplementation, which both demonstrated positive recovery benefits in the respective studies discussed. Although there has been substantially more research conducted with TMCJ as a recovery aid following strenuous exercise compared to pomegranate and NZBC, it is promising to see that of the studies that have been conducted, both pomegranate and NZBC have demonstrated efficacy as recovery aids following strenuous exercise. Whilst the main form of polyphenol found within pomegranate is ellagitannins (Trombold et al. 2010), both TMCJ and NZBC are abundant in anthocyanins, which have been suggested to be the key polyphenols associated with the purported physiological benefits (Cook and Willems, 2018). Given that the anthocyanin profile of TMCJ and NZBC is not too dissimilar, with both containing cyanidin-3-glucoside albeit in varying proportions (Slimestad and Solheim, 2002; Vitaglione et al. 2007), it is reasonable to propose that the available *in vivo* evidence points toward NZBC being an efficacious supplementation strategy to facilitate recovery from EIMD. Thus, Chapters 3 and 5 will build on the previous research carried out and examine whether NZBC extract supplementation is able to facilitate recovery following two different muscle damaging exercise protocols (half-marathon and 100-DJP, respectively).

However, it is important to consider when attempting to draw comparisons about the efficacy of polyphenol supplementation across studies, that a number of reasons may cause disparity in the experimental findings. Some of the potential reasons are, but not limited to, participant sex (males vs. females), training status (resistance trained vs. non-resistance trained), supplementation protocol (duration, dose and frequency of supplementation), sample size (whether there was sufficient statistical power within the study to observe meaningful changes), baseline polyphenol status (high habitual consumer vs low habitual consumer of the polyphenol of interest), timing around

experimental measures (follow up time points after EIMD protocol) and the markers of EIMD used to observe polyphenol supplementation efficacy.

With regard to the last of these, it is clear a number of markers are often used to quantify the presence and magnitude of EIMD whilst supplementing with polyphenols, but it is debatable whether these measurement tools are appropriate (Warren et al. 1999; Owens et al. 2018; Cobley et al. 2017; Baird et al. 2012; Damas et al. 2016; Clarkson and Hubal, 2002). These markers are typically categorised as 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 blood, inflammation and oxidative stress (Duffield et al. 2008; Paulsen et al. 2012; Cobley et al. 2017). Most often, a combination of direct and indirect markers are used to quantify the magnitude of EIMD response with polyphenol supplementation, perhaps due to the ease of obtaining them as opposed to histological markers that are typically more invasive or time consuming to gather (Clarkson and Hubal, 2002; Paulsen et al. 2012). However, direct markers of EIMD such as muscle force production are considered the gold-standard measure to quantify the effect of and recovery from EIMD (Warren et al. 1999; Damas et al. 2016). Within the literature, the terms indirect and direct are used interchangeably, thus for the purpose of reader clarity, when the terms indirect and direct are used within this thesis, the following definitions will be used:

- Indirect – e.g. alterations in systemic markers of inflammation, oxidative stress and intramuscular proteins as measured through blood samples (CK)
- Direct – e.g. changes in muscle function, soreness and collecting and analysing sections of muscle tissue

2.14 Indirect markers of EIMD

Systemic markers of intramuscular protein secretion as markers of membrane damage are often reported within the literature such as lactate dehydrogenase, aspartate

aminotransferase, carbonic anhydrase isoenzyme-2 and CK. Measures of CK are often reported more frequently than others perhaps due the magnitude of increase being so great relative to other proteins after strenuous exercise, and the cost of the assay is comparatively modest (Clarkson and Hubal, 2002; Baird et al. 2012). There are three isoforms of CK that can be measured in the plasma, which originate from skeletal muscle (CK-MM), brain (CK-BB) and cardiac (CK-MB) tissues. It is important to highlight that often when CK is measured following an EIMD protocol, the CK isoform is not specifically measured due to the assumption that CK increase following an EIMD exercise is likely derived from the injured skeletal muscle tissue as opposed to CK-BB or CK-MB (Baird et al. 2011). However, one of the main issues with exercise induced CK efflux is that it is highly variable among individuals (Clarkson and Ebbeling, 1988; Nosaka et al. 2002). The reason for the variability is poorly understood, and it is possible that training status, gender, genetics and fibre type distribution, as well as the exercise task being carried out play a role (Baird et al. 2012). Furthermore, concentration of CK-MM in the blood has been shown to correlate well with decrements in muscle function following EIMD (Warren at al. 1999) and the high inter-subject variability associated with CK, even amongst a relatively homogenous cohort, makes accurate interpretations problematic (Paulsen et al. 2012). Similarly, examining exercise-induced inflammation with measures of change in limb-girth due to local oedema (Warren et al. 1999) and/or by directly measuring specific systemic inflammatory markers such as cytokines, leukocytes and acute phase proteins in the blood can lead to equivocal findings (Paulsen et al. 2012). Cytokines are frequently chosen to assess the inflammatory cascade, particularly IL-6, which could be due to the fact that it is secreted in greater volumes than other cytokines, especially during, and in the immediate hours after exercise (Peake et al. 2015). However, interpretation of results with cytokines can be troublesome due to their apparent dual roles in the biological processes with macrophage and fibroblast activation and, therefore, muscle regeneration after EIMD (Del Giudice and Gangestad, 2018; Tidball and Villalta 2010; Paulsen et al. 2012). Therefore, making it problematic to discern

whether they are a cause or merely a consequence of muscle damage. Lastly, as exercise can induce a transient state of cellular oxidative stress and oxidative stress may be harmful and contribute to the secondary damage cascade, it is frequently assessed in EIMD studies (Margaritelis et al. 2015; Nikolaidis et al. 2012). However, it is important to note that there is not currently an accepted 'gold standard' method or single biomarker that accurately reflects the oxidative state of an individual (Close et al. 2004; Powers et al. 2010). As such use of a single biomarker is discouraged and a variety of different methods that include different cellular targets such as lipids and proteins should be used to estimate the state of oxidative stress in an individual (Close et al. 2005; Powers et al. 2010; Cobley et al. 2017). Furthermore, quantifying oxidative stress is inherently challenging given 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/s used, valid and reliable results are difficult to obtain (Fisher-Wellman and Bloomer, 2009; Powers et al. 2010). Akin to the proposed dual role of inflammation in muscle damage, oxidative stress has been shown to be fundamental in exercise adaptations (Margaritelis et al. 2016) and thus, an increase in reactive oxygen nitrogen species (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 and associated with a variety of morphological alterations (Close et al. 2004; Nikolaidis et al. 2008).

2.15 Direct markers of EIMD

The muscle function measure that is most widely accepted as the best measure for evaluating muscle damage following strenuous exercise is one that describes the power or force generating capacity of skeletal muscle (Warren et al. 1999; Damas et al. 2016; Paulsen et al. 2012). One of the key advantages of this measure over other EIMD markers, is that muscle function is the most relevant to athletes and those in arduous occupations whose principal goal during recovery is to restore optimal muscular

performance as quickly as possible (Byrne et al. 2004; Cockburn et al. 2010). Maximal voluntary contractions (MVC) are one of the most widely used measures to assess muscle function (Warren et al. 1999). Further, when participants are stratified based upon the magnitude of their MVC decline post-exercise, it greatly increases the precision in quantifying changes in EIMD by proxy markers such as muscle soreness, ROM and CK efflux (Damas et al. 2016). Damas et al. (2016) concluded that based on this stratification process, it indicates that the most commonly used markers of EIMD are valid and MVC orchestrates their responses, consolidating the role of MVC as the best EIMD direct marker. Maximal voluntary contractions are typically performed as isometric, concentric and/or eccentric actions. Isometric muscle contractions appear to be routinely used and following eccentrically biased exercise they have been shown to significantly decrease immediately, 24, 48 and 72 h post-exercise (Warren et al. 1999; Paulsen et al. 2012). However, the use of MVC as a marker of muscle function does have its limitations. Given that MVC 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. sprinting, jumping, squatting) that require several muscle groups to work synergistically (Gathercole et al. 2015; Komi, 2000). Therefore, it is recommended that in order to obtain a more complete picture of the magnitude and time course of EIMD and whether polyphenol supplementation plays a role in the recovery of EIMD, changes in both isometric and dynamic movements should be evaluated.

A dynamic movement concept that has been suggested as a good model to investigate normal and damage muscle is that of the stretch-shortening cycle (SSC) as it is involved in many sporting movements, including running and jumping (Komi, 2000). Vertical jumps such as countermovement and drop jumps are commonly used within research looking to assess the magnitude of EIMD on the SSC (Byrne and Eston, 2002b). Byrne and Eston (2002a) reported that CMJ, drop-jump and static jump performance are all affected by EIMD, although static jumps appear to be affected to a greater extent than

CMJ or drop jumps, possibly due to the lack of an SSC component. It has been suggested that the SSC used during both the CMJ and drop jumps may provide potentiating mechanisms that attenuate the detrimental effects of EIMD, thus, attenuating performance decrements (Byrne and Eston, 2002a). However, static jumps are not necessarily representative of real-world sporting scenarios as they are considered a pure measure of concentric muscle performance as they have the SSC component removed (Byrne and Eston, 2002b). Taken together, as dynamic movements, such as CMJ, incorporate the SSC and are used in real-world settings (i.e. a jumping in basketball) and isolated muscle contractions (such as MVC) are rarely used, using a panel of functional markers in conjunction with one another should be recommended to assess the applicability of the experimental findings to a real-world scenario.

From a muscle function perspective, measures of self-perceived muscle soreness or muscle pain (used interchangeably throughout the literature) are frequently used to assess EIMD magnitude (Warren et al. 1999). This is typically assessed on a VAS when the individual is asked to perform a movement that is likely to allow them to feel soreness (i.e. 90^{o} static squat, elbow flexion, palpation of muscle belly, pain pressure threshold test [PPT]). The concept of measuring muscle soreness is based on the observation that often, following an EIMD bout, individual's report feeling increased levels of soreness between 24 to 72 h post-exercise and not immediately post (e.g. DOMs) (Clarkson and Hubal, 2002). However, as with other markers of EIMD, there is substantial variability amongst individuals and often, irrespective of how it is measured, its intensity and time course does not necessarily correlate well with changes in histological muscle damage or other indirect markers of muscle function, CK efflux or inflammation (Hyldahl and Hubal, 2014; Nosaka et al. 2002; Crameri et al. 2007). Therefore, it has been recommended to measure muscle soreness in conjunction with other EIMD markers to quantify the magnitude of muscle damage (Paulsen et al. 2012). Nevertheless, it has

been observed that muscle soreness may heighten an individual's risk of injury due to pain-related alternations in movement patterns during activity (Smith, 1992), and influence the degree of effort exerted (Fletcher et al. 2016). Thus, evaluating muscle soreness is still considered a useful tool for assessing the efficacy of a polyphenol supplement on recovery and an individual's preparedness in the days after exercise (Ranchordas et al. 2017).

Despite the complexity with some of the aforementioned systemic markers of EIMD, having a selection of OS, inflammation and muscle damage biomarkers coupled with muscle function measures such as MVC and CMJ should allow for potential mechanistic insights into how a polyphenol supplementation may be exerting its physiological effects (Gathercole et al. 2015; Howatson and van Someren, 2008; Owens et al. 2018; Powers et al. 2010). Lastly, it is beyond the scope of this review to describe and critique all of the available indirect and direct markers of EIMD in-depth, but further information is available elsewhere (Duffield et al. 2008; Paulsen et al. 2012; Cobley et al. 2017).

2.16 Potential underpinning mechanisms of the effects of dietary polyphenols on exercise-induced muscle damage

From a mechanistic perspective, precisely how polyphenols exert their effects is unclear. However, Myburgh (2014) suggests that the potential rationale for examining dietary polyphenol supplementation on exercise performance and/or recovery is based on one or more of the following:

1. Mitochondrial adenosine triphosphate production is not 100% efficient, so that free radical species are formed in increased quantities during exercise. The more oxygen is utilised during exercise, the more free radical species are formed that would need to be quenched.
2. Exercise induced muscle damage results in excess free radical production during the secondary cascade, and this hinders adequate recovery.

3. The endogenous antioxidant defence mechanisms for the removal of the excess free radical species are insufficient and antioxidant/polyphenol supplements should mitigate the deletrious consequences of excess accumulation within the damaged tissue/s by either quenching them or by upregulating the endogenous antioxidant defence system itself.

On the other hand, it has been suggested that polyphenols play a role with alterations in specific cardiovascular and vasoactive parameters such as cardiac output, stroke volume and decreased peripheral resistance (Cook et al. 2017a) and influence of vascular smooth cell migration *in vitro* (Keane et al. 2016) and that this is how they may exert their effects. With this in mind, two potential mechanistic pathways have been suggested in the literature for how polyphenols may exert their physiological benefit *in vivo* during and/or following EIMD: (1) free radical scavenging and (2) improvements in blood flow through increasing availability of nitric oxide or affecting vascular smooth muscle cells.

Firstly, *in vitro,* polyphenols have been shown to react with free radicals (e.g. superoxide anion, peroxyl radical, aloxyl radical and hydroxyl radical) to yield polyphenol radicals (Rahman et al. 2006). It has been suggested that perhaps the polyphenols are able to scavenge free radicals, such as RNOS, that are produced in response to muscle damaging exercise (Powers and Jackson, 2008; Myburgh, 2014). While polyphenols generally have favourable kinetics, whether they accumulate in sufficient amounts to scavenge free radicals is debateable (not to mention whether scavenging free radicals is even desirable in the view of hormesis) (Owens et al. 2018).

An example that has been put forward previously is that of the free radical copper zinc superoxide dismutase (CuZnSOD) (Owens et al. 2018). The free radical CuZnSOD reacts rapidly with superoxide anion and is present in tissues at ~20 μM (Halliwell and

Gutteridge, 2015). Plasma concentrations of “free” polyphenols rarely exceed 1 μM, so even assuming a tissue concentration of 1 μM CuZnSOD still outcompetes polyphenols, which questions the plausibility of scavenging mechanisms (Schaffer and Halliwell, 2012). It is unlikely that tissue polyphenol concentrations occur above the nanomolar range (Forman et al. 2014; Schaffer and Halliwell, 2012). A situation supported by the fact that polyphenol metabolism via methylation, sulphation and glucuronidation hinders their activity towards free radicals (Goszcz et al. 2017; Halliwell and Gutteridge, 2015).

Further, it is unlikely that polyphenols accumulate at sites of free radical generation in an EIMD setting because inflammatory cell infiltrates release superoxide anion and other reactive species into the phagosome (Winterbourn et al. 2016), which imposes a spatial restriction. For these reasons, Owens et al. (2018) concluded that they disfavour polyphenol scavenging as a mechanism, instead, favouring the hypothesis that small amounts of polyphenols are metabolised to electrophiles (e.g. quinones), that then activate the cyto-protective endogenous antioxidant response via nuclear factor erythroid 2-related factor 2 (Nrf-2) signalling (Forman et al. 2014; Goszcz et al 2017). The Nrf-2 pathway is a master regulator of the antioxidant response through the regulation of a wide range of antioxidant and phase II detoxification genes and protects cells from stressors including ROS (Bowtell and Kelly, 2019). As reviewed in Forman et al. (2014), electrophiles can activate Nrf-2 signalling by conjugating reactive cysteine residues within kelch-like ECH associated protein 1 (KEAP-1), an inhibitory protein responsible for sequestering Nrf-2 in the cytoplasm, via Michael addition. Previous research has demonstrated how polyphenol supplementation can increase the endogenous antioxidant system capacity via signalling through Nrf-2 and ARE pathways in a similar fashion to exercise adaptation (Huang, Li, Su and Kong, 2015; Ji, 2008). Thus, it is plausible to suggest that if polyphenols are able to upregulate the endogenous antioxidant capacity via their conversion to electrophiles and subsequently activate the Nrf-2/antioxidant response element (ARE) pathway, in turn they would be able to

indirectly reduce the amount of ROS produced during the secondary phase of the EIMD cascade, which may enhance exercise recovery. However, more mechanistic *in vivo* research with polyphenol supplementation is warranted to shed light on parent polyphenol and their metabolite's effects on the Nrf-2/ARE pathway. For further comprehension of the proposed mechanism, readers are referred to other reviews (Cobley et al. 2015a; Cobley et al. 2015b; Margaritelis et al. 2015, 2016; Forman et al. 2014).

As alluded to previously, whether free radical scavenging is desirable or not from the perspective of exercise adaptations, can be conceptualised through the proposed concept of hormesis (Peake et al. 2015; Mattson, 2008). Within the concept of hormesis, it is 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. a bell-shaped curve response) (Nikolaidis et al. 2012; Peake et al. 2015). Applying the theory to EIMD and polyphenol supplementation, it could be suggested that an increase in RNOS could be an important driver of acute or chronic molecular adaptations but, excessive or chronic production could lead to an impaired adaptive response (Peake et al. 2015; Martin and Appel, 2010).

Figure 2.4 highlights the region (yellow) where it has been proposed a dietary polyphenol intervention could aid recovery from excessive exercise stress before the stress leads to impaired exercise adaptations.

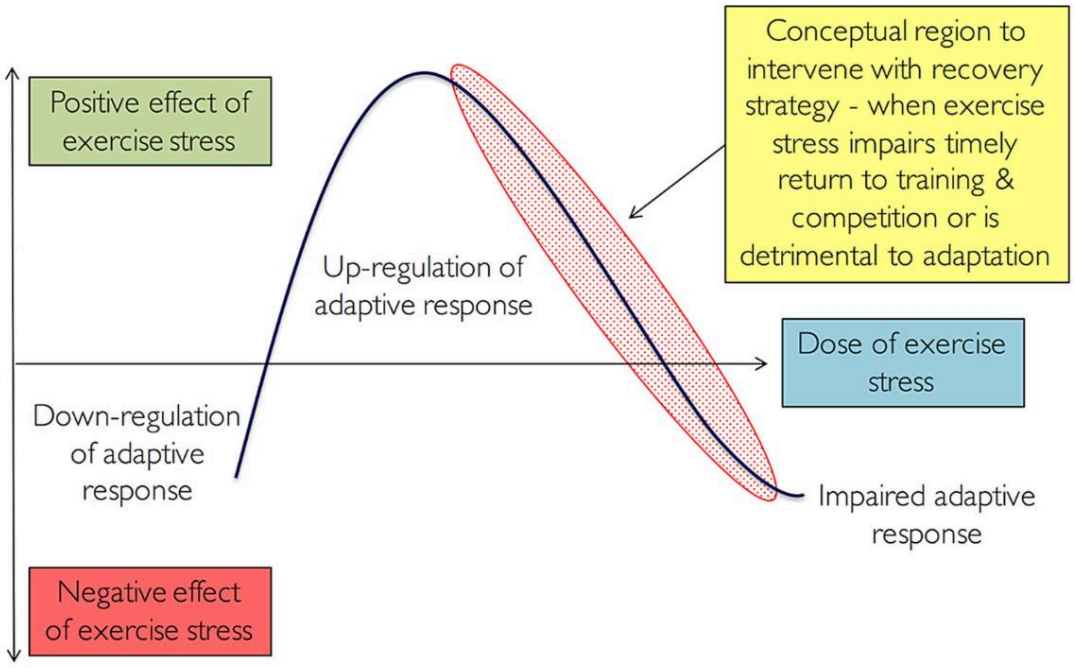


Figure 2.4. Adapted model of hormesis in view of EIMD. Yellow box highlights the conceptual region where polyphenol supplementation may play a key role in exercise recovery (Owens et al. 2015; Peake et al. 2015b).

Secondly, as muscle blood flow is of critical importance in oxygen delivery for muscle metabolism, an impairment of blood flow will likely hasten the onset of fatigue (Cook et al. 2017b). A nutritional intervention that can offset any decline in blood flow will likely be of interest for athletes and those in arduous occupations given that the greater muscular fatigue experienced by an individual, the more susceptible they may be to injury during heightened training periods (Jones et al. 2017).

It has been previously documented that flavonoid polyphenols can increase the activity of endothelial nitric oxide synthase (eNOS) in animal and cell studies (de Nigris et al. 2007; Edirisinghe et al. 2013; Nicholson et al. 2010). Endothelial nitric oxide synthase (eNOS) is responsible for the generation of the vasodilator nitric oxide (NO) in the vascular endothelium (Epstein, Moncada and Higgs, 1993). Previous *in vitro* studies

have shown that incubation of bovine arterial cells with the anthocyanin, cyanidin-3-glucoside, increased eNOS expression in a dose-dependent manner (Xu et al. 2004a). In rats, Rodriguez-Mateos et al. (2013) demonstrated that increases in circulating anthocyanins from blueberry juice consumption were associated with dose-dependent increases in flow-mediated dilation (FMD), a marker of endothelial dysfunction, with up to 310 mg of blueberry anthocyanins. Furthermore, Ziberna et al. (2013) demonstrated how bilitranslocase, a plasma membrane flavonoid transporter expressed in the vascular endothelium, mediates a critical step in endothelium-dependent vasodilation and relaxation induced by anthocyanins. Importantly, when this transporter was challenged with specific antibodies, reduced vasodilation activity was observed. Whilst the same responses have yet to be replicated in human trials, it is possible that polyphenols (or their metabolites) are able to influence vasodilation and relaxation, by increasing production of NO (Xu et al. 2004a; Xu et al. 2004b). Several human trials have demonstrated how various polyphenol supplementation strategies have been able to improve surrogate markers of blood flow and vascular function (Cook et al. 2017b; Fryer et al. 2020; Keane et al. 2016; Matsumoto et al. 2005; Rodriguez-Mateos et al. 2016).

Matsumoto et al. (2005) was one of the first to demonstrate a potential benefit of polyphenol supplementation on blood flow to potentially enhance typing work. The authors demonstrated that 2 h after blackcurrant ingestion, forearm blood flow was significantly increased relative to pre-values compared to PLA. A 1.22±0.13-fold increase was observed with blackcurrant ingestion in comparison to a 0.83±0.06-fold increase for placebo from the baseline values after releasing a venous occlusion from a brachial cuff. However, there were no differences in forearm blood flow compared to placebo at 1, 3, or 4 h following ingestion. Furthermore, no observed effect was seen for forearm oxygen consumption between conditions despite the increased flow at 2 h. A second study by the same group, where both male and female participants consumed blackcurrant concentrate capsules at a dose of 7.7 $mg \cdot kg \cdot bm^{-1}$ or PLA before completing a typing

work task, observed that blackcurrant intake prevented the decrease in oxygenated haemoglobin significantly compared to PLA as measured through Near-Infrared Spectroscopy (NIRS) (Matsumoto et al. 2005). It must be acknowledged that whilst NIRS provides feedback regarding tissue oxygenation, it is a surrogate marker for peripheral blood flow. Furthermore, Rodriguez-Mateos et al. (2016) demonstrated how cranberry juice supplementation can cause dose-dependent increases in FMD at 1, 2, 4, 6 and 8 h with a peak at 4 h. Furthermore, the authors observed a total of 60 downstream metabolites in plasma after cranberry juice consumption, with only 12 metabolites significantly correlating to the increases observed in FMD. Cook et al. (2017b) found that seven-days of NZBC extract supplementation (600 $mg \cdot day^{-1}$) was able to increase femoral artery diameter during a submaximal (30% MVIC) 120-s sustained isometric contraction of the *m.quadriceps femoris.* In addition, systolic and diastolic blood pressure, mean arterial blood pressure and total peripheral resistance with a concomitant increase in cardiac output and stroke volume were also observed. In contrast, Fryer et al. (2020) found that seven-days NZBC extract supplementation (600 $mg \cdot day^{-1}$) did not alter brachial artery blood flow despite the observed increase in forearm muscle oxygenation as measured through NIRS in male intermediate-level rock climbers. Keane et al. (2016) found that in pre-hypertensive males, 60 mL of TMCJ was able to reduce systolic blood pressure by 7±2 mmHg 2 h following intake. Interestingly, this occurred at the same time point as peak increases in the downstream metabolites of anthocyanin, protocatechuic acid (PCA) and vanillic acid (VA) within plasma. In the same study, Keane et al. (2016) observed no effect on plasma nitrate and nitrite (a proxy measure of NO production), suggesting the metabolites are responsible for the observed vascular responses. Additionally, Keane et al. (2016) also showed that in response to a combination of the metabolites PCA and VA, human vascular smooth cells migrated further *in vitro* compared to isolated incubation. As polyphenols, particularly anthocyanin, are reported to have low bioavailability (McGhie and Walton, 2007; Scalbert and Williamson, 2000), the bioactivity is likely mediated by the metabolites, that exist within

the circulation at much higher concentrations (Czank et al. 2013; de Ferrars et al. 2014). As reported by Rodriguez-Mateos et al. (2016), the increase in FMD 1 h post cranberry juice consumption occurred alongside a peak in the anthocyanin metabolites, ferulic acid, vanillic acid, isoferulic acid, 2-hydroxybenzoic acid and caffeic acid. However, Czank et al. (2013) reported 24 metabolites from the anthocyanin cyanidin-3-glucoside in human serum, which may suggest that many metabolites have yet to be examined for cardiovascular bioactivity. Despite this, Edwards et al. (2015) highlighted that the anthocyanin cyanidin-3-glucoside could increase eNOS expression, whereas the metabolites PCA and VA could not. Therefore, whilst it was observed the parent anthocyanin can increase eNOS expression, metabolism of the parent anthocyanin into its downstream metabolites loses this effect. However, the metabolites can maintain vascular homeostasis possibly by increasing NO as previously shown (Xu et al. 2004a; Xu et al. 2004b).

Based on the available evidence to date, the most plausible mechanism(s) for polyphenol action on increasing blood flow having a positive effect on the rate of recovery from EIMD is via two possible pathways.

Firstly, by a reduced production of ROS during the secondary phase of the EIMD cascade where reduced exposure to ROS within the damaged tissue may improve the bioavailability of the vasodilator, NO, due to a reduced production of peroxynitrite via the reaction of superoxide and NO (Bowtell and Kelly, 2019), in combination with the aforementioned effects of polyphenols on eNOS activity. Both *in vivo* and *in vitro* evidence have demonstrated that phenolic metabolites, such as PCA, can reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, which is one of the key sources of superoxide production during exercise (Maraldi, 2013). Consequently, one could speculate that reduced ROS production would also lead to a mitigated inflammatory cascade, which in turn could accelerate the rate of recovery from EIMD.

Second, it has been suggested that eccentric exercise and/or myocyte swelling and altered intramuscular pressures can inflict damage to the microcirculation (e.g., myocyte capillary network) (Crenshaw et al. 1994; Kano et al. 2005), which could then contribute to localised oedema and compromised blood flow, thus exacerbating the magnitude of EIMD (Jones and Round, 1997). It has been previously shown how following an ultramarathon race, where muscle soreness was present and evidence of ultrastructural fibre damage during the muscle biopsy analysis was observed, that the majority of the capillaries within the biopsied myocytes exhibited disturbed endothelial cells (Crenshaw et al. 1994). Furthermore, Kano et al. (2005) demonstrated that downhill running, impaired both muscle microcirculatory flow and also the balance between oxygen delivery and oxygen utilisation at the onset of contractions as evidenced by the accelerated fall of microvascular oxygen pressure within the spinotrapezius muscle in rats. The authors suggested that a consequence of the lowered oxygen pressure during the first 20 – 40 s of the muscle contractions would be an impaired blood myocyte oxygen diffusion. Indeed, these findings complement and may help explain the extensive and prolonged structural damage and impaired muscle function that often follow a single bout of eccentric exercise. Thus, as polyphenols have been shown to enhance muscle oxygenation (Fryer et al. 2020) and upregulate eNOS activity (de Nigris et al. 2007; Edirisinghe et al. 2013; Nicholson et al. 2010), it could be speculated that polyphenols may be able to hasten recovery from EIMD by mitigating the effects of the muscle damaging exercise on the microvascular networks within skeletal muscle by maintaining a degree of vascular homeostasis. However, it is clear that more conclusive *in vivo* research is needed to establish exactly how polyphenols are able to support cardiovascular function parameters, whether the parent polyphenols are responsible or the downstream metabolites and in turn how this specifically affects the rate of recovery from EIMD.

In summary, the information from *in vivo* work presented above demonstrates that NZBC increases blood flow during and following exercise (Cook et al. 2017a; Cook et al. 2017b; Fryer et al. 2020; Matsumoto et al. 2005). The *in vitro* work suggests that NZBC extracts mechanisms of action may act via, 1) upregulation of the endogenous antioxidant capacity through the metabolites conversion to electrophiles, which subsequently activate the Nrf-2/antioxidant response element (ARE) pathway and/or, 2) by increasing the activity of eNOS and subsequently the generation NO leading to greater vasodilation within the endothelium and/or 3) by improving muscle oxygenation and in turn a degree of vascular homeostasis within the microcirculation. Thus, in theory it is plausible to suggest that if NZBC metabolites were able to upregulate the Nrf-2/antioxidant response element (ARE) pathway and in turn, indirectly reduce the amount of ROS produced during the secondary phase of the EIMD cascade, combined with the increase in eNOS activity leading to greater vasodilation and muscle oxygenation, the metabolites could then indirectly further dampen the amount and/or reduce the exposure of ROS infiltrating the damaged tissue following EIMD. Subsequently, this may facilitate an improved recovery following muscle damaging exercise. However, this is purely speculation based on the applied *in vivo* and *in vitro* work that has been completed to date. Future research is warranted using *ex vivo* and *in vivo* models being used, along with the applied work with NZBC supplementation to shed light on the exact mechanisms of action.

2.17 Individual variation in responses to polyphenols and exercise-induced muscle damage

One area that needs careful consideration when evaluating the potential effectiveness of polyphenols as recovery strategies from EIMD, is the inter-individual responses apparent within the literature. The rationale for differing inter-individual responses have previously been associated with, but not limited to, previous maximal or submaximal exercises (Chen et al. 2012), training status (Newton et al. 2008), bioavailability of the polyphenol of interest (Koli et al. 2010; Manach et al. 2005; de Ferrars et al. 2014), gut

microbiota status (Tomas-Barberan et al. 2018; Dueñas et al. 2015), effort exerted during the exercise (Sayers et al. 2003; Chen et al. 2012), female contraceptive use (Hicks et al. 2017) and genotype (Baumert et al. 2016). It is beyond the scope of this review to cover each in detail, but those that are thought to be most influential with the magnitude of EIMD symptoms experienced and polyphenol supplementation are briefly discussed below.

2.18 Training status

Establishing an individual's training status and experience can be troublesome, but previously it has been suggested to define this based on years in sport participation, the level of competition, and the frequency of individual training sessions and duration (Jeukendrup, Craig and Hawley, 2000). This is an important consideration in study design given that it has previously been suggested that resistance training provides a unique stimulus to the neuromuscular system culminating in neural, muscular and connective tissue adaptations (Carroll, Riek and Carson, 2002; Jones, Rutherford and Parker, 1989). Consequently, when a chronically resistance trained individual is then exposed to an eccentrically strenuous exercise task, they exhibit a smaller magnitude of change in both direct and indirect markers of EIMD compared to their untrained counterparts (Newton et al. 2008). However, Newton et al (2008) highlighted that muscle soreness was not rated different between trained and untrained in this study, but it is widely accepted that soreness often demonstrates poor correlations with changes in muscle function following eccentric exercise (Warren et al. 1999). Nevertheless, the magnitude of EIMD response to the same exercise stimulus, even amongst individuals of similar training status, can vary widely (Hubal, Rubenstein and Scott, 2007; Sayers and Clarkson, 2001). Intuitively, this could also impact how an individual might respond to a recovery intervention, such as with polyphenol supplementation. 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.

2.19 Genotype

Intra-study EIMD response variation within a homogenous cohort warrants further consideration, with some evidence suggesting that genetic variability may play a role in the response to EIMD and polyphenol supplementation (Baumert et al. 2016). Some genes have common variations in sequence, known as polymorphisms, which, depending on where this polymorphism occurs within the gene, can directly affect gene expression and ultimately the amount of protein produced (Baumert et al. 2016). The most common type of sequence variation is a SNP, where one nucleotide substitutes another. Another type of common variation is the insertion/deletion (indel) polymorphism, in which a specific nucleotide sequence is present (insertion) or absent (deletion) from the allele [such as the angiotensin-I converting enzyme (ACE)] (Valdivieso et al. 2017). Some polymorphisms can modify the protein product, thus potentially altering function (Del Coso et al. 2018; Stebbings et al. 2017). Thus, polymorphisms of genes encoding key proteins in the muscle-tendon unit (such as the alpha-actinin-3 [ACTN3] R577X SNP) may have implications for the ability to recover from strenuous exercise, potentially influencing injury risk (Del Coso et al. 2018; Clos et al. 2019; Maffulli et al. 2013). Furthermore, specific gene polymorphisms (e.g., collagen type 1 alpha 1 gene [COL5A1] rs12722) have been associated with tendon/ligament injury prevalence (e.g. Achilles tendinopathy/rupture and anterior cruciate ligament rupture) (Bell et al. 2012; September et al. 2009; September et al. 2016). However, very little is known about the potential genetic association with muscle damage and muscle regeneration in response to muscle damaging exercise, either in young or older people, male or female, or the mechanisms that underpin that association.

Furthermore, it has been previously observed that some polyphenols can increase the activity of endothelial nitric oxide synthase (eNOS) in animal and cell studies (Edirisinghe et al. 2011; Nicholson et al. 2008). Endothelial nitric oxide synthase (eNOS) is responsible for the generation of the vasodilator nitric oxide (NO) in the vascular

endothelium (Maxwell, 2002). Acute and chronic studies with polyphenol supplementation have shown considerable heterogeneity in vascular responses (e.g. Matsumoto et al. 2005; Cook et al. 2017). A potential mechanism for this inter-individual variability may be a polymorphism in the eNOS gene, where a modification in its coding sequence occurs when guanine (G) is substituted for thymine (T) at position 894 in exon 7, resulting in the replacement of glutamic acid for aspartic acid at codon 298 (Glu298Asp) (Leeson et al. 2002). Consequently, individuals are identified as either homozygous for guanine (GG, wild type), thymine (TT), or heterozygous (GT). The frequency of the ASP 298 allele is approximately 33% in Caucasian populations (Leeson et al. 2002). Previously, George et al. (2012) observed that this SNP differentially affects the endothelium-dependent vasodilation response within the forearm (measured by Laser Doppler with iontophoresis) to a fruit and vegetable puree drink (FVPD) in healthy males and females. A nutrient-gene interaction was observed with the GG group showing a significant increase in endothelium-dependent vasodilation after 180 min post consumption of a FVPD compared to the GT individuals. It is possible then that observable inter-individual variability in physiological responses to different polyphenol supplements may partially be due to individuals being heterozygous for the Glu298Asp SNP. Therefore, Chapters 4 and 6 will attempt to address this gap in the literature by examining the association between the SNPs ACTN3, ACE and titin (TTN) and the responses to EIMD induced by a half-marathon event and 100-DJP, respectively, with NZBC or placebo supplementation.

2.20 Bioavailability and plasma uptake of polyphenols

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 circulation, in sufficient quantities to be utilized by cells (Toutain and Bousquet-Melou, 2004). 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 and 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). With this in mind, it is often reported that polyphenols have poor bioavailability and that it is the downstream metabolites (phase 1 metabolism), that are more likely the cause of the associated *in vivo* observational health benefits (Keane et al. 2016; de Ferrars et al. 2014; Williamson and Clifford, 2010). Therefore, when interpreting the results from studies examining the effect of polyphenol supplementation on recovery aetiology, it is important to consider that it is unlikely that the food ingested is responsible for any physiological outcome, and that it is more likely to be a resultant downstream metabolite product of polyphenol metabolism (Williamson and Clifford, 2010). Furthermore, it has been recently shown that baseline antioxidant status can be a determinant in the effectiveness of supplementing with antioxidants (i.e. vitamin C and E). For example, individuals with a low baseline status of vitamin C (Paschalis et al. 2016) and glutathione (Paschalis et al. 2018) improved their $\dot{V}O_{2max}$ following supplementation with vitamin C and N-acetylcysteine, respectively, however those with higher baseline levels did not respond. It is possible that a similar event occurs when utilising polyphenol supplementation and that an individual with a low baseline status of a given polyphenol metabolite (i.e. PCA, a key metabolite of anthocyanins) (de Ferrars et al 2014) may experience a greater magnitude of a given physiological response compared to an individual with a higher baseline status. Therefore, highlighting the potential benefit to stratify participants into supplementation groups based upon their baseline levels of the polyphenol metabolite of interest.

To date, no study, which has utilised a NZBC extract supplementation strategy has provided data regarding the plasma uptake of the phenolic acids of anthocyanins at rest

or alongside exercise. Understanding the plasma uptake of both acute and chronic intakes of NZBC extract would better inform optimal dosing strategies and intake guidelines and move dosing protocols towards a more rigorous pharmacokinetic approach. Furthermore, most plasma uptake studies incorporate dietary polyphenol restriction in their design in an attempt to reduce the background noise that may be introduced by variation in dietary polyphenol intake (Matsumoto et al. 2001; Keane et al. 2016). However, this approach may also maximise the effects produced by polyphenol supplementation (Bowtell and Kelly, 2019). Therefore, it has been suggested to ensure ecological validity (Bowtell and Kelly, 2019), superimposing polyphenol supplementation onto a habitual diet is the most appropriate method to assess the plasma uptake of anthocyanin-rich berries and the potential applicability to exert a health or physiological effect *in vivo*. Despite a number of reports indicating beneficial effects of NZBC extract supplementation, the plasma uptake of key phenolic acids from anthocyanin, vanillic acid (VA), gallic acid (GA) and protocatechuic acid (PCA) following NZBC extract ingestion, when supplemented during a non-polyphenol restricted diet, has yet to be investigated. In addition, it has been suggested that future work in this field should ideally quantify exercise performance outcomes alongside measurement of plasma phenolic metabolites to enable identification of the bioactives metabolites and inform optimisation of the polyphenol blends consumed (Bowtell and Kelly, 2019). Therefore, to address the aforementioned gaps in the literature, Chapter 7 and 8 will examine the time course of VA, GA and PCA following acute and 10-days ingestion of NZBC extract in individuals following a habitual diet, respectively.

2.21 Gut microbiota status and polyphenols

As aforementioned, polyphenols are reported to be rapidly absorbed but have low bioavailability (Manach et al. 2005; Koli et al. 2010), thus they are transferred during digestion to the small intestine and colon where they encounter the gut microbes. Colonic microbiota degrades the unabsorbed polyphenols leading to metabolites (phase I

metabolism) that are better absorbed than their food precursors (Tomas-Barberan et al. 2018) and have been associated as the drivers of the purported health benefits observed *in vivo* (Keane et al. 2016; de Ferrars et al. 2014; Williamson and Clifford, 2010). However, the unambiguous determination of the specific health benefits of polyphenols has been revealed to be a very problematic task (Tomas-Barberan et al. 2018) with large inter-individual variation in the biological response to polyphenols being outlined as a key component (Manach et al. 2005). Part of the inter-individual response to polyphenol intake may be due to an individual's production of bioactive compounds by the gut microbiota, which depends on the disparities in microbial ecology that colonises the colon of each individual (McGhie and Walton, 2007; Del Rio et al. 2010; Williamson and Clifford, 2010; Tomas-Barberan et al. 2018). Therefore, a given polyphenol can lead to different metabolites depending on each individual's specific gut microbiota composition, and to potentially different biological effects (Williamson and Clifford, 2010; Tomas-Barberan et al. 2018). One example within the gut microbiome polyphenol interaction literature is the production of S-equol, the gut microbiota metabolite of daidzein after soy isoflavone consumption. It has previously been found that 30-40% of the Western people excrete significant quantities of S-equol after the consumption of soybean isoflavones (Setchell et al. 2002), whilst the Japanese excrete 50-60% (Morton et al. 2002). Indirect studies to correlate the production of a specific gut microbiota metabolite with health biomarkers (i.e. lipid profiles, highly sensitive c-reactive protein (hs-CRP)) have also been elusive, particularly for soybean isoflavones. When S-equol was given orally to individuals of the equol-producing phenotype, the response was improvement of some cardiovascular risk biomarkers (Hazim et al. 2016). This improvement, however, was not observed when S-equol was given to individuals of the equol nonproducing phenotype, therefore leading to questioning its biological effects *in vivo* and suggesting an effect mediated through gut microbiota. Lampe (2009) previously reported that approximately 30-50% of humans have the bacteria capable of producing equol and 80-90% do not. Factors that influence the capacity to produce equol are not clearly established; however,

gut physiology, genetics and, diet are reported to contribute to inter-individual differences in conversion of daidzein to equol (Lampe, 2009). Therefore, it is possible that a similar event occurs when an individual consumes berry polyphenols (i.e. NZBC, tart Montmorency cherry, blueberry), given the gut microflora are responsible for converting polyphenols into phenolic acids (Cook and Willems, 2018).

2.22 Summary and conclusions

It is clear that the aetiology of EIMD is complex and components of the secondary cascade, such as oxidative stress and inflammation, have a dual function (Peake et al. 2015). On the one hand, oxidative stress and inflammation may amplify muscle tissue damage, but on the other hand both processes play important roles in the resolution of function and in adaptation (Margaritelis et al. 2016). However, being able to maintain a status of hormesis or by avoiding a state of excessive exposure of oxidative stress and/or inflammation, through implantation of a dietary intervention is of interest to practitioners and athletes alike. The use of polyphenol rich supplements is of growing interest (see Figure 2.2) and represents a realistic alternative for in managing muscle damage and exercise recovery (Owens et al. 2018). A caveat to the polyphenol literature discussed in this review is that many of the studies have used low-polyphenolic diets in order to control the study and observe whether the potential effects are due to the polyphenol supplement. Whether these effects would persist as a supplement to a habitual diet is not clear. At best, polyphenols provide vital nutrients; at worst, exercise recovery could be augmented (in accordance with hormesis) (Martin and Appel, 2010; Peake et al. 2015). Furthermore, for the polyphenol supplementation strategy to be able to exert any health and/or physiological benefit, it needs to be bioavailable *in vivo* (Rein et al. 2013). The successful delivery of personalised dietary advice may depend on researchers being able to elucidate the underlying factors that are responsible for the inter-individual variability that is so often reported within the EIMD and polyphenol supplementation literature (Damas et al. 2016; Paschalis et al. 2018). One of these areas may lie with

genotypes and researcher's ability to identify phenotypes that are responsive to dietary interventions (Baumert et al. 2016; Paschalis et al. 2018).

Therefore, as stated previously, the overarching aim of this thesis is to investigate the effects of NZBC extract supplementation on recovery following EIMD. This will be addressed through six studies (Figure 2.5), with the specific aims to examine the:

1. Effect of NZBC extract supplementation taken 7-days before and 2-days following running a half-marathon race on markers of EIMD (Chapter 3).
2. Inter-individual variability in response to EIMD induced by a half-marathon event with NZBC extract or placebo supplementation (Chapter 4).
3. Effect of NZBC extract supplementation taken 7-days before and 3-days following a 100-DJP on markers of EIMD. (Chapter 5).
4. Intra- and inter-individual variability in response to EIMD induced by a 100-DJP with NZBC extract or placebo supplementation (Chapter 6).
5. Time course of the plasma concentration of the phenolic acids; vanillic acid (VA), gallic acid (GA) and protocatechuic acid (PCA), following acute ingestion of a single dose of NZBC extract in individuals following a non-polyphenol restricted diet (Chapter 7).
6. The time course of plasma concentrations of the phenolic acids; VA, PCA and GA following 10-days of NZBC extract supplementation alongside a non-polyphenol restricted diet and the relationship between these phenolic acids and muscle function measures of MVIC and CMJ pre and following a 100-DJP (Chapter 8).

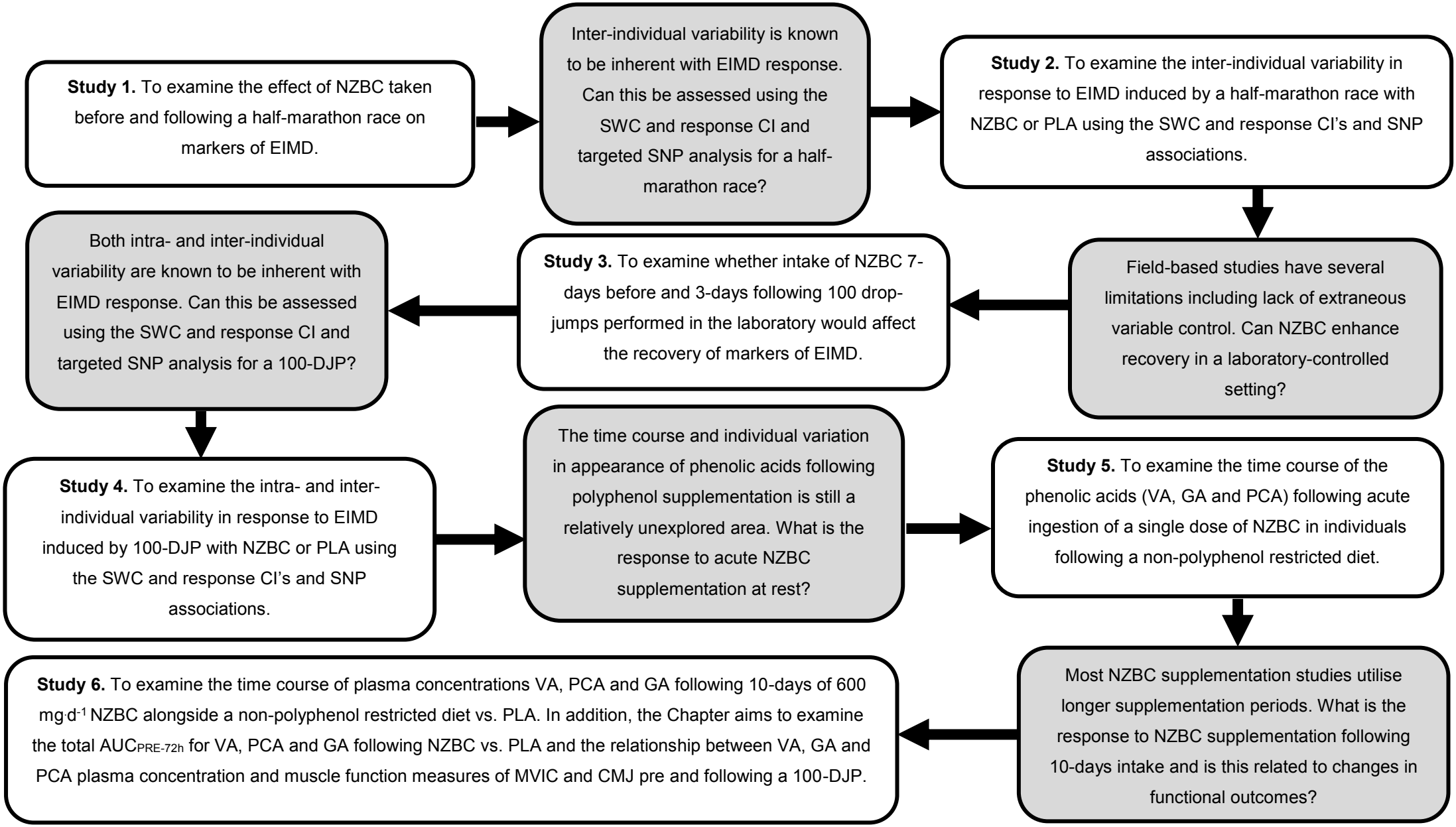


Figure 2.5. Overview of aims (white boxes) and proposed questions (grey boxes) in Studies 1 to 6 within the thesis. NZBC, New Zealand blackcurrant; EIMD, exercise-induced muscle damage; SWC, smallest worthwhile change; CI, confidence interval; SNP, single nucleotide polymorphism; PLA, placebo; VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid; 100-DJP, 100-drop jump protocol; MVIC, maximal voluntary isometric contraction; CMJ, countermovement jump; AUCPRE-72h, total area under the curve pre to 72 h.

3. No Effect of New Zealand Blackcurrant Extract on Recovery of Muscle Damage Following Running a Half-Marathon

Publication arising from this Chapter:

Costello, R., Willems, M.E.T., Myers, S.D., Myers, F., Lewis, N.A., Lee, B.J., & Blacker, S.D. (2020). No effect of New Zealand blackcurrant extract on recovery of muscle damage following running a half-marathon. *International Journal of Sports Nutrition & Exercise Metabolism, 30(4),* 287-294*.* doi:10.1123/ijsnem.2019-3012.

3.1 Abstract

New Zealand blackcurrant (NZBC) contains anthocyanins, known to moderate blood flow and display anti-inflammatory properties that may improve recovery from exercise-induced muscle damage (EIMD). We examined whether NZBC extract supplementation enhances recovery from EIMD after a half-marathon race. Following a randomized, double-blind, independent groups design, 20 (8 women) recreational runners (age 30±6 years, height 1.73±0.74 m, body mass 68.5±7.8 kg, half-marathon finishing time 1:56:33±0:18:08 h:min:s) ingested either 600 mg·day^{-1} capsules of NZBC extract (CurraNZ™; containing a total of 210 mg anthocyanins) or a visually matched placebo (PLA), for 7-days prior to and 2-days following a half-marathon. Countermovement jump (CMJ) performance variables, urine interleukin-6 (IL-6), perceived muscle soreness and fatigue were measured pre-, post-, and at 24 h and 48 h after the half-marathon and analysed using a mixed linear model with statistical significance set a priori at $P<0.05$. The CMJ performance variables were reduced immediately post-half-marathon ($P<0.05$) with all returning to pre half-marathon by 48 h levels except concentric and eccentric peak force and eccentric duration, with no difference in response between groups ($P>0.05$). Urine IL-6 increased 48 h post-half-marathon in the NZBC group only ($P<0.01$) and remained unchanged compared to pre half-marathon levels in PLA group ($P>0.05$). Perceived muscle soreness and fatigue increased immediately post-half-marathon ($P<0.01$) and returned to pre half-marathon by 48 h, with no difference between groups ($P>0.05$). Supplementation with NZBC extract had no effect on the recovery of CMJ variables and perceptions of muscle soreness or fatigue following a half-marathon in recreational runners.

3.2 Introduction

Exercise-induced muscle damage (EIMD) occurs following exercise that involves eccentric contractions (Paulsen et al. 2012). A biphasic response to EIMD is typically observed, where initially metabolic and mechanical disruptions are followed by a secondary phase initiated by a disruption in intracellular Ca^{2+} homeostasis (Howatson and van Someren, 2008). Half-marathons have been shown to cause EIMD (Duthie et al. 1990; Withee et al. 2017). The magnitude of EIMD can be assessed through direct measures of structural damage and force deficits (Warren et al. 1999; Clarkson and Hubal, 2002) and via indirect markers measured systemically in plasma such as CK and inflammatory cytokines (e.g. IL-6) and muscle soreness (Hydahl and Hubal, 2014; Clarkson and Hubal, 2002).

Recently, foods and supplements that are rich in polyphenols such as berries and fruits have been shown to enhance exercise performance and recovery (for a review see Cook and Willems, 2018). Montmorency tart cherry juice (MCJ) has been shown to enhance recovery of muscle function and reduce inflammation and lipid peroxidation following a marathon race (Howatson et al. 2009). However, beetroot juice supplementation did not affect recovery following a marathon race (Clifford et al. 2016). The difference may be related to the profile of the polyphenolic compounds, e.g., the anthocyanins. Although the precise mechanisms are not clear, it has been speculated that anthocyanins may exert their recovery benefits by reducing ROS production during the secondary phase of the EIMD cascade, thus increasing the bioavailability of the vasodilator NO, due to a reduced production of peroxynitrite via the reaction of superoxide and NO and subsequently upregulating eNOS activity and blood flow to the affected tissues (Bowtell and Kelly, 2019). Alternatively, by increasing muscle oxygenation (Fryer et al. 2020) and maintaining a degree of eNOS activity despite the likely disruption to the endothelium from eccentric exercise (Kano et al. 2005) anthocyanins, therefore, may indirectly help to clear cellular debris within the microvascular network and dampen the degree of localised swelling within the myocytes (Clarkson and Hubal, 2002) during recovery from EIMD.

New Zealand blackcurrant (NZBC) is unique due to its high anthocyanin content and has been shown to enhance exercise performance (for a review see Cook and Willems, 2018) and recovery from EIMD (Coelho et al. 2017) in laboratory settings. The effects of NZBC extract on recovery following more ecologically valid events in the field, such as a half-marathon race, are not known.

The aim of this study was to examine the effect of NZBC extract supplementation taken before and following running a half-marathon race on markers of EIMD.

The following hypothesis was tested:

- Participants who supplemented with NZBC extract, when compared to placebo (PLA), would demonstrate a quicker recovery, by an accelerated return of muscle function, reduced muscle soreness and fatigue, and a mitigated exercise-induced inflammatory cascade response.

3.3 Methods

3.3.1 Participants

Twelve healthy men and eight healthy women (Table 3.1) who were runners taking part in the 2018 Chichester Half-Marathon, Chichester, UK volunteered to participate in the study. Based on a similar previous study focusing on recovery with a polyphenol-rich supplement following a running event (Clifford et al. 2016), established on CMJ height we calculated (G*Power; Faul et al. 2007) that at 80% power, and an α of 0.05, at least eight volunteers were required to detect a group difference of 5% (using change from pre-half marathon data) (3.5% SD) at any time points post the half-marathon event. Participants completed a health history questionnaire, were non-smokers, had no known food allergies and were not taking anti-inflammatory therapies. Females completed a menstrual cycle questionnaire (Köhne et al. 2016). Participants abstained from strenuous exercise and alcohol for 48 h prior, and caffeine-containing products on the day of the half-marathon. Participants were also asked to avoid all

additional means that could affect recovery and adhere to their normal activity schedule. The study was approved by the University of Chichester Research Ethics Committee with protocols and procedures conforming to the 2013 Declaration of Helsinki.

Table 3.1 Descriptive data of the volunteer Half-Marathon runners in the NZBC and placebo groups

Participant Characteristics	NZBC (n=10)	Placebo (n=10)
Age (years)	30±4	29±7
Sex (M/F)	6/4	6/4
Height (m)	1.72±0.78	1.74±0.67
Body Mass (kg)	69.0±8.1	68.0±7.8
Estimated female menstrual cycle phase		
Luteal	3	2
Follicular	1	2
Years running	6±5	11±5
Average weekly mileage	12±8	14±7
Longest training run (miles)	11±6	11±6
Previous half-marathons	5±3	6±4
Predicted finish time (h:min:s)	1:56:30±0:15:40	1:58:18±0:22:52
Actual finish time (h:min:s)	1:58:12±0:17:53	1:54:54±0:18:15
Average Heart Rate (bpm)	166±16	162±27

Values are mean±SD, n=20. There were no differences between groups for any variable (P>0.05); NZBC, New Zealand blackcurrant.

3.3.2 Experimental design

The study followed a double-blind, placebo-controlled, randomised, independent-groups study design. Groups were matched according to predicted half-marathon finish times by pairing participants with equivalent times (Howatson et al. 2009; Clifford et al. 2016). Blinding of the placebo and supplement was carried out by an independent researcher who had no involvement with this investigation. Packets were made up with visually identical NZBC and placebo capsules for each participant and labelled with a random letter. Each participant in a matched pair was randomly assigned to one of the letters and provided with that packet of capsules. The blinding codes were revealed following data analysis. The participants completed one familiarisation visit, and four experimental visits pre- and immediately post-half-marathon (in the race holding area), 24 and 48 h (laboratory; Figure 3.1). For the familiarisation visit, participants were briefed on the study, explained all the procedures and had their height and body mass recorded. CMJ, VAS for muscle soreness and fatigue and a urine sample were completed in this order during each experimental visit. Heart rate was collected during the half-marathon (Polar Team 2, Polar Electro Ltd, UK) and race distance confirmed using GPS (Polar M430 GPS, Polar Electro Ltd, UK).

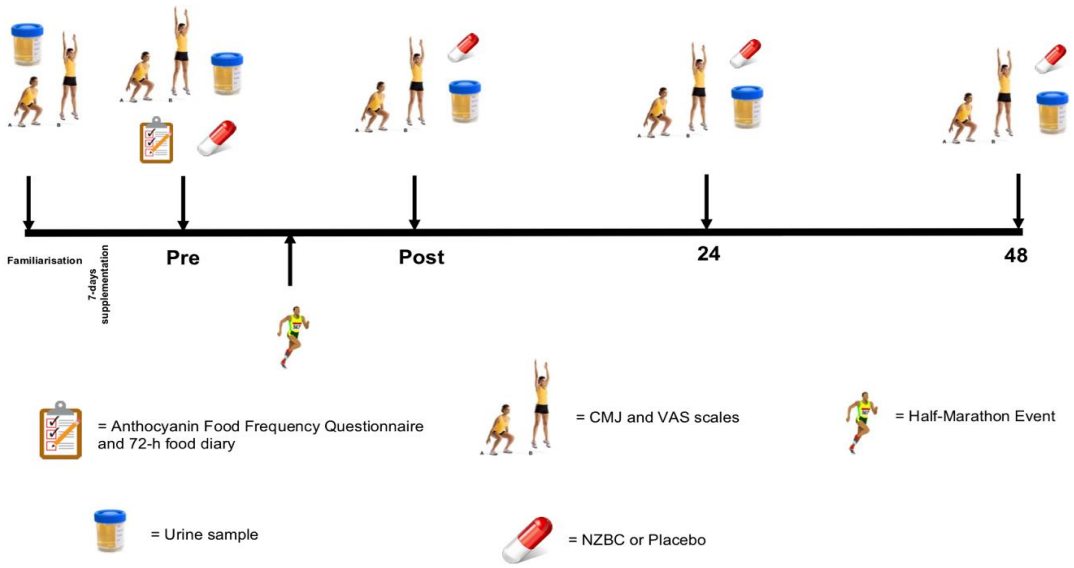
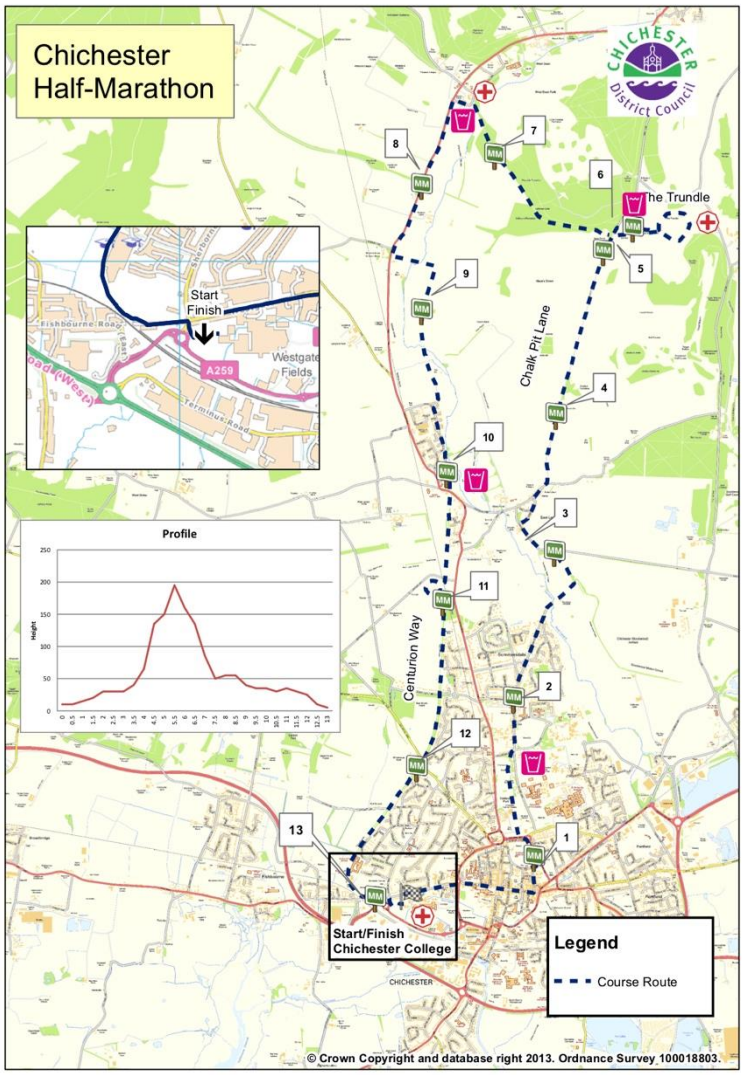


Figure 3.1 Study design.

3.3.3 Half-marathon

The half-marathon took place on 19th October 2018 in Chichester (West Sussex, UK). The course was mostly flat, across a mix of concrete terrain, grass and chalk (Figure 3.2). However, mile 4 to 8 consisted of a steep incline and decline (total route ascent: 239 m; total route descent: 232 m). At the start of race at 9:00, the air temperature was 8ºC, humidity 81%, barometric pressure 1023 hPa, and air speed 10 mph. It remained dry and mostly overcast with intermittent sunny spells for the duration of the race.

A



B

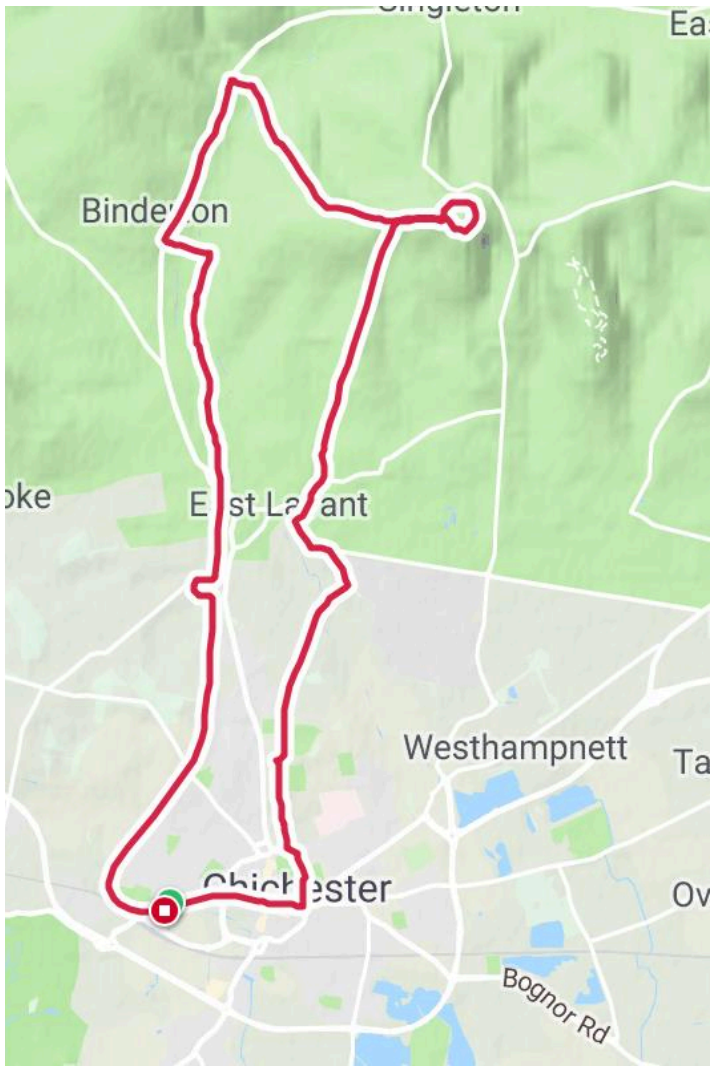


Figure 3.2 Half-Marathon race route. A. Advertised race route used with permission of Chichester District Council. B. Recorded race route from a participant's Polar M400 GPS watch.

3.3.4 Supplementation protocol

Participants ingested two capsules of NZBC extract (2 x 300 mg CurraNZ™) each containing 105 mg of anthocyanins (CurraNZ™, Health Currancy Ltd, Surrey, United Kingdom) or two capsules of identical looking placebo capsules (2x300 mg microcrystalline cellulose M102; CurraNZ™, Health Currancy Ltd, Surrey, United Kingdom) with breakfast every morning for 7-days and 2-days following the half-marathon. On the morning of the half-marathon, participants consumed their supplement 2 h prior to starting the race. This supplementation regime was based on previous work where anthocyanins peak in systemic circulation ~2 h after ingestion (Matsumoto et al. 2005). Full compliance with intake was achieved. Blinding was not broken until after analysis was completed and a follow-up questionnaire revealed 40% of participants accurately guessed which supplementation they received.

3.3.5 Dietary intake

For ecological validity, participants maintained their habitual diet prior to and post- the half-marathon (Bowtell and Kelly, 2019) and recorded their 72 h dietary intake in food diaries which were analysed (Nutritics Ltd, Dublin, Ireland) for carbohydrate, fat and protein, and total energy intake. The habitual anthocyanin food frequency questionnaire recorded the amount and frequency of anthocyanin containing foods eaten within the last three months from the Phenol Explorer database (Neveu et al. 2010). The intake of anthocyanin was calculated as the sum of the consumption frequency of each anthocyanin containing food, multiplied by the content of the anthocyanin content for the portion sizes.

3.3.6 Indices of muscle function

The CMJ were performed on a force plate (PASPORT force plate, PS-2141, PASCO Scientific, California, USA) sampling at 1000 Hz (Lake et al. 2018). Participants were instructed to jump as high and as fast as possible, without specific information on squat depth to avoid altering natural jump patterns (Jidovtseff et al. 2014). Three maximal efforts were performed, separated by 30 seconds of passive (standing) recovery. Outcome variables: jump

height (JH), reactive strength index modified (RSImod), time to take-off and neuromuscular variables; concentric phase average peak force, net impulse, power, duration and eccentric phase average peak force, net impulse, displacement (braking phase) and duration are reported (Gathercole et al. 2015). Reactive strength index modified was calculated as (JH/time to take off). The neuromuscular variables are expressed relative to body mass and outcome variables JH and RSImod are expressed as a percentage change from pre-half marathon to account for inter-individual variability. The coefficient of variation (CV) for the outcome variables, JH, RSImod and time to take off was 6, 9 and 6%, respectively.

3.3.7 Muscle soreness and fatigue

Whilst in a 90^{o} degree squat position, participants rated their self-perceived muscle soreness and fatigue using a 0-10 VAS, where 0 represented *no soreness* and 10 represented *extreme soreness* and 0 represented *no fatigue* and 10 represented *extreme fatigue*, respectively (Jakeman et al. 2017).

3.3.8 Urine sampling, handling and biochemical analysis

Second evacuation, mid-stream urine samples were collected into 50-mL Falcon® conical tubes. At all four time points (pre, post, 24 h post and 48 h post), urine was collected and kept on ice for no more than 2 h prior to being centrifuged at 1000x*g* for 10 minutes. The urine was subsequently stored in 2-mL aliquots at -80^{o}C and thawed on the morning of the analysis. Urinary IL-6 concentration was determined in duplicate using a quantitative sandwich enzyme immunoassay ELISA technique (Quantikine, R&D Systems Europe Ltd., Abingdon, UK). Normal reference ranges for this assay are reported at <3 pg·mL. The urine intra- and inter-assay precision determined by CV was 4 %. Urinary cytokine levels were expressed as ratios of IL-6 to creatinine (pg·mg creatinine) to avoid dilution effects, to be able to compare results from different participants, and to standardise the samples in light of differences in post-race hydration status. Urine creatinine was measured using a colorimetric assay (CR510, Randox, County Antrim, Northern Ireland).

3.3.9 Data analysis

Statistical analyses were completed using GraphPad Prism V8 (Graphpad software, San Diego, California). In accordance with many previous studies examining the effects of a nutrition intervention on EIMD (Clifford et al. 2015; Howatson et al. 2010; Trombold et al. 2010; 2011), data analysis for CMJ and VAS was conducted on data corrected to percentage change from baseline. This was to account for the large inter-individual variability typically observed with these markers in response to EIMD (Sayers and Clarkson, 2002). Dependent variables (CMJ, VAS and IL-6 analyses) were analysed using a mixed linear model with two independent group levels (NZBC vs. PLA) and four repeated measures time points (pre, post, 24 and 48 h post). The Levene's test was used to check homogeneity of variance for all variables and any violations of the assumption were corrected using the Greenhouse-Geisser adjustment. Significant main effects or interactions were assessed using Bonferroni adjustment post hoc analysis. Differences in half-marathon finish times and group dietary intake were analysed with independent samples *t*-tests. The alpha level for statistical significance was set at 0.05 a priori. All data are reported as mean±SD for n=10 for each group, unless otherwise stated.

3.4 Results

Half-marathon finish times did not differ between groups (P=0.67). Average energy intake (KJ) in the day before the half-marathon until the cessation of the study did not differ between groups (P=0.90) nor did the proportions coming from carbohydrate (P=0.51), protein (P=0.36) or fat (P=0.63). Habitual anthocyanin intake did not differ between groups (P=0.99) (Table 3.2).

Table 3.2 Absolute and relative to body mass average daily intake macronutrient intake prior to and for the 2-day following the half-marathon and habitual anthocyanin intake as indicated from the anthocyanin food frequency questionnaire (n=10 per group, Mean±SD).

Nutritional component	NZBC	Placebo
Total energy intake (kJ)	9091±3319	8903±2198
(kJ·body mass^{-1})	133±46	134±38
Carbohydrate (g)	226±73	249±68
(g·kg body mass^{-1})	3.3±1.1	3.8±1.1
Protein (g)	107±37	92±23
(g·kg body mass^{-1})	1.6±0.5	1.4±0.4
Fat (g)	93±46	84±23
(g·kg body mass^{-1})	1.3±0.6	1.3±0.4
Habitual anthocyanin intake (mg·day^{-1})	153±122	172±81

NZBC, New Zealand blackcurrant.

3.4.1 Indices of muscle function

CMJ outcome variables (JH and RSImod) and neuromuscular variables (concentric average relative peak force, concentric net impulse, concentric average power, eccentric average relative peak force, eccentric net impulse) showed a main effect of time ($F_{(3,54)}$=11.40, P=0.01; $F_{(3,54)}$=8.25, P=0.01; $F_{(1.6,29.6)}$=4.649, P=0.02; $F_{(2.1,37.5)}$=10.52, P=0.02; $F_{(3,54)}$=8.55, P=0.01; $F_{(1.6,29.9)}$=17.95, P=0.01; $F_{(3,54)}$=9.07, P=0.01, respectively), indicating muscle damage after the half-marathon (Figures 3.2a, 3.2b; Table 3.3). Relative to pre-half marathon, JH and RSImod decreased to a similar extent in the NZBC and PLA groups immediately post half-marathon (91.3±11.5 vs 85.6±19.5%, respectively) and had returned to pre half-marathon levels by 24

h (97.2±11.1 vs 101.6±10.7%, respectively). Apart from TTT, no group or interaction effects were present at any time point for any of the CMJ outcome or neuromuscular variables (all $P>0.05$) (Table 3.3).

Table 3.3 Indices of muscle function and damage for both New Zealand blackcurrant and placebo groups before and following Half-Marathon race

CMJ variable	Pre Half-Marathon	Post Half-Marathon	24 h post Half-Marathon	48 h post Half-Marathon
Time to take off (s)#				
NZBC	0.96±0.12	1.03±0.20	0.95±0.13	0.91±0.11
PLA	0.93±0.17	0.98±0.16	1.02±0.17	1.03±0.19
Concentric phase peak force (N/kg)*				
NZBC	11.32±1.56	10.40±1.72	10.16±2.02	10.51±1.99
PLA	11.33±3.34	10.32±2.07	10.05±2.04	10.03±2.27
Concentric phase net impulse (Ns/kg)*				
NZBC	2.06±0.36	1.94±0.28	2.02±0.32	2.10±0.31
PLA	2.06±0.33	1.87±0.28	2.06±0.25	2.13±0.27
Concentric phase average power (W/kg)*				
NZBC	20.06±4.31	17.98±3.35	18.99±4.04	19.83±3.66
PLA	19.81±4.03	16.64±3.29	20.68±6.56	19.78±4.39
Concentric phase average duration (s)				
NZBC	0.32±0.05	0.32±0.06	0.33±0.06	0.32±0.05
PLA	0.33±0.06	0.33±0.06	0.34±0.07	0.33±0.07

CMJ variable	Pre Half-Marathon	Post Half-Marathon	24 h post Half-Marathon	48 h post Half-Marathon
Eccentric phase peak force (N/kg)				
NZBC	10.16±2.16	7.12±1.14***	7.99±1.41***	8.42±1.68***
PLA	10.79±3.56	6.49±1.30***	7.24±1.73***	7.97±2.56***
Eccentric phase net impulse (Ns/kg)				
NZBC	1.01±0.26	0.89±0.20**	0.94±0.23	0.98±0.20
PLA	1.06±0.20	0.77±0.13**	0.83±0.16	0.91±0.15
Eccentric phase displacement (braking phase) (m)*				
NZBC	0.21±0.03	0.26±0.05	0.24±0.05	0.23±0.04
PLA	0.30±0.17	0.29±0.08	0.27±0.06	0.30±0.10
Eccentric phase average duration (s)*				
NZBC	0.21±0.03	0.26±0.05	0.24±0.05	0.23±0.04
PLA	0.25±0.06	0.29±0.08	0.27±0.06	0.30±0.10

Values are mean±SD, n=10 per group. #Time*Supplement interaction (P=0.02). *Main effect of time but not statistically significant when Bonferroni correction applied (P>0.05). **Elevated above pre-half marathon at immediately post (time effect, P<0.05*)*. ***Elevated above pre-half marathon immediately post, 24 and 48 h post (time effect, P<0.05) No other group or interaction effects observed (P>0.05). NZBC, New Zealand blackcurrant; PLA, placebo; CMJ, countermovement jump.

3.4.2 Muscle soreness and fatigue

Muscle soreness and fatigue both showed a main effect of time ($F_{(3,54)}$=66.43, *P*=0.01; $F_{(3,54)}$=77.12, *P*=0.01, respectively) (Figures 3.3a, 3.3b). However, no group or interaction effects were present at any time point for muscle soreness or fatigue (*P*>0.05).

3.4.3 Inflammatory cytokine response

At 48 h after the half-marathon, IL-6 urine concentrations corrected to creatinine increased compared to pre-half marathon in the NZBC group only ($F_{(1,18)}$=7.96, *P*=0.01) and remained unchanged at all time points in the placebo group compared to pre-half marathon (*P*>0.05). No time or interaction effects were present (*P*>0.05) (Figure 4).

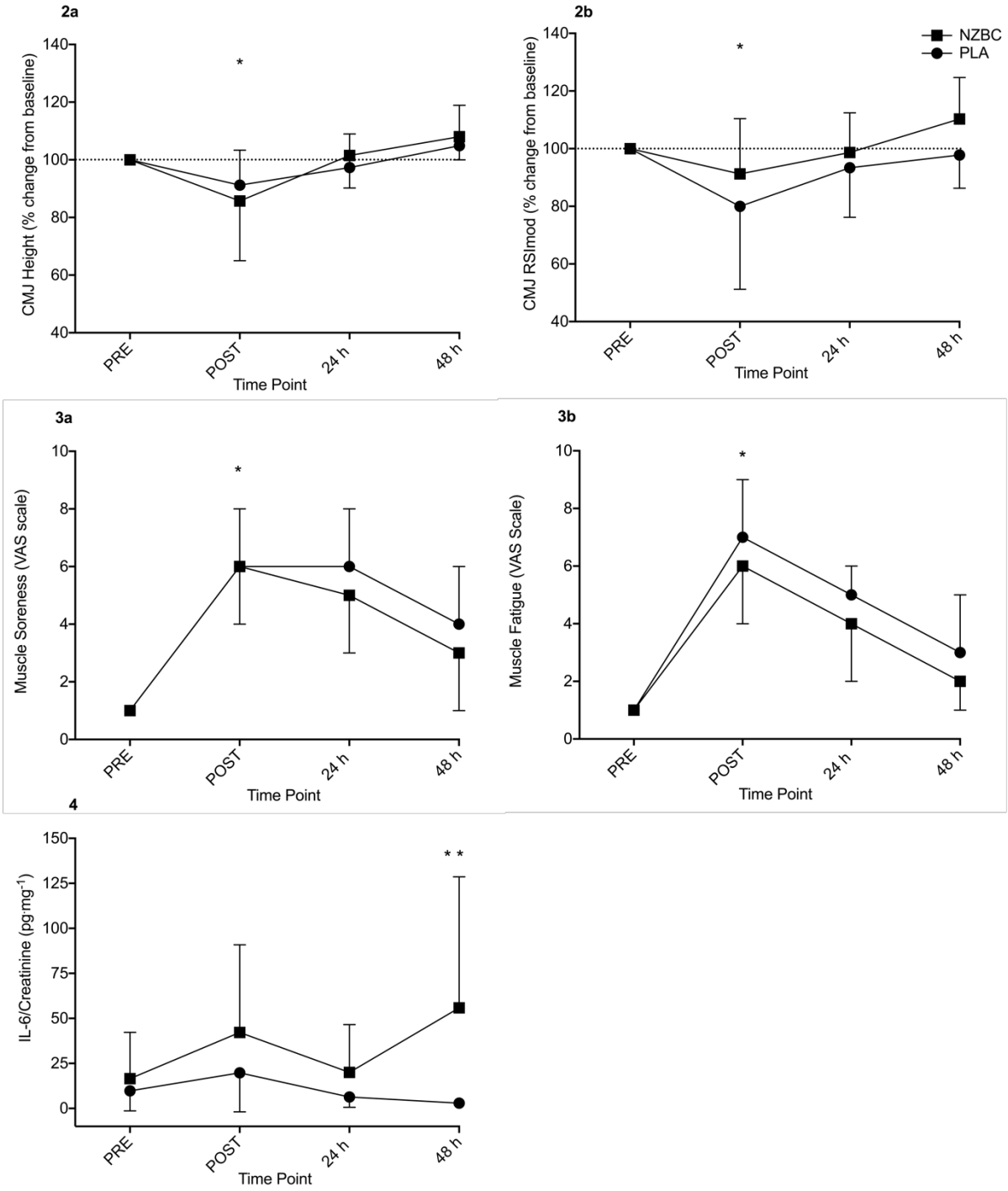


Figure 3.2a, 3.2b, 3.3a, 3.3b and 3.4 – 3.2a. Percentage change from pre half-marathon in countermovement jump (CMJ) height and post half-marathon (*pre to post; *P*<0.01). 3.2b. Percentage change from pre half-marathon in reactive strength index modified (RSImod) and post half-marathon (*pre to post; *P*<0.01). 3.3a. Muscle soreness ratings pre and post half-marathon (*pre to post; *P*<0.01). 3.3b. Muscle fatigue ratings pre and post half-marathon (*pre to post; *P*<0.01). 3.4. Interleukin-6 urine concentrations with creatinine correction pre and post half-marathon (**pre to 48 h; *P*<0.01). Values are mean±SD (n=10 per group for 3.2a, 3.2b, 3.3a, 3.3b and 3.4).

3.5 Discussion

This is the first study to investigate the effect of NZBC extract supplementation on recovery from EIMD following a half-marathon running race. However, contrary to our hypothesis, NZBC extract did not affect the recovery of muscle function, reduce muscle soreness or attenuate the acute inflammatory response in the 48 h after the half-marathon.

The reduction in the CMJ variables (concentric phase average peak force, net impulse, average power and eccentric phase average peak force and average duration) immediately and in the days after the half-marathon running race demonstrated that the event caused EIMD. However, the similar response for each condition over time indicates that NZBC extract did not affect post-race muscle recovery. The lack of observable difference between groups may be due to the half-marathon race only inducing modest changes in all of the CMJ outcome and neuromuscular variables. Future research could investigate whether NZBC extract is able to modulate declines in contractile properties following exercise with a greater effect on EIMD.

The results of the present study are in contrast to those previous ones where anthocyanin rich supplements have been provided following running exercise. Howatson et al. (2010) showed that an MCJ supplement enhanced recovery of muscle function following a marathon and observed attenuation of biomarkers of inflammation (serum C-reactive protein, CRP; IL-6 and uric acid) and oxidative stress (thiobarbituric acid reactive species, TBARS) in the 48 h following the marathon; effects that were associated with an accelerated recovery of muscle function as determined by maximal voluntary isometric contraction (MVIC). Differences in findings between the present study and Howatson et al. (2010) may be attributable to the different anthocyanins in each supplement, the mode of delivery (capsules vs. juice) and the exercise protocol (half-marathon vs marathon). Supplements were provided before and after the half-marathon both in in the present study (7-days pre, 2-days post), and by Howatson et al. (2010) (5-days pre, 3 days post). The NZBC in the present study was provided in capsules containing 210 mg of anthocyanins per day, and the main anthocyanin was delphinidin-3-rutinoside (Rothwell et al. 2013). In contrast, MCJ was provided in a juice containing 80 mg of

anthocyanins per day and the main anthocyanin was cyanidin-3-glucosylrutinoside (Howatson et al. 2009). *In vitro* models have demonstrated that cyanidin-3-glucoside upregulates eNOS activity (Edwards et al. 2015). As the main anthocyanin in NZBC is delphinidin-3-rutinoside, it is possible that the cyanidin-3-glucoside in MCJ is better able to upregulate eNOS activity, thus influencing blood flow through flow mediated dilation (Cook et al. 2017) during strenuous exercise and reducing the susceptibility to injury (Jones et al. 2017). Further, polyphenol scavenging has been purported as a potential mechanism by which, polyphenols could help support redox status by dampening the oxidative stress response following EIMD (Powers and Jackson, 2008). However, this notion has recently been debated with polyphenol metabolism to electrophiles and a cyto-protective endogenous antioxidant response via nuclear factor erythroid 2-related factor 2 (Nrf-2) signalling having been suggested as a more plausible mechanism (Owens et al. 2018). For example, Cimino et al. (2013) previously showed in an *ex vivo* model how an anthocyanin-rich supplement composed of purified bilberry and blackcurrants (160mg anthocyanin) was able to significantly increase Nrf-2 nuclear accumulation activation, heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase-1 (NQO-1) gene expression within human umbilical vein endothelial cells (HUVECs) under normoxic conditions. The authors suggested that this *ex vivo* model had shown for the first time that intake of the anthocyanin-rich supplement was able to produce serum levels of anthocyanin and/or metabolites great enough to induce physiological effects and protect against hyperoxia-induced endothelial damage via activation of the Nrf-2 pathway.

However, other studies have also reported no benefit from supplementation with nitrate-rich, beetroot juice (Clifford et al. 2016) and anthocyanin-rich, bilberry juice (Lynn et al. 2018) on markers of EIMD following marathon and half-marathon running, respectively. Clifford et al. (2016) observed that beetroot juice supplemented for the 3-days following a marathon, was unable to attenuate declines in CMJ and MVIC, and elevations in markers of inflammation, (leucocytes, neutrophils, monocytes, hs-CRP, IL-1ra, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and interferon-γ). On the other hand, Lynn et al. (2018) concluded that consumption of bilberry

juice 5-days prior to, on race day, and for 2-days following a half-marathon, evoked moderate increases in exercise-induced muscle soreness and markers of inflammation (CRP) and muscle damage (determined by CK concentrations). Similarly, the lack of benefit observed may be attributable to the different supplementation strategies used (beetroot juice 3-days following the marathon only vs. bilberry juice 5-days prior to, on race day and 2-days following the half-marathon), leading to different biological activities of the phytonutrients.

Using a different exercise model, Coelho et al. (2017) examined the effect of NZBC extract on recovery from EIMD induced by 60 maximal eccentric contractions of the biceps brachii in 13 healthy young women. No effects on muscle function and plasma IL-6 were reported but muscle soreness and serum CK were attenuated in the recovery period with NZBC. Compared to the present study, differences in exercise protocol (half-marathon vs. repeated isolated forearm flexor exercise), techniques used to quantify EIMD (CMJ vs. MVIC) and participant characteristics (mixed men and women vs. women only), between the present study and Coelho et al. (2017) are all factors that could provide a potential explanation for these equivocal findings.

Urinary IL-6 has previously been observed to increase following long distance running events (Sugama et al. 2013; Mrakic-Sposta et al. 2015). However, there was no increase in IL-6 immediately post and 24 after the half-marathon for either PLA or NZBC (Figure 3.4). Large inter-individual variability was present due to four participant's data skewing the NZBC group average. These data suggest that IL-6 is unlikely to have significant role in the secondary damage process in the days after a half-marathon in recreational runners. The increase in urine IL-6 observed at 48 h in the NZBC only could be indicative of the known anti-inflammatory role of the cytokine. However, this is purely speculative without a broader range of biomarkers indicative of pro- and anti-inflammation and oxidative stress response to compare with (Owens et al. 2018).

A limitation of the present study was that participants were not provided with standardised meals prior to and immediately following the half-marathon event. As the participants appeared to have low habitual carbohydrate intake compared to the recommended guidelines of 6-10 $g \cdot kg \cdot d^{-1}$ (Thomas et al. 2016), it is possible that this may have influenced our results. Future research should look to implement standardised meals to ensure that optimal intake of macronutrients prior to exercise are met. Further, participants were permitted to maintain their habitual anthocyanin intake in an effort to increase the ecological validity of the findings. However, it is possible that by increasing ecological validity we may have limited our ability to detect any meaningful benefit of NZBC extract supplementation on recovery.

3.6 Conclusion

In conclusion, NZBC extract supplementation for 7-days prior to and 2-days following a half-marathon, does not affect the recovery of muscle function, muscle soreness and fatigue or markers of inflammation in recreational half-marathon runners. As muscle function was only modestly reduced immediately following the half-marathon and had largely returned to baseline by 2 days post, the CMJ variables utilised may have not been sensitive enough to detect any subtle changes in muscle function indicative of EIMD, thus, NZBC extract provided no benefit as far as we could observe. However, utilising the CMJ neuromuscular variables provided greater insight into how participants may adopt different CMJ strategies following half-marathon running, potentially highlighting aspects of relevance to real-world sporting performance that may be masked when only considering variables such as jump height.

The overall aim of this thesis was to determine whether NZBC extract is effective at attenuating EIMD and enhancing recovery following strenuous exercise. Based upon the laboratory data demonstrating the performance and recovery benefits of NZBC extract (Cook and Willems, 2018; Coelho et al. 2017), it was anticipated that NZBC extract might also help to protect against EIMD following a half-marathon. However, the results in the present Chapter suggest that NZBC extract is not an effective recovery aid following half-marathon running. Muscle function, as measured by CMJ outcome (jump height, RSImod, time to take off) and

neuromuscular variables (concentric average peak force, net impulse, average power, duration, eccentric average peak force, net impulse, displacement and duration), and muscle soreness and fatigue, did not differ between the NZBC extract and PLA groups in the 2 days after the half-marathon. Further, urinary IL-6 was unaffected by NZBC extract supplementation.

There are several factors that could explain the discrepant results between the present study and that of Coelho et al (2017), which observed an attenuation of muscle soreness and serum CK in the 96 h following EIMD. These include, but are not limited to, differences in the exercise model (60 maximal eccentric contractions of the biceps brachii vs half-marathon running), participant sex (female only vs mixed male and female) timing of measures (up to 96 h post- vs up to 48 h post half-marathon). The measurements used in each of the studies were also different with Coelho et al. (2017) using MVIC, perceived muscle soreness, range of movement (ROM), mid-arm circumference (MAC), serum CK and plasma IL-6 and present study using CMJ, perceived muscle soreness and fatigue and urinary IL-6. Perhaps the biggest difference in the present study is that the magnitude of EIMD was modest. This could be because the half-marathon runners in the present study were relatively experienced (6 half-marathons completed on average per group) and, therefore, possibly sufficiently accustomed to protect against muscle soreness and prolonged deficits in muscle function. Alternatively, not having what is widely considered the gold standard for indirectly measuring the magnitude of EIMD, MVIC, and only CMJ, may have limited our ability to detect the presence of EIMD. Irrespective of the exact reasoning, this study does not support the use of NZBC extract to alleviate EIMD or effect recovery in recreational runners. Furthermore, although the original hypothesis was that NZBC extract might enhance recovery by attenuating the cascade in post-exercise inflammation, there was no evidence of this in the present study.

However, what was apparent was the inter-individual variation with both NZBC and PLA groups for CMJ responses following the half-marathon. It is possible that this variation may have impacted our ability to detect a meaningful change for each CMJ neuromuscular and

outcome variable between NZBC and PLA. Inter-individual variation within studies has been previously reported within the EIMD literature (Nosaka and Clarkson, 1996) and a number of investigations suggest that multiple genetic variations may play a role in influencing an individual's susceptibility to EIMD (Clarkson et al. 2005; Baumert et al. 2016). Therefore, to ascertain whether NZBC extract supplementation has any potential recovery benefits at the individual level following running a half-marathon, Chapter 4 will examine the effects of NZBC extract supplementation on recovery from a half-marathon with the SWC and targeted SNPs analysis.

4. Inter-Individual Variability in Countermovement Jump Response to Half-Marathon Running with NZBC Extract Supplementation: Statistical and Genotype Insights

4.1 Abstract

Introduction. New Zealand blackcurrant (NZBC) extract was unable to improve recovery from EIMD following a half-marathon race. However, large inter-individual variability was apparent. Therefore, the smallest worthwhile change (SWC) approach and genotype analysis was used in an attempt to elucidate some of the potential sources for the observed variability. **Method.** Using data collected in Chapter 3, mean individual observed scores with true response 95%CI and SWC for group (NZBC vs PLA) inter-individual variation were graphically constructed to determine individual responses and participants were retrospectively genotyped for candidate SNPs, α-actinin-3 (*ACTN3*), angiotensin-I converting enzyme (*ACE*) and titin (*TTN*) and associations with CMJ variables were examined pre-, post-, and at 24 h and 48 h after the half-marathon using a mixed model ANCOVA, with statistical significance set *a priori* at $P<0.05$. **Results.** The SWC and response CI's demonstrated that, in general, more individuals within the NZBC group appeared to recover eccentric peak force, net impulse, phase duration and concentric power quicker following the half-marathon than PLA. Secondly, the *ACTN3* and *ACE* genotypes appeared to influence the recovery of eccentric peak force, eccentric net impulse and concentric power, respectively, but no differences could be detected between NZBC and PLA groups. **Conclusion**. Implementation of the SWC and response CI's highlighted that several participants appeared to recover quicker with NZBC extract supplementation following a half-marathon event. The *ACTN3* and *ACE* may explain some of the variability observed within CMJ neuromuscular variables. However, future research is required to explore this observation further.

4.2 Introduction

In Chapter 3, it was apparent that NZBC extract supplementation for 7-days prior to- and 2-days following a half-marathon event did not affect recovery of muscle function and inflammation. However, despite both groups being homogenous, large inter-individual variability in response was apparent for the CMJ outcome (jump height, RSImod, time to take off) and neuromuscular variables (concentric average peak force, net impulse, average power, duration, eccentric average peak force, net impulse, displacement and duration). It is probable, that even though the *a priori* power analysis indicated sufficient power was obtained to detect group differences of 5% (see Chapter 3), with ten participants per group, the large variability in response may have masked potential effects in recovery with NZBC extract supplementation, particularly at the individual level.

An approach to interpreting the inter-individual variability that has gained popularity within the last two decades is the dichotomous classification of individuals to an intervention as 'responders' or 'non-responders' using predetermined thresholds (Astorino and Schubert, 2014; Montero and Lundby, 2017; Ross et al. 2015; Scharhag-Rosenberger et al. 2012; Paulsen et al. 2012) such as two times the typical error (2x TE) (Bouchard et al. 2012; Bonafiglia et al. 2016; Gurd et al. 2016; Raleigh et al. 2016; Alvarez et al. 2017; de Lannoy et al. 2017; Astorino et al. 2018). However, dichotomous classification fails to consider the continuous range of probabilities of a positive response and likely misclassifies individuals as 'non-responders' (Bonafiglia et al. 2018). Furthermore, use of the 2x TE is frequently applied relative to zero (Bonafiglia et al. 2016; Gurd et al. 2016; Astorino et al. 2018), which fails to consider 'non-response' within the context of meaningful benefit/change (Swinton et al. 2018; Bouchard et al. 2012; Hecksteden et al. 2015). In order to accurately interpret individual data collected from group-based interventions, it is essential that researchers consider a range of concepts including the confounding influence of measurement error and biological variability (Swinton et al. 2018; Bonafiglia et al. 2018; Mann et al. 2014). In addition, the ability to interpret practical and statistical significance are enhanced by concepts such as smallest worthwhile

(meaningful) change (SWC) and response confidence intervals (CIs) and have been suggested to be more informative than threshold-based dichotomous classification (Swinton et al. 2018; Bonafiglia et al. 2018). No previous studies have attempted to utilise this particular statistical framework to look past the group mean response in determining the effect of an anthocyanin-rich supplement, such as NZBC extract supplementation, following a half-marathon event.

Previous studies have determined a wide range of inter-individual variability in response to EIMD, between (Clarkson et al. 2005; Vincent et al. 2010) and within studies (Nosaka and Clarkson, 1996). Variations between and within studies can occur due to age (Fielding et al. 1991), sex (Sewright et al. 2008), ethnic origin (Sherwood et al. 1996), training status (de Lima et al. 2018) and nutrition (Howatson and van Someren, 2008). However, even when all of these factors are taken into account, it still cannot entirely explain the large variation in response to EIMD, and a number of investigations suggest that multiple genetic variations may play a role in influencing an individual's susceptibility to EIMD (Baumert et al. 2016; Clarkson et al. 2005). The most common genetic sequence variation is a SNP, where one nucleotide substitutes another (Baumert et al. 2016). Another type of common sequence variation is the insertion/deletion (indel) polymorphism, in which a specific nucleotide sequence is present (insertion) or absent (deletion) from the allele (Baumert et al. 2016). Several SNPs that encode proteins (i) in skeletal muscle (Ma et al. 2013), tendon and muscle extracellular matrix (Collins and Raleigh, 2009; September et al. 2016), or (ii) are related with the inflammatory response (Baumert et al. 2016), have been associated with changes in various biomarkers of EIMD following eccentric exercise. Thus, it is unlikely that any one SNP is responsible for observed variations in the magnitude of EIMD, instead, that any genetic association with EIMD is likely to be polygenic (Baumert et al. 2016).

The α-actinins are a family of actin-binding proteins encoded by two genes: *ACTN2* and *ACTN3.* The sarcomeric α-actinins are major components of the Z line and they crosslink the thin actin filaments, whilst coordinating myofibril contraction (Yang et al. 2003). *ACTN*2 is

present in all muscle fibre types, whereas, *ACTN3* is only present in type II fibres (MacArthur and North, 2004; Mills, 2001). A common SNP in the *ACTN3* gene produces a premature stop codon that leads to the production of a truncated non-functional *ACTN3* (North et al. 1999). Roughly 18% of the world population is homozygous for this nonsense mutation (Ivarsson and Westerblad, 2015). With regards to EIMD, *ACTN3* has been the most heavily investigated (Baumert et al. 2016) and SNP homozygotes (XX) have been found to show elevated levels of serum CK activity and self-perceptual measures of pain compared to wild-type homozygotes (RR) (Vincent et al. 2010). At present, only two studies have investigated a pre-selected candidate SNP with regard to muscle damaging exercise in a 'real-world' setting (Del Coso et al. 2016; Del Coso et al. 2017). Both studies investigated the association of *ACTN3* (rs1815739) with plasma CK concentration and CMJ height immediately before and post a half-ironman and marathon events, respectively. Due to the low number of XX homozygotes in both studies, the authors grouped XX homozygotes with those heterozygous (RX) for the *ACTN3* polymorphism. The authors observed that X-allele carriers in both studies presented with a greater magnitude of EIMD as indicated by reduced CMJ height and increased serum CK immediately post the half-ironman and marathon events. Del Coso et al. (2017) suggested that the absence of a functional *ACTN3* produced by the X-allele carriers may have induced higher levels of muscle breakdown during the marathon event. Although it has been suggested that XX homozygotes for *ACTN3* compensate for the absence of this protein with increased expression of *ACTN2* (Seto et al. 2011; Yang et al. 2003), given the evidence to date which highlights that the magnitude of EIMD experienced is greater in XX homozygotes (Clarkson et al. 2005; Deuster et al. 2013; Pimenta et al. 2012; Seto et al. 2011; Venckunas et al. 2012; Vincent et al. 2010), *ACTN3* could still play a role in the inter-individual variability apparent within EIMD responses (Garton et al. 2013).

With regards to human physical performance, the angiotensin-I converting enzyme (*ACE*) gene has been studied more extensively than any other (Ma et al. 2013). ACE is a zinc metallopeptidase and is recognised as being integral to the renin-angiotensin system (RAS)

(Rigat et al. 1990). ACE activity has a key role in the regulation of blood pressure in humans (Kem and Brown, 1990) and a functional polymorphism of *ACE* has been identified within intron 16 of the gene on chromosome 17 (Rigat et al. 1990). ACE is an *indel* polymorphism, where an absence (deletion, D) rather than presence (insertion, I) of a 287 amino acid base pair ALU repeat sequence is associated with increased concentrations of tissue serum ACE activity (Rigat et al. 1990; Danser et al. 1995). Accordingly, this polymorphism results in the II, ID and DD genotypes with the respective frequencies among Caucasian adults of approximately 25%, 50% and 25% (Myerson et al. 1999). Previously, one study has found associations with the *ACE* I/D polymorphism and EIMD in humans (Yamin et al. 2007), where it was observed that II homozygotes elicited the highest CK response after strenuous exercise, compared to the DD homozygotes. However, in contrast, Heled et al. (2007) did not find any association between the *ACE* I/D polymorphism and CK response following EIMD. As *ACE* D-allele carriers have a decreased bradykinin half-life, it has been suggested that they may have an attenuated nerve growth factor expression following EIMD and therefore a decreased pain sensitivity. Further, the octapeptide hormone angiotensin-II, which is synthesized from angiotensin-I, may indirectly mediate skeletal muscle damage by influencing angiogenesis in response to exercise (Vaughan et al. 2013). Given the potential influence of the *ACE* I/D genotype on recovery from EIMD, it may help explain some of the inter-individual variability observed following strenuous exercise.

The *TTN* gene is responsible for encoding the largest documented protein to date, which is the third most abundant protein within the myofilament of human skeletal and cardiac muscle (Stebbings et al. 2017; Vikhlyantsev and Podlubnaya, 2012; Fürst et al. 1988). The *TTN* filament has many functional roles within human striated muscle including contraction and cell signalling (Chauveau et al. 2014). Within human striated muscle, seven splice isoforms of *TTN* exist, each differing in size and elasticity (Chauveau et al. 2014; Vikhlyantsev and Podlubnaya, 2012). Although several skeletal muscle diseases have been associated with mutations in the *TTN* gene (Chauveau et al. 2014), one non-pathological polymorphism has been identified

and demonstrated associations with an exercise-related phenotype in healthy, untrained Caucasians (Timmons et al. 2010; Rankinen et al. 2004). A missense C > T transition identified within *TTN* (rs10497520) has been reported to contribute to the variability in the training response of maximal oxygen consumption (Timmons et al. 2010) and stroke volume (Rankinen et al. 2003). Further, associations have been found for shorter skeletal muscle fascicle lengths within T-allele carriers and faster marathon running times in habitually trained men (Stebbings et al. 2017). Fascicle length has a significant influence on the force-velocity and force-length relationships and, by extension, may alter muscle function (Timmins et al. 2016). As a previously injured muscle possesses shorter fascicles in comparison to an uninjured muscle (Timmins et al. 2015), this could lead to a reduced maximal shortening velocity of the injured muscle (Timmins et al. 2016). It is also hypothesised that muscle fascicle lengths have some influence on the force-length relationship; however, evidence in humans is limited (Lieber and Ward, 2011; Lieber and Friden, 2000). It has been suggested that a previously injured muscle, which is identical to an uninjured muscle, will have a reduced working range as a result of fewer sarcomeres in-series (Brockett et al. 2001). Thus, this may increase the volume of work being completed on the descending limb of the force-length relationship, where a reduced force-generating capacity may result in an increased potential for EIMD (Lieber and Ward, 2011; Lieber and Friden, 2000). To date, no evidence exists as to whether the C > T transition within the *TTN* gene (rs10497520) plays a role in the inter-individual variability of EIMD response following half-marathon running.

Therefore, the aims of this Chapter are; (1) to examine the inter-individual variability in response to EIMD induced by a half-marathon event with NZBC or placebo supplementation using the SWC and response CI's. (2) To examine the association between the SNPs *ACTN3*, *ACE* and *TTN* and the responses to EIMD induced by a half-marathon event with NZBC or placebo supplementation.

The following hypothesis were tested:

- As no group benefit was observed in Chapter 3 with NZBC extract supplementation on recovery from EIMD following a half-marathon event, that in utilising the SWC and response CI's, this would elucidate that some participants did experience positive responses on recovery from EIMD.
- Runners with the null X allele for ACTN3, T allele for TTN and D (deletion) for ACE would show greater losses in muscle function when compared to the homozygous RR, CC and II counterparts, respectively.

4.3 Methods

4.3.1 Participants, experimental design, half-marathon event, supplementation, dietary control and countermovement jumps

Please refer to Section 3.3.1 and 3.3.2 for the participant characteristics and experimental design, respectively. Details on the half-marathon event are described in Section 3.3.3 Information regarding the timing of NZBC extract supplement intake and blinding procedures can be found in Section 3.3.4. Details on the dietary control are described in Section 3.3.5 and details on the CMJ measurement are described in the previous Section 3.3.6.

4.3.2 Saliva collection

Participants provided a saliva sample into the issued Oragene® DNA Self-Collection Kit (OG-500 Disc Format, DNA Self-Collection Kit, Genotek, Ottawa, Ontario, Canada). Participants were instructed to follow the manufacturer's instructions for saliva collection, which were to 1) avoid food or fluid consumption 30 minutes before providing a saliva sample into the tube; 2) spit enough saliva to reach an indicated level on the container; 3) push down securely the preservative cap; 4) unscrew the collection cap and screw on the container cap securely; 5) invert the container for at least 5 seconds. Saliva samples were then stored at room temperature until genotyping analysis. The yield of DNA from saliva obtained through self-

collection kits has been shown to be comparable of that through blood samples (Abraham et al. 2012). All participants completed saliva collection successfully and collection tubes were coded and labelled to ensure participant anonymity in accordance with the Human Tissue Act (2004).

4.3.3 DNA isolation

The isolation of DNA from the saliva samples was carried out in accordance with the manufacturer guidelines (Oragene® DNA Self-Collection Kit, OG-500 Disc Format, DNA Self-Collection Kit, Genotek, Ottawa, Ontario, Canada). Accordingly, the saliva collection tubes were incubated at 50^{o}C in a water incubator (Grant Instruments™, Cambridge, UK) for 1 h to ensure that the DNA was adequately released and that nucleases were permanently inactivated. A 500 µL aliquot of each sample was pipetted into a 1.5 mL microcentrifuge tube and 20 µL of PrepIT (PT-L2P, Oragene®, Genotek, Ottawa, Ontario, Canada) was added and vortexed for 5 s, ensuring that any impurities and inhibitors were precipitated. The 500 µL aliquots were then placed on ice for 10 min and centrifuged at room temperature for 5 min at 15,000 x *g* to remove impurities. The clear supernatant was transferred into a fresh microcentrifuge tube, with the remaining pellet containing any impurities being discarded. A 600 µL of room temperature 98% ethanol was added to 500 µL of supernatant and inverted 10 times to mix. The samples were then allowed to stand at room temperature for 10 minutes, allowing the DNA to be precipitated. The samples were centrifuged for 2 min at 15,000 x *g* before the supernatant was removed and discarded, leaving the DNA pellet in the microcentrifuge tube. The samples were then washed with 250 µL of 70% room temperature ethanol and allowed to stand for 1 min, before having all the ethanol removed. The remaining pellet was allowed to stand and dry completely before having 100 µL of tris-ethylenediamine tetraacetic acid (TE) solution added to dissolve the DNA pellet. The samples were vortexed for 5 s to ensure complete dissolvement of the DNA pellet and incubated at 50^{o}C for 1 h in a water incubator. Finally, the fully rehydrated DNA sample aliquots were stored at -20^{o}C until later analysis.

4.3.4 DNA genotyping

The DNA yield in samples was quantified using a NanoDrop® 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) with absorbance being read at 320, 280 and 260 nm. The corrected A_{280} and A_{260} values were obtained from subtracting the 320 nm (A_{320}) from the A_{280} and A_{260} values, with the DNA yield in ng·µL being calculated from the ratio of the A_{280} and A_{260} values following Beer-Lambert law. The average DNA yield was 10.6±0.3 ng·µL (mean±SD), which was sufficient for both polymerase chain reaction (PCR) and real-time polymerase chain reaction (qPCR). For all SNP assays, 10 ng of extracted DNA was used for amplification. All samples were analysed in duplicate to minimise the occurrence of genotyping errors known to negatively affect the statistical power of genetic association studies (Tintle et al. 2009) and 100% agreement between all duplicate samples was achieved. Participant genotypes for the retrospective SNP (*ACTN3, ACE* and *TTN*) analysis can be located in Table 4.1.

4.3.5 α-actinin-3 (ACTN3)

The 291 bp fragment of exon 16 of the *ACTN3* gene was amplified by PCR using the forward primer 5'-CTGTTGCCTGTGGTAAGTGGG-3' and the reverse primer 5'-TGGTCACAGTATGCAGGAGGG-3' as recommended by Mills et al. (2001). The PCR was carried out in 0.5 mL PCR tube strips with each tube containing a reaction volume of 25 µL using an Applied Biosystems™ Veriti™ 96-well thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR reaction cycle for both sets of primers was: 95ºC for 5 min, 95ºC for 45 s, 60ºC for 45 s, 72 ºC for 45 s, for 35 cycles, with a final extension of 72 ºC for 10 min. The amplified PCR fragments were subsequently digested with *DdeI* restriction enzyme (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK) and the digested PCR fragments were separated on a 6% native polyacrylamide gel electrophoresis, which was then stained with ethidium bromide and photographed under UV light (G:BOX®, Syngene, Cambridge, UK) and viewed in GeneSnap® (Syngene, Cambridge, UK). The alleles 577R and 577X were distinguished by the presence (577X) or absence (577R) of a *DdeI* restriction site. Digestion

of PCR products of the 577X allele yields bands 108, 97 and 85 bp fragments, whereas digestion of PCR products of the 577R allele yields bands of 205 and 86 bp fragments (Figure 4.1).

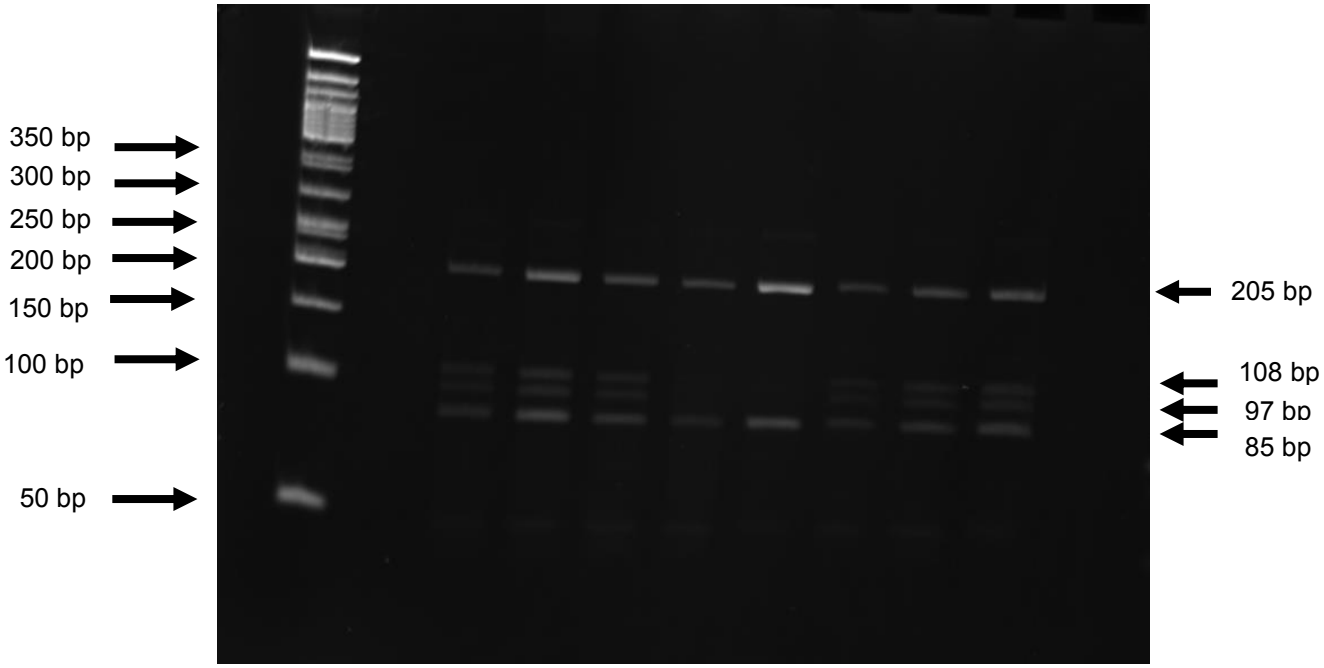


Figure 4.1 ACTN3 gel electrophoresis with 50 bp DNA ladder (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK). Lane 1, DNA maker; Lane 2, negative control; Lane 3, cut participant; Lane 4, cut participant; Lane 5 cut participant; Lane 6, cut participant; Lane 7, cut participant; Lane 8, cut participant; Lane 9, cut participant; Lane 10, cut participant.

4.3.6 Angiotensin-I converting enzyme (ACE)

The 288 bp ALU fragment in intron 16 of the *ACE* gene was amplified by PCR using the forward 1 primer 5'-CCCATTTCTCTAGACCTGCT-3', the forward 2 primer 5'-TGGGATTACAGGCGTGAT-3' and reverse primer 5'-AGAGCTGGAATAAAATTGGC-3' as recommended by Minucci et al. (2013). The PCR was carried out in 0.5 mL PCR tube strips with each tube containing a reaction volume of 25 µL using an Applied Biosystems™ Veriti™ 96-well thermal cycler (Thermo Fisher Scientific, Waltham, MA, USA). The PCR reaction cycle for sets of primers was: 95ºC for 5 min, 95ºC for 30 s, 55ºC for 30 s, 72 ºC for 30 s, for 35 cycles, with a final extension of 72 ºC for 5 min. After the PCR step, total volume was loaded

on a 6% native polyacrylamide gel, electrophoresed and then stained with ethidium bromide, photographed under UV light (G:BOX®, Syngene, Cambridge, UK) and viewed in GeneSnap® (Syngene, Cambridge, UK). The three major haplotypes; homozygotic insertion (II), heterozygotic (ID) and homozygotic deletion (DD) were then identified. II homozygotes had an insertion at 356 and 60 bp. ID heterozygotes had an insertion at 356, 68 and 60 bp. DD homozygotes had an insertion at 68 bp (Figure 4.2).

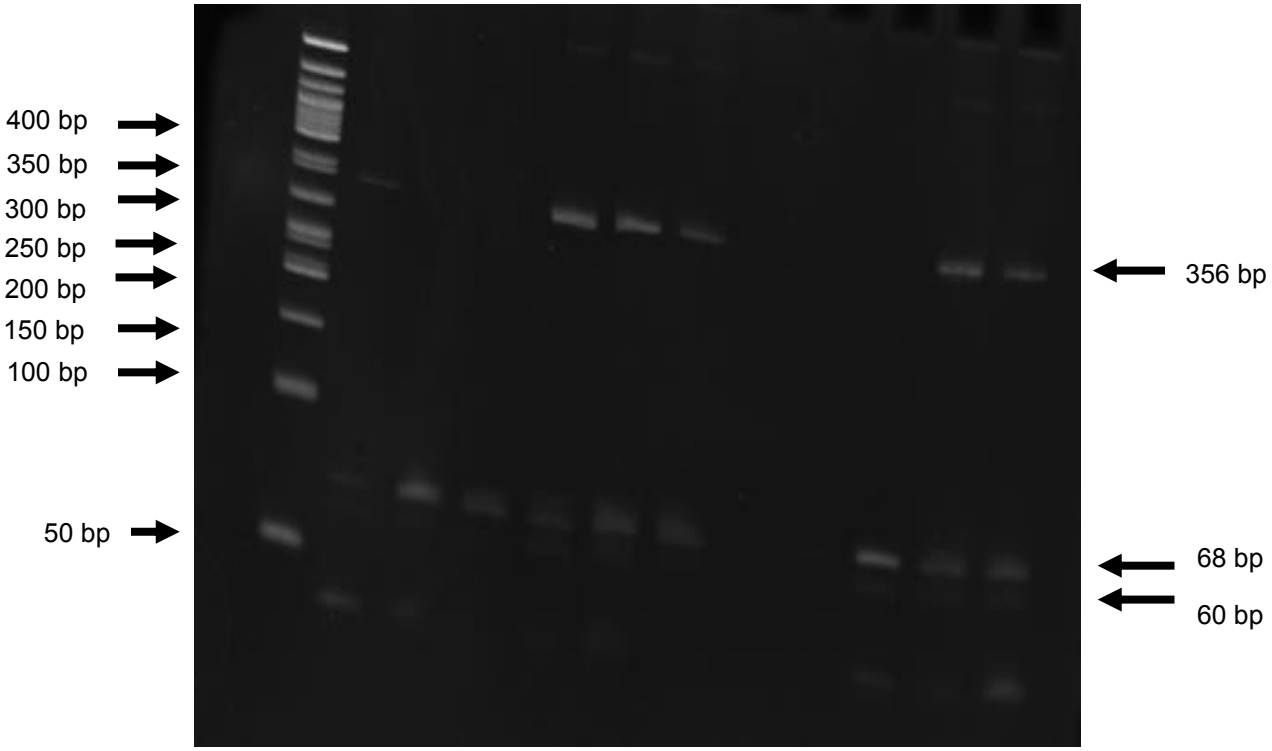


Figure 4.2 ACE gel electrophoresis with 50 bp DNA ladder (New England Biolabs UK Ltd., Hitchin, Hertfordshire, UK). Lane 1, DNA maker; Lane 2, cut participant; Lane 3, uncut participant; Lane 4, uncut participant; Lane 5 cut participant; Lane 6, cut participant; Lane 7, cut participant; Lane 8, negative control; Lane 9, negative control; Lane 10, uncut participant; Lane 11, cut participant; Lane 12, cut participant.

4.3.7 Titin (TTN)

Each participant was genotyped for the *TTN* rs10497520 polymorphism using the fluorophore-based detection technique of TaqMan® qPCR. This technique requires the amplification of a segment of genomic DNA overlapping the specific polymorphism being genotyped. To achieve amplification, forward primers were used to identify the starting point of the genomic DNA segment and reverse primers to identify the endpoint (Primer Design®, Chandlers Ford, UK). Allele-specific probes, identified by either VIC® or ROX® fluorophore attached to their respective complementary sequences (CC and TT homozygote, respectively). After amplification the emitted fluorescent dye that was detected by the qPCR machine (ViiA™ 7 qPCR, Applied Biosystems®, Life Technologies™, USA). Real-time PCR was carried out in 96-well plates with each well containing a reaction volume of 10 µL. The reaction volume for genotyping *TTN* contained 2.5 µL of participant DNA, 5 µL of Precision®PLUS genotyping master mix (Primer Design®, Chandlers Ford, UK), 2 µL of nuclease-free H_2O (Primer Design®, Chandlers Ford, UK), 0.5 µL of primer/probe mix (Primer Design®, Chandlers Ford, UK). Control wells were included on each 96-well plate, in which nuclease-free H_2O (Primer Design®, Chandlers Ford, UK) replaced the DNA sample for the negative control and wild-type and homozygous SNP DNA replaced the DNA sample for the positive controls (Primer Design®, Chandlers Ford, UK). Each 96-well plate was sealed with an optical adhesive seal (Primer Design®, Chandlers Ford, UK) and DNA amplification completed on the ViiA™ 7 96-well qPCR (Applied Biosystems®, Life Technologies™, USA). DNA amplification was completed using the following qPCR protocol: denaturation for 2 mins at 95° C, followed by 15 cycles of incubation for 10 s at 95° C, 35 cycles of primer annealing at 95° C for 10 s, extension for 1-minute at 55° C and plate read. Genotypes were determined by measurement of the end-point fluorescence of VIC® and ROX® detected by the qPCR machine. Results were subsequently analysed using a computer interfaced with software supplied by the manufacturer of the qPCR machine (QuantStudio®, Applied Biosystems®, Life Technologies™, USA) (Figure 4.3).

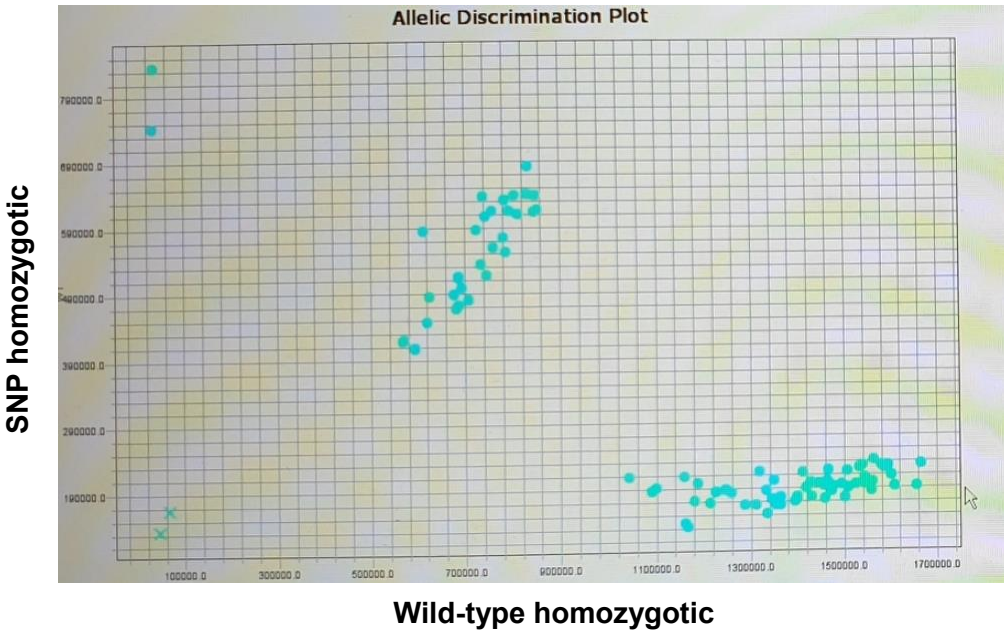


Figure 4.3 Allelic discrimination plot for determination of TTN homozygote wildtype (CC), heterozygote mutant (CT) and homozygote mutant (TT). Note: two observable points on SNP homozygotic axis are positive controls and two crosses are negative controls.

Table 4.1 Summary of participant genotypes.

Participant	*ACTN3*	*ACE*	*TTN*
1	RX	II	CC
2	XX	ID	CC
3	RX	ID	CC
4	RX	ID	CC
5	RR	II	CT
6	RX	ID	CC
7	RX	ID	CC
8	RX	II	CT
9	RX	ID	CC
10	RX	ID	CT
11	RR	II	CC
12	RX	II	CT
13	RX	II	CC
14	RX	ID	CC
15	RX	DD	CC
16	RR	DD	CT
17	RR	ID	CC
18	RX	ID	CT
19	RX	ID	CT
20	RR	ID	CT

Total for each single nucleotide polymorphism (SNP), alpha-actinin-3 (*ACTN3*) RR = 5, RX = 14, XX = 1; angiotensin-I converting enzyme (*ACE*) II = 6, ID = 12, DD = 2; titin (*TTN*) CC = 12, CT = 8, TT = 0; RR II, CC, homozygous dominant; RX, ID, CT, heterozygous dominant; XX, DD, TT, homozygous recessive.

4.3.8 Data analysis

To address the first aim of this present Chapter, data for each parameter were assessed for normal distribution with the Shapiro-Wilk test and the homogeneity of variance of each genotype was assessed using Levene's test. Hardy-Weinberg equilibrium was determined for

the *ACTN3, ACE* and *TTN* SNPs using a Chi-Square (X^2) test. As no group differences were apparent for NZBC vs. PLA from the repeated measures ANOVA in Chapter 3, supplementation groups were treated as the covariate to allow for independent assessment of any potential associations of genotype with the independent variables across time. Two-way mixed ANCOVAs [within-subjects factor: time (pre-exercise, post-exercise, 24 and 48 h post exercise; between-subjects factor: genotype (RR, RX; II, ID; CC, CT, respectively); covariate: group (NZBC or PLA)] with Bonferroni post-hoc tests were used to detect associations between the *ACTN3*, *ACE* and *TTN* SNPs and CMJ outcome and neuromuscular variables, muscle soreness and fatigue and urinary IL-6 response before and following a half-marathon event. Differences in participant group and genotype characteristics and predicted and actual half-marathon times were analysed with independent samples Student's *t*-tests. Statistical analyses were completed using Statistical Package for Social Sciences 23.0 (SPSS, Chicago, Illinois). The alpha level for statistical significance was set at 0.05 *a priori*. Partial-eta^2 (ηp^2) effect sizes (ANOVA) with respective 90%CI are reported to indicate the magnitude of observed effects (Lakens, 2013). Partial-eta^2 (ηp^2) effect sizes of 0.01–0.06, 0.06–0.14 or ≥0.14 are considered small, medium and large changes, respectively (Lakens, 2013).

To address the second aim of this present Chapter, in a separate trial conducted before data collection, the absolute test re-test reliability of each CMJ outcome and neuromuscular variable was established (Table 4.2). The typical error of measurements (TE) was determined using the methods recommend by Swinton et al. (2018), and true score 95% confidence intervals (CI) were then constructed using the TE corrected by sample size (Swinton et al. 2018). To aid inference of the graphical representations, a descriptive interpretation is provided (Bonafiglia et al. 2018). The SWC was calculated as 0.2 x *SD*, representing a 'small' effect size and the smallest meaningful decrement in performance (Swinton et al. 2018; Copay et al. 2007), plus the SEM (Table 4.2).

Data in text and tables are reported as mean (95%CI) and data in figures as mean with individual data points (Weissgerber et al. 2015) for group and genotype effects and mean individual observed score with true 95%CI and SWC for group inter-individual variation (Swinton et al. 2018; Bonafiglia et al. 2018), unless otherwise stated.

4.4 Results

There were no differences in participant characteristics and predicted half-marathon finishing time (P>0.05). Data relating to group differences and dietary intake is in Chapter 3, Table 3.1 and Table 3.3, respectively.

4.4.1 Countermovement jump inter-individual responses to half-marathon running

Table 4.2 Reliability statistics for CMJ outcome and neuromuscular variables.

CMJ variable	CV (%)	ICC	SEM
Outcome			
Jump height (m)	6	0.87	0.01
RSImod (index)	8	0.84	0.02
Time to take off (s)	5	0.88	0.05
Neuromuscular			
Concentric peak force (N)	5	0.69	0.38
Concentric average power (W)	4	0.69	0.90
Concentric net impulse (Ns/kg^{-1})	4	0.87	0.08
Concentric phase duration (s)	5	0.83	0.02
Eccentric peak force (N)	9	0.83	0.56
Eccentric net impulse (Ns/kg^{-1})	6	0.92	0.07
Eccentric displacement (braking phase) (m)	9	0.83	0.01
Eccentric phase duration (s)	7	0.79	0.01

CMJ, countermovement jump; RSImod, reactive strength index modified; CV, coefficient of variation; ICC, interclass coefficient; SEM, standard error of measurement.

Immediately post the half-marathon, 2 of the participants in the PLA group demonstrated a negative meaningful response for CMJ height (Figure 4.4. panel A). Further, 2 participants in the PLA group demonstrated a negative meaningful response immediately post the half-marathon for CMJ TTT (Figure 4.4. panel A). No other negative or positive meaningful responses to the half-marathon were apparent for CMJ height or TTT in either NZBC or PLA (Figure 4.4). All other responses for CMJ height and TTT, remained unchanged for both NZBC and PLA (Figure 4.4).

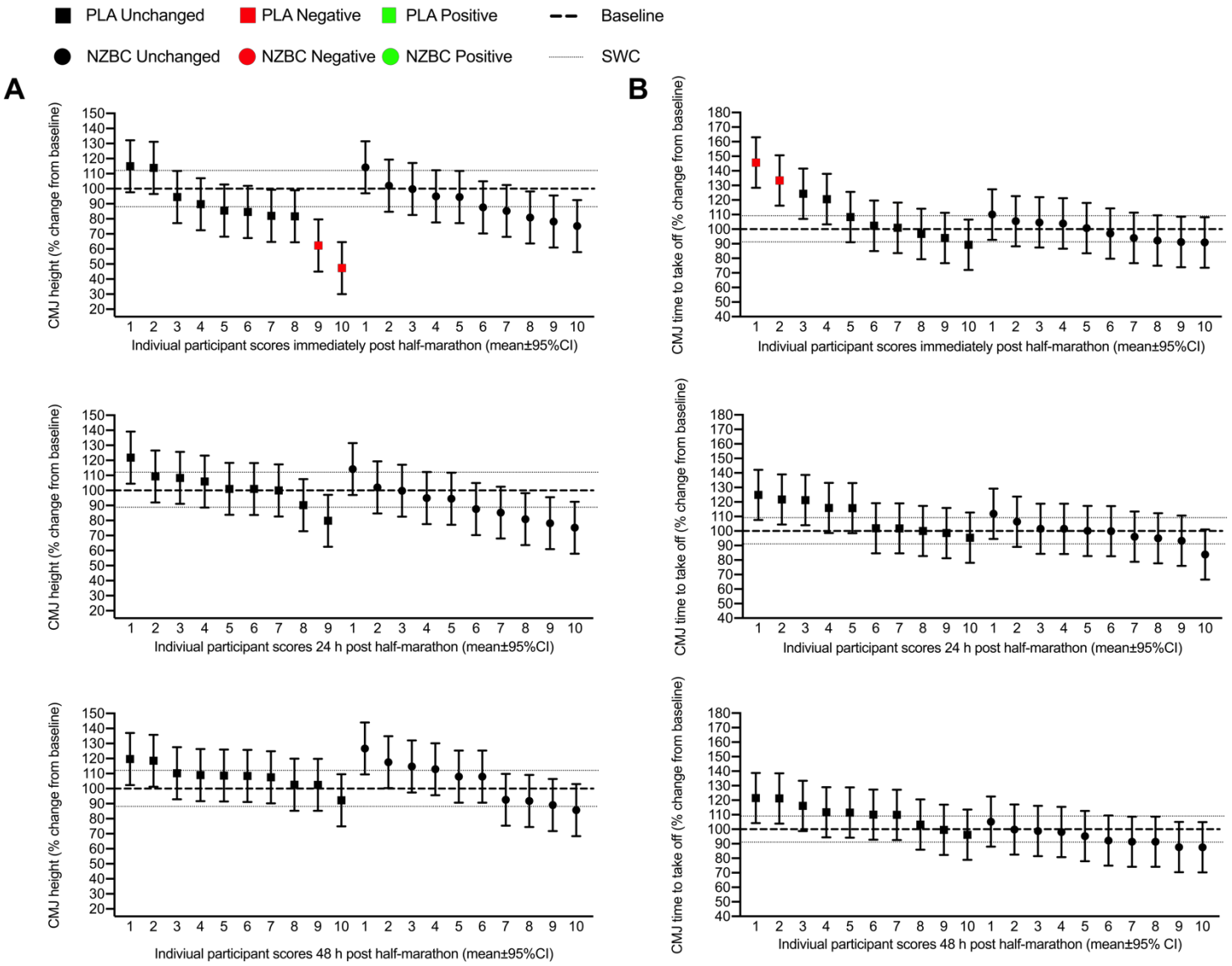


Figure 4.4 Individual observed values for CMJ height (panel A) and TTT (panel B) immediately post, 24 and 48 h after a half-marathon event with 95% true score CI's. Smallest worthwhile change (SWC) are calculated as 12% and 9% for CMJ height and time to take off, respectively. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched across panels and are presented in ascending order based on response estimates. Data are n=10 per group.

Immediately post the half-marathon, 2 participants in PLA and 1 participant in the NZBC group demonstrated a negative meaningful response for CMJ concentric power (Figure 4.5. panel A). At 24 h post the half-marathon, 1 participant in both the PLA and NZBC group demonstrated negative meaningful responses for CMJ concentric power (Figure 4.5. panel A). At 48 h post the half-marathon, 1 participant in the NZBC group demonstrated a positive meaningful response for CMJ concentric power (Figure 4.5. panel A). Immediately post the half-marathon, 2 participants in PLA and 10% in the NZBC group demonstrated a negative meaningful response for CMJ eccentric peak force (Figure 4.5. panel B). At 24 h post the half-marathon, 3 participants in PLA demonstrated a negative meaningful response for CMJ eccentric peak force (Figure 4.5. panel B). At 48 h post the half-marathon, 1 participant in both the PLA and NZBC group demonstrated negative meaningful responses for CMJ eccentric peak force (Figure 4.5. panel B). All other CMJ concentric power and eccentric peak force responses remained unchanged for both NZBC and PLA (Figure 4.5).

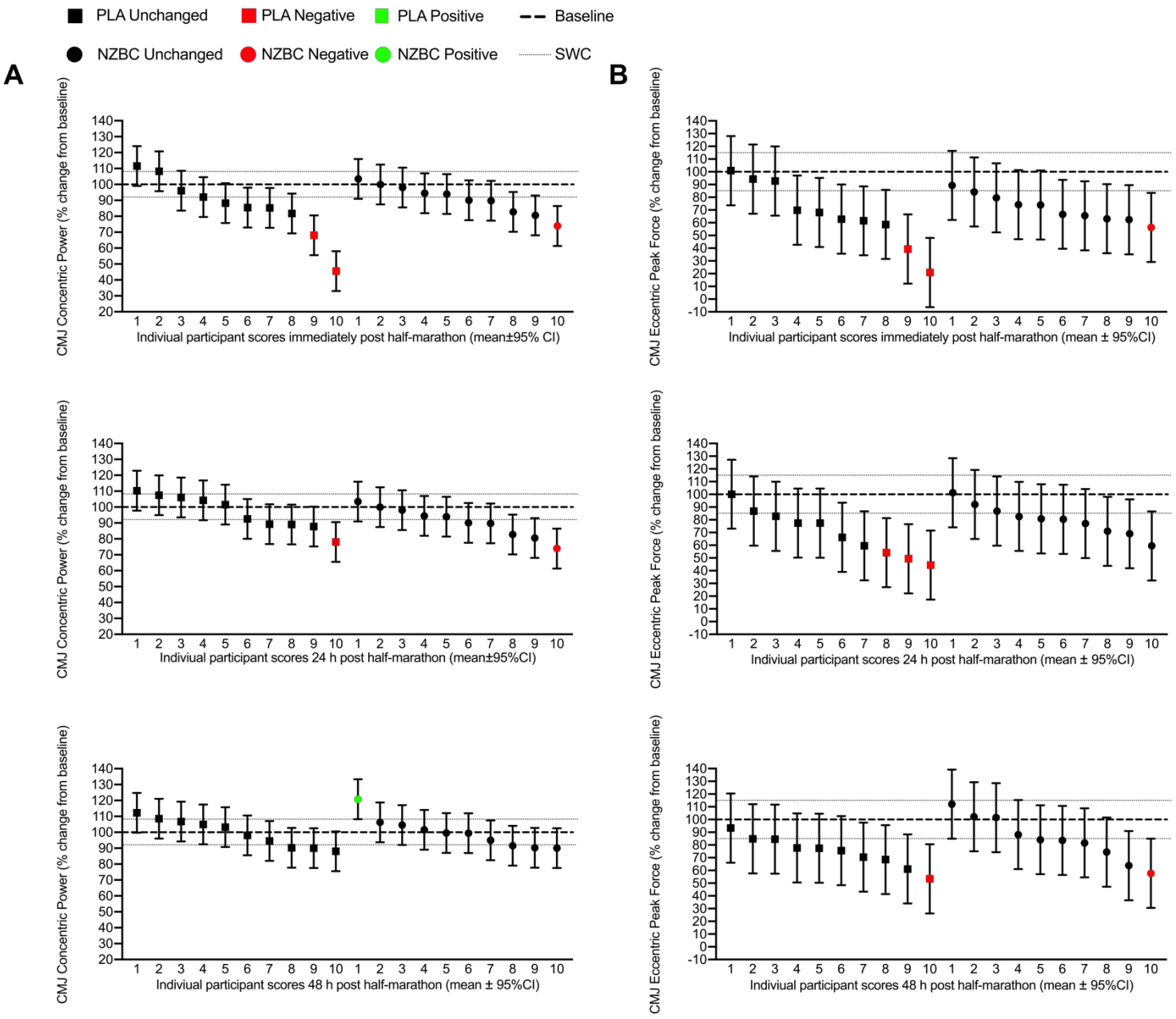


Figure 4.5 Individual observed values for CMJ concentric average power (panel A) and eccentric peak force (panel B) immediately post, 24 and 48 h after a half-marathon event with 95% true score CI's. Smallest worthwhile change (SWC) are calculated as 8% and 15% for CMJ concentric average power and eccentric peak force, respectively. **Red**, **black** and **green** circles represent **negative**, **unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched across panels and are presented in ascending order based on response estimates. Data are n=10 per group.

At 24 h post the half-marathon, 2 participants in PLA group demonstrated negative meaningful responses for CMJ eccentric net impulse (Figure 4.6. panel A). Further, at 24 h post the half-marathon, 1 participant in NZBC group demonstrated a positive meaningful response for CMJ eccentric net impulse (Figure 4.6. panel A). At 48 h post the half-marathon, 1 participant in NZBC demonstrated a negative meaningful response for CMJ eccentric net impulse (Figure 4.6.e). Immediately post the half-marathon, 3 participants in PLA and 4 participants in NZBC group demonstrated negative meaningful responses for CMJ eccentric phase duration (Figure 4.6. panel B). At 24 h post half-marathon, 3 participants in PLA and 1 participant in NZBC group demonstrated negative meaningful responses for eccentric phase duration (Figure 4.6. panel B). At 48 h post half-marathon, 4 participants in PLA and 1 participant in NZBC demonstrated negative meaningful responses for eccentric phase duration (Figure 4.6. panel B). All other CMJ eccentric net impulse and phase duration responses remained unchanged for both NZBC and PLA (Figure 4.6).

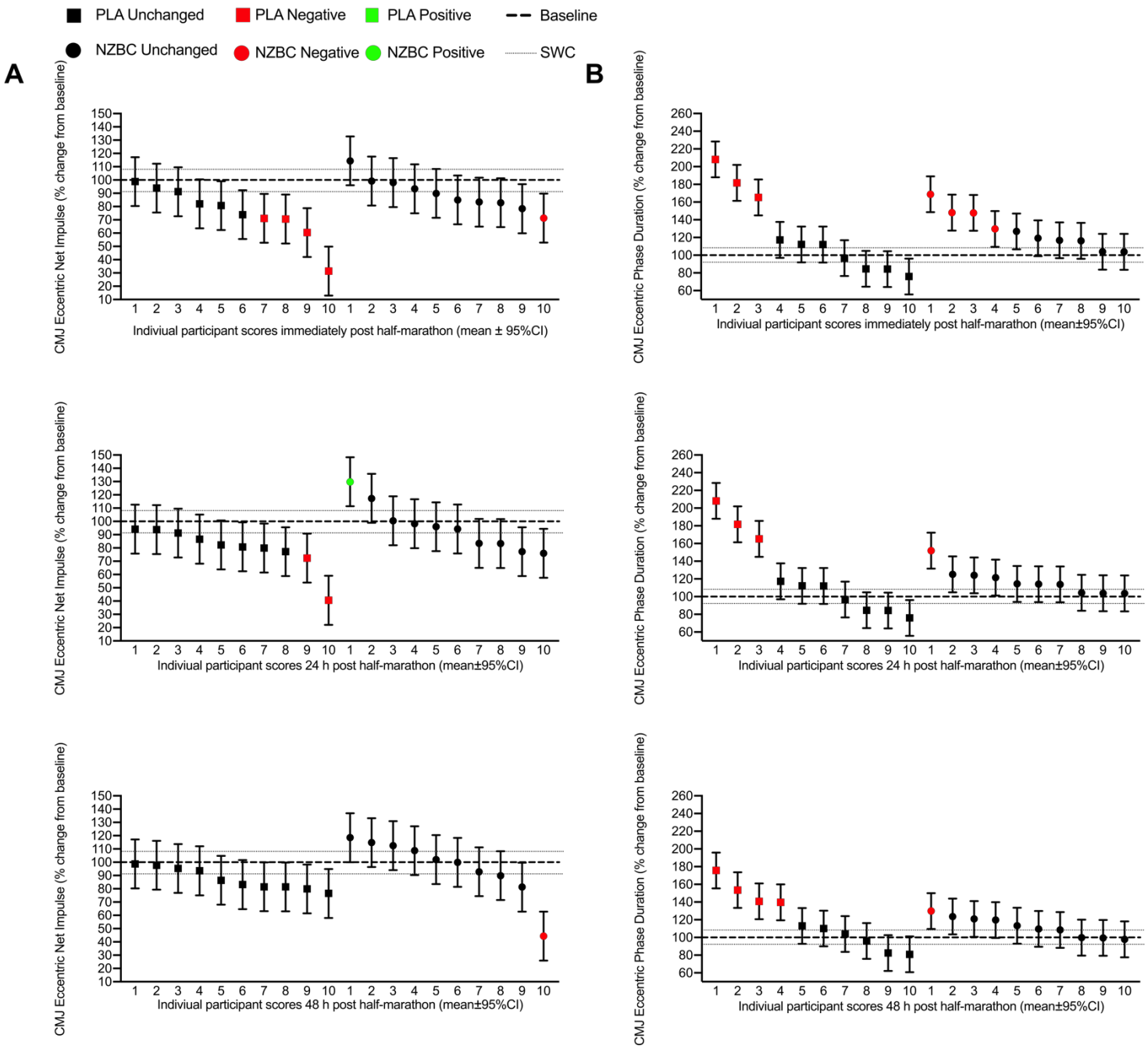


Figure 4.6 Individual observed values for CMJ eccentric net impulse (panel A) and eccentric phase duration (panel B) immediately post, 24 and 48 h after a half-marathon event with 95% true score CI's. Smallest worthwhile change (SWC) are calculated as 8% for both CMJ eccentric net impulse and eccentric phase duration. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched across panels and are presented in ascending order based on response estimates. Data are n=10 per group.

4.4.2 SNP associations with markers of EIMD following half-marathon running

Genotype frequency distribution for the *ACTN3* (rs1815739, X^2=3.505, *P*=0.173), *ACE* (rs4341, X^2=2.400, *P*=0.301) and *TTN* (rs10497520, X^2=0.800, *P*=0.371) SNPs were in Hardy-Weinberg equilibrium. Due to the low numbers of *ACTN3* XX homozygotes (n=1), *ACE* DD homozygotes (n=2) and *TTN* TT homozygotes (n=0) present in the sample population, these data were combined with those heterozygotes (RX, ID and CT, respectively) as has been previously recommended (Stebbings et al. 2017; Del Coso et al. 2017a; Del Coso et al. 2017b). No differences between *ACTN3, ACE, TTN* genotypes were observed for predicted (*P*=0.825, *P*=0.631, *P*=0.208, respectively) and actual run time (*P*=0.167, *P*=0.288, *P*=0.761, respectively).

There were main effects of time for *ACE* with CMJ height (Figure 4.7. panel A), *ACTN3, ACE,* and *TTN* with eccentric peak force (Figure 4.9. panel B), *ACTN3* and *TTN* with eccentric phase duration (Figure 4.10. panel B), in both NZBC and PLA groups. Post-hoc comparisons revealed that CMJ height was reduced immediately post the half-marathon compared to baseline (*P*=0.007) for *ACE* only. Further, post-hoc comparisons showed that eccentric peak force was reduced immediately post, 24 and 48 h following the half-marathon for *ACTN3* (*P*=0.001, *P*=0.001, *P*=0.001, respectively)*, ACE* (*P*=0.001, *P*=0.001, *P*=0.001, respectively*,* and *TTN* (*P*=0.002, *P*=0.001, *P*=0.001, respectively). Post-hoc comparisons revealed that the time taken to complete the eccentric phase of the CMJ increased immediately post, 24 and 48 h post for *ACTN3* (*P*=0.007, *P*=0.017, *P*=0.0034, respectively) and *TTN* (*P*=0.010, *P*=0.014, *P*=0.023, respectively). There were no significant genotype main effects or associations with any CMJ variable for *ACTN3, ACE* o*r TTN* (*P*>0.05) (Figure 4.7, 4.8, 4.9 and 4.10).

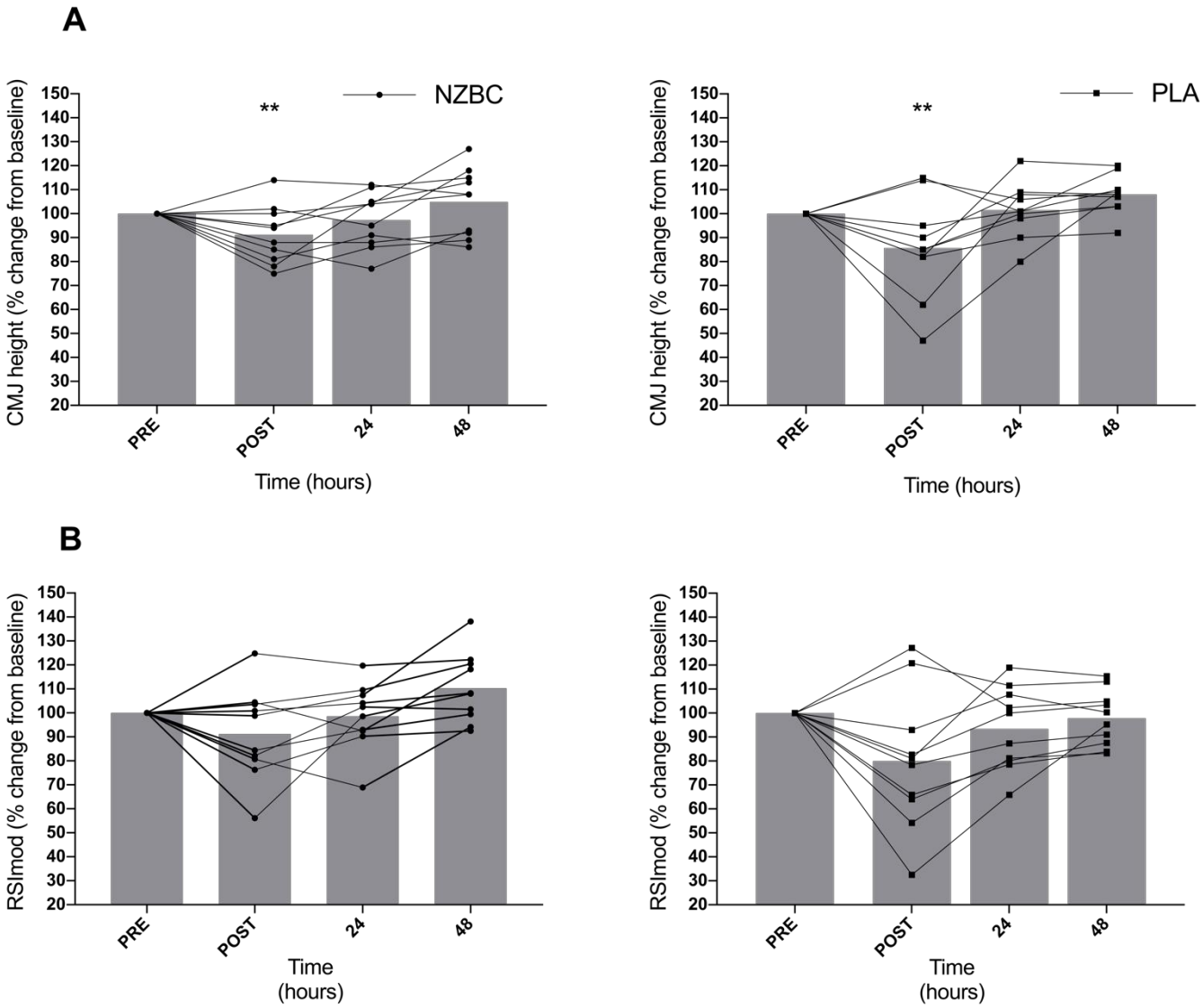


Figure 4.7 Percentage changes in CMJ height (panel A) and RSImod (panel B), immediately pre-, post-, 24 and 48 h following a half-marathon event. Double asterisk (**) indicates a main effect of time for ACE genotype (P<0.05); data are presented as mean (bars) and individual data points (n=10 per group).

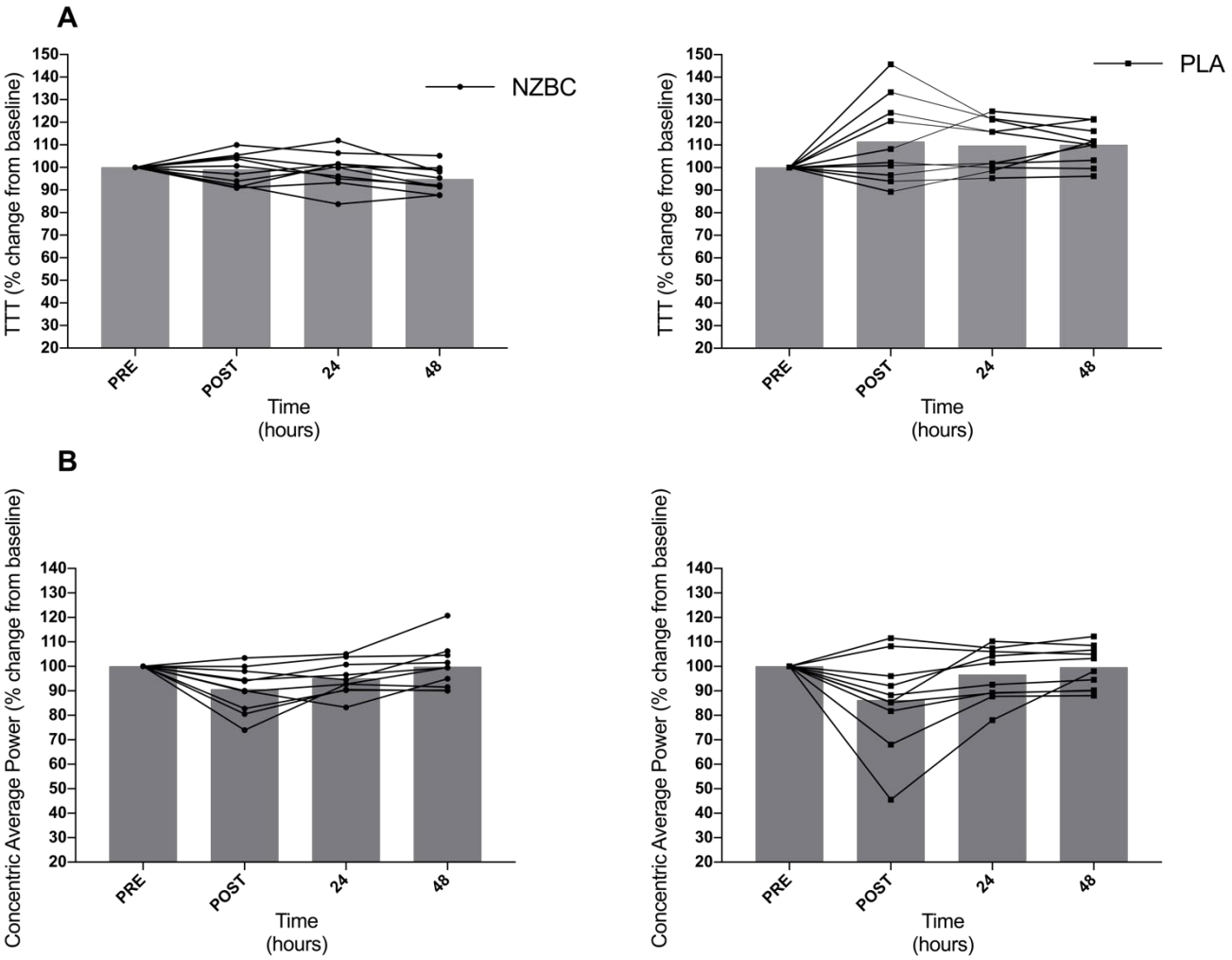


Figure 4.8 Percentage changes in CMJ TTT (panel A) and Concentric Average Power (panel B) immediately pre-, post-, 24 and 48 h following a half-marathon event. Double asterisk (**) indicates a main effect of time for ACE genotype (P<0.05); data are presented as mean (bars) and individual data points (n=10 per group).

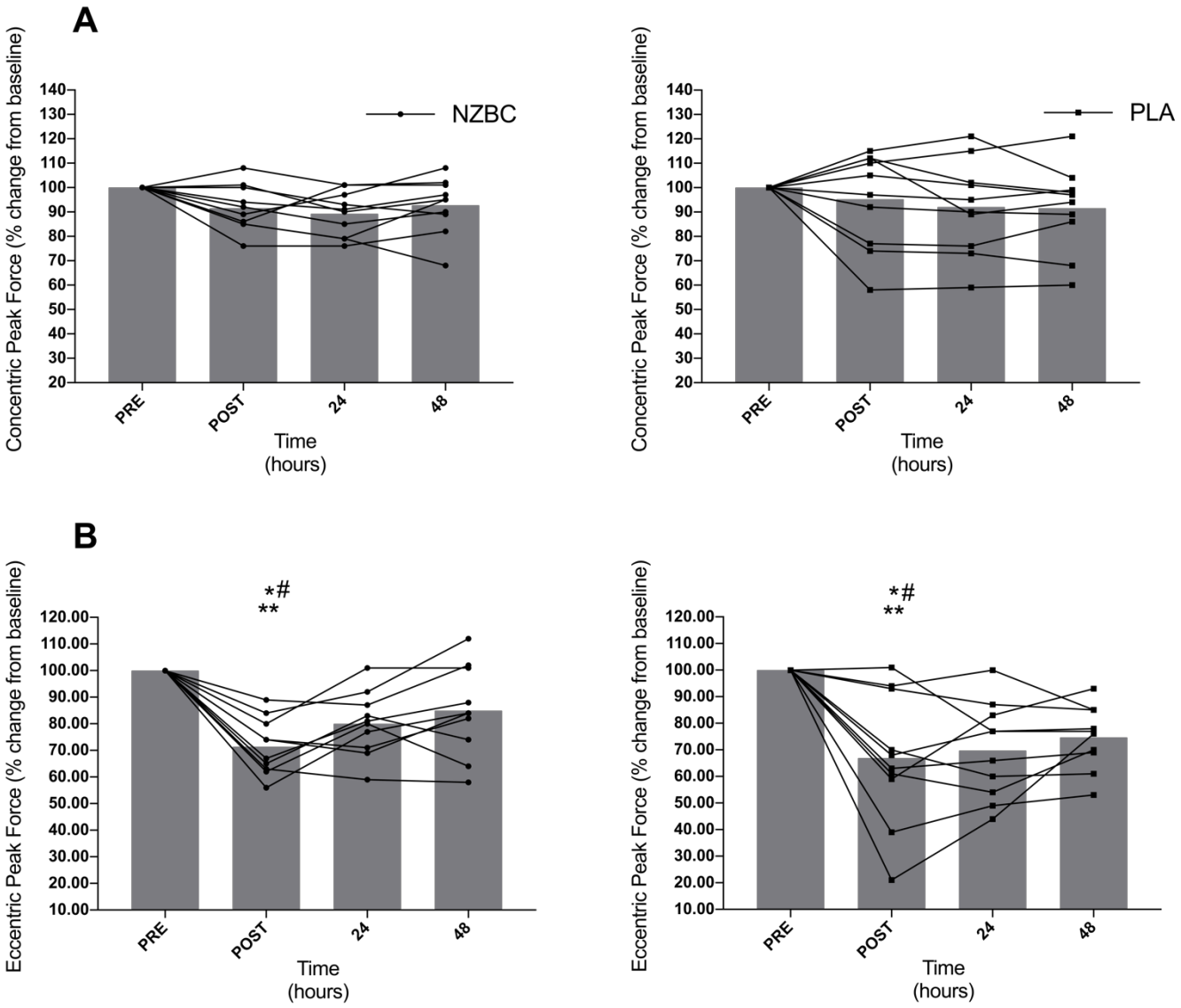


Figure 4.9 Percentage changes in CMJ concentric peak force (panel A) and eccentric peak force (panel B) immediately pre-, post-, 24 and 48 h following a half-marathon event. *, **, and # indicate a main effect of time for ACTN3, ACE and TTN genotype, respectively (P<0.05); data are presented as mean (bars) and individual data points (n=10 per group).

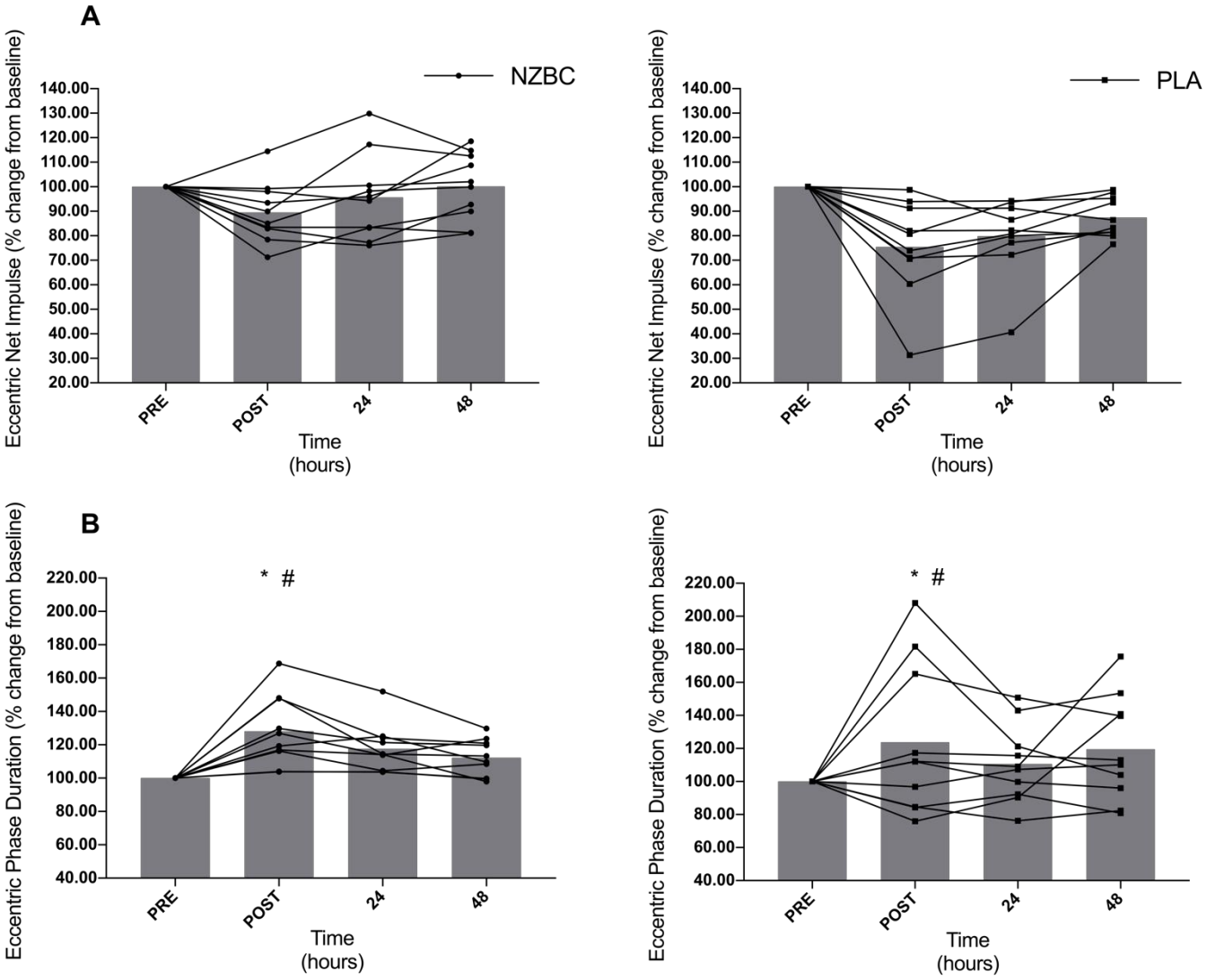


Figure 4.10 Percentage changes in CMJ eccentric net impulse (panel A) and eccentric phase duration (panel B) immediately pre-, post-, 24 and 48 h following a half-marathon event. *, **, and # indicate a main effect of time for ACTN3, ACE and TTN genotype, respectively (P<0.05); data are presented as mean (bars) and individual data points (n=10 per group).

Between-subjects effects were apparent for *ACTN3* with eccentric average peak force and net impulse and *ACE* with concentric average power (Table 4.3). There were no other between subject effects for *ACTN3, ACE* or *TTN* for any other CMJ variables (P>0.05).

Table 4.3 ACTN3 and ACE genotype significant between-group responses for CMJ performance and neuromuscular variables following half-marathon.

SNP	rs-number	Between subjects-factor	Beneficial (*n*)	Detrimental (*n*)	*P*-Value	Effect Size (ηp^2) (90% CI)
ACTN3	1815739	Eccentric average peak force	RR (NZBC: 3; PLA: 2)	RX (XX) (NZBC: 7; PLA: 8)	0.031	0.244 (0.01–0.467)
		Eccentric net impulse			0.026	0.259 (0.02–0.480)
ACE	4341	Concentric average power	II (NZBC: 4; PLA: 2)	ID (DD) (NZBC: 6; PLA: 7)	0.029	0.249 (0.01–0.471)

SNP, single nucleotide polymorphism; CMJ, countermovement jump; *ACTN3*, alpha-actinin-3; *ACE*, angiotensin-I converting enzyme; RR, homozygous dominant; RX, heterozygous dominant; XX, homozygous recessive; II, homozygous dominant; ID, heterozygous dominant; DD, homozygous recessive.

Table 4.4 ACTN3 and ACE genotype group responses pre and post half-marathon running. Values are mean (95%CI). 95%CI have been adjusted for sample size. ACTN3 (n=5, RR; n=15, RX) and ACE (n=6, II; n=14, ID).

SNP (rs-number)	CMJ Variable	Pre Half-Marathon	Post Half-Marathon	24 h post Half-Marathon	48 h post Half-Marathon
ACTN3	Eccentric average peak force (N/kg)				
(1815739)	RR	11.94 (8.44–15.44)	7.65 (6.32–8.97)	9.07 (8.02–10.12)	9.69 (7.62–11.76)
	RX	9.99 (8.38–11.59)	6.52 (5.85–7.19)	7.08 (6.26–7.90)	7.69 (6.51–8.87)
	Eccentric net impulse (Ns/kg)				
	RR	1.16 (0.86–1.45)	0.93 (0.77–1.09)	1.07 (0.84–1.31)	1.10 (0.85–1.35)
	RX	0.99 (0.87–1.11)	0.79 (0.69–0.89)	0.82 (0.73–0.92)	0.86 (0.78–0.95)
ACE	Concentric average power (W/kg)				
(4341)	II	22.27 (17.58–26.97)	19.51 (15.59–23.44)	21.53 (17.54–25.53)	21.84 (17.91–25.76)
	ID	18.93 (16.74–21.13)	16.37 (14.69–18.05)	17.91 (15.94–19.89)	18.94 (16.63–21.24)

SNP, single nucleotide polymorphism; CMJ, countermovement jump; *ACTN3*, alpha-actinin-3; *ACE*, angiotensin-I converting enzyme; RR, homozygous dominant; RX, heterozygous dominant; II, homozygous dominant; ID, heterozygous dominant.

4.5 Discussion

The purpose of the present Chapter was to investigate the inter-individual variability in response to half-marathon running with NZBC extract or placebo supplementation. Firstly, by examining the inter-individual variability in response to EIMD induced by a half-marathon event with NZBC extract or placebo supplementation using the SWC and response CI's. Secondly, by examining the association between the candidate SNPs ACTN3, ACE and TTN and the responses to EIMD induced by a half-marathon event with NZBC or placebo supplementation. The main findings from this study were that, in general, more individuals within the NZBC group appeared to recover eccentric peak force, net impulse, phase duration and concentric power quicker following the half-marathon than PLA, supporting the first hypothesis tested. Secondly, in partial support of the second hypothesis, the *ACTN3* and *ACE* genotypes appeared to influence the recovery of eccentric peak force, eccentric net impulse and concentric power, respectively, but no differences could be detected between NZBC and PLA groups. However, this observation is a preliminary finding and requires further studies with a much larger sample size than in the present investigation, to enable definitive conclusions to be drawn.

4.5.1 Inter-individual responses on recovery from half-marathon running with NZBC supplementation

Typically, in sports medicine research, analysis of the presented data is carried out at the group level focusing on the mean response (Buford et al. 2013). Whilst this approach allows researchers to determine whether an intervention has been 'successful' or not, this approach does not recognise that contributions documented at the group level may not fully apply to each individual member of that group (Burford et al. 2013). Further, the importance of assessing individual responsiveness to various treatments including food supplements is often overlooked both in research and on the field (Burke, 2019). Evaluation of the individual participant data may reveal more information than simply looking at the group-level data (Buford et al. 2013). Hecksteden et al. (2017) attempted to develop individualized reference

ranges to individualise monitoring of muscle recovery in athletes by using a Bayesian approach comparable to that developed for the Athlete Biological Passport (Sottas et al. 2007; Sottas et al. 2012). However, truly Bayesian statistics are not yet in mainstream use and its application requires learning a new framework of data analysis that is complex (Hecksteden et al. 2018).

Alternatively, other researchers have adopted highlighting the extent of individual variation by reporting 'absolute' individual response ranges to a given intervention by using bar graphs to depict rank-ordered individual response (Bouchard and Rankinen, 2001; Hautala et al. 2006; McPhee et al. 2010). However, two factors that are typically omitted when presenting these results are the measurement error and the SWC associated with the particular measurement (Mann et al. 2014). Firstly, measurement error may explain a degree of individual variation in responses when comparing pre versus post values; individual changes that fall within the typical error of a measurement may be as a result of noise in the measurement and thus, cannot necessarily be interpreted as individual variation in EIMD responses (Mann et al. 2014; Swinton et al. 2018). Second, the SWC, sometimes referred to as the minimum clinically important difference (MCID), can be defined as a threshold value that lies beyond zero and represents the smallest change required to be practically relevant (Copay et al. 2007; Swinton et al. 2018). Although using these values together can be deemed a conservative approach (Bonafiglia et al. 2018), they are required to avoid individuals obtaining observed score changes greater than the SWC due to the randomness of measurement error alone (Swinton et al. 2018). Furthermore, it has been recommended that the use of confidence intervals (CI) of certain widths alongside the observed score, can help guide statistical inference as CI provide information to quantify uncertainty in estimates that cannot be directly measured (Bonafiglia et al. 2018; Swinton et al. 2018). Therefore, a 95%CI provides a range of plausible values given the observed data where the true value is likely to be obtained 84.3% of the time in the long run (Cumming and Maillardet, 2006).

Utilising the recommended approaches of Swinton et al. (2018) and Bonafiglia et al. (2018), it was observed that, despite there being no observed effect of NZBC extract supplementation at the group level on recovery following a half-marathon with NZBC, individuals receiving NZBC extract supplementation appeared to recover CMJ eccentric peak force, duration, net impulse, and jump height quicker than individuals in PLA. However, when considering the combined SWC and TE of the different CMJ variables selected (i.e., jump height; time to take off), it is clear by visual inspection of the 95%CI plots, that many individuals in both NZBC and PLA group did not exceed these thresholds and so this brings into question whether the CMJ is a sensitive enough tool to detect meaningful changes in the population in the present study. Nonetheless, this Chapter documents for the first time the inter-individual responses to EIMD induced by a half-marathon event with NZBC extract or placebo supplementation utilising the SWC and 95%CI response. Large CI (95%) were utilised in this Chapter to increase the confidence with which individuals could be classified as having 'negative, unchanged or positive meaningful response' but this likely increased the proportion of individuals who were classified as having 'unchanged responses'. However, this decision was made due to the fact that utilising narrower CI, such as 50%, inflates the type 1 error rate (i.e., classifying individuals as having had a positive or negative meaningful response, when in fact there may not have been). As it is not possible to use a single CI width that simultaneously protects against both type 1 and type 2 errors, future studies need to decide which type of error they are more willing to risk making when classifying individual responses (Bonafiglia et al. 2018). Further, the 95%CI constructed use the TE to provide a range of plausible true scores given the observed data. However, what cannot be said is that if these same individuals were to repeat this experiment, they would reproduce the same responses/values again (Senn, Rolfe and Julious, 2011).

4.5.2 Genotype associations with time

In Chapter 3, main effects of time were apparent for CMJ jump height, eccentric peak force and phase duration indicating muscle damage was present but as no group or interaction

effects were evident for NZBC or PLA, supplementation group was used as a covariate herein, so that genotype association on recovery from half-marathon running could be independently assessed.

4.5.3 ACTN3 genotype associations on recovery from half-marathon running

In addition to being associated with a greater susceptibility to EIMD, XX homozygotes of the *ACTN3* gene are thought to possess the unfavourable genotype for fast and powerful muscle contractions (Del Coso et al. 2018). Yang et al. (2003) was one of the first to document the underrepresentation of *ACTN3* 577XX genotype in sprint athletes, when compared to a control population of healthy untrained individuals. This observation has since been replicated in several elite athlete cohorts where there is a higher frequency of the R allele in sprint and power disciplines (Eynon et al. 2009, 2013; Alfred et al. 2011; Kikuchi et al. 2015; Roth et al. 2008; Ginszt et al. 2018; Niemi and Majamaa, 2005). Further, untrained RR homozygotes have been found to possess a higher baseline strength compared to XX homozygotes (Walsh et al. 2008; Clarkson et al. 2005; Erskine et al. 2014). Previously, Bosco and Komi (1979) observed that in the CMJ, jump height and net impulse outcome were related to the percentage of fast twitch muscle fibres in the vastus lateralis. Further, a previous cross-sectional study revealed that individuals with greater levels of lower-body strength, were able to produce higher peak force, power, velocity and displacement outputs (Cormie et al. 2009). These theoretical differences stem from the neuromuscular adaptations evident with prolonged strength-power training including increased cross-sectional area of type II fibres, selective motor unit recruitment, improved firing frequency, and synchronisation (Fitts et al. 1991; Komi, 1986; Kyröläinen et al. 2005). All together, these findings may explain our observations where RR homozygotes appeared to have higher levels of relative eccentric peak force and net impulse at all time points compared to RX heterozygotes (Table 4.2). As *ACTN3* expression is found only in the type II skeletal muscle fibre (Mills et al. 2001) and those who are homozygous for the X allele are unable to express *ACTN3* in those fibres, it is plausible that RX heterozygotes have a tenuous disadvantage in the CMJ movement pattern

compared to RR homozygotes. However, this is purely speculation and further research with a larger sample size, particularly with more XX homozygotes, is needed.

With regards to EIMD following the half-marathon, it would appear that RX heterozygotes experienced the greatest losses in force immediately post, 24 and 48 h following the half-marathon in eccentric peak force (~30%, 27%, and 22% of baseline, respectively) compared to RR homozygotes (~32%, 20% and 16% of baseline, respectively) (Table 4.4). However, this is a preliminary observation and future studies are warranted with larger sample sizes before any firm conclusions can be made. These findings are in accordance with previous literature where X-allele carriers presented with greater reductions in jump height and higher concentrations of serum CK following a marathon (Del Coso et al. 2017) and a half-ironman (Del Coso et al. 2016). Despite it being widely accepted that slow-twitch muscle fibres are preferentially recruited during endurance-based tasks (Asp et al. 1999; North, 2008), it has been suggested that *ACTN3* plays a role during the eccentric phase of endurance exercise that confers a higher capacity to the muscle to resist EIMD (Del Coso et al. 2018). However, previous research studying the association of *ACTN3* genotypes on markers of EIMD following an eccentric elbow flexion exercise, did not find any associations between genotypes (Clarkson et al. 2005). As EIMD typically occurs following unaccustomed exercise that involves large amounts of eccentric contractions (Clarkson and Hubal, 2002), it is perhaps not surprising that decrements in the eccentric phase of the CMJ were apparent following the half-marathon event. Within the CMJ, the eccentric phase is a fundamental component of the stretch shortening cycle (SSC) and neuromuscular (NM) function (Nicol et al. 2006). The SSC fatigue immediately decreases NM function through metabolic disturbances, impaired EC coupling, and a stretch-reflex sensitivity-related reduction in muscle stiffness (Nicol et al. 2006; Avela et al. 1999). Decreased reflex sensitivity is one process that is believed to protect fatigued muscle fibres from further damage (Avela et al. 1999). These mechanisms primarily affect eccentric function and so may have contributed to the decreased eccentric peak force and net impulse at post, 24 and 48 h following half-marathon in both RR homozygotes and

RX heterozygotes. However, *ACTN3* genotype for eccentric peak force and net impulse only account for 24% and 26%, respectively (Table 4.3), of the total variance observed, thus may only be a small part of a much bigger picture.

4.5.4 ACE genotype associations on recovery from half-marathon running

Most of the evidence to date examining *ACE* insertion/deletion (I/D) polymorphism has focused on associations with physical performance such as endurance capacity (Montgomery et al. 1998; Ma et al. 2013), where the I-allele has been deemed superior, and with muscular strength (Williams et al. 2005), where the D-allele has been deemed superior. When considering susceptibility to EIMD, to date only two studies have investigated the influence of the *ACE* I/D polymorphism on contraction-induced damage in humans (Heled et al. 2007; Yamin et al. 2007). Yamin et al. (2007) observed different concentrations of plasma CK between *ACE* genotypes after eccentric exercise: II homozygotes elicited the highest CK response, whilst DD homozygotes elicited the lowest CK activity after strenuous exercise, therefore suggesting that the I-allele is associated with a greater susceptibility to EIMD. However, Heled et al. (2007) did not find any association between *ACE* I/D polymorphism and CK response following strenuous exercise. The different outcome is likely attributed to the moderate-intensity exercise test and higher activity level and different ethnicities of the participants in Heled et al (2007). A limitation of both of these studies is that only CK concentration was measured as a marker of EIMD, which is only one of several indirect biomarkers of EIMD. As a result, it is difficult to directly compare our findings with that in the literature. Nevertheless, considering Yamin et al. (2007) conclusion that II homozygotes were more susceptible to EIMD than their DD homozygote counterparts, our findings contradict this with II homozygotes exhibiting less concentric average power loss than ID heterozygotes (Table 4.2). *ACE* II homozygotes exhibited marginally smaller decrements in concentric average power at all time points (post, 24 h and 48 h following half-marathon) compared to ID heterozygotes (11%, 3% and 1% vs. 12%, 5% and 0%, respectively). However, the *ACE*

genotype for concentric average power only accounted for 25% (Table 4.2), of the total variance observed, thus this outcome is likely to only play a small role in the observation.

Previous rodent model studies have demonstrated how blocking of the angiotensin-II receptor type 1 improves regeneration of injured skeletal muscle (Bedair et al. 2008) and suppresses reactive oxygen species (ROS) production following strenuous exercise (Sim et al. 2014). Furthermore, nerve growth factor up-regulation through activation of B_2 bradykinin receptors is strongly associated with increased pain sensitivity (Murase et al. 2010; Babenko et al. 1999). A possible benefit of the *ACE* enzyme D-allele, which causes a decreased bradykinin half-life, is that it could have attenuated nerve growth factor expression following EIMD and therefore decreased pain sensitivity. Attenuated substance P and bradykinin in the inflammatory process may explain the high frequency of D-allele carriers among elite strength/power athletes (Costa et al. 2009). However, within this current study, no associations between *ACE* genotype and perceptions of muscle soreness or fatigue were observed. Our inability to detect any other associations with other markers of EIMD may have been down to small sample size in question, particularly with the low frequency of DD homozygotes, thus, further research is needed to explore the potential role of *ACE* genotype in susceptibility to EIMD.

4.5.5 Limitations

The present Chapter presents some limitations derived from the experimental design chosen that have to be discussed to improve the scope and applicability of the outcomes. Firstly, examining individual responses utilising the methods proposed by Swinton et al. (2018) and Bonafiglia et al. (2018), a limitation of distribution-based approaches such as the SWC, is the fact that they are sample specific in the sense that the SWC value depends on the variability of the scores in the studied samples. Further, the calculation of individual 95%CI is dependent on the certainty of the TE estimate and assumes that the effect of TE on observed measures is random (i.e., repeated measures normally distribute around the true value and that there is no fixed or proportional bias) (Bonafiglia et al. 2018; Ludbrook, 1997).

Second, this study retrospectively studied the genotype of *ACTN3, ACE* and *TTN* for each of the participants in an independent groups design (i.e., between-subject comparisons). Thus, the sample size is far from optimal and participants were not matched on their genotypes. Given the low frequency of the minor allele for *ACTN3, ACE* and *TTN* homozygotes (i.e., XX, DD, TT) in Caucasian populations, it is perhaps not surprising that we were unable to find many, if any, in our sample. For example, based on the T-allele for the *TTN* genotype that was observed within the present study, future studies would require at least 200 participants to obtain approximately 50 TT homozygotes. However, this Chapter did not intend on evaluating the multitude of interactions that may occur between involved genes and their protein products (Noble, 2011), but instead, aimed to examine any possible associations between the candidate SNPs and markers of EIMD to help elucidate some of the individual variation observed within the data from Chapter 3. Conversely, using this approach meant using the supplementation groups as a covariate in the analysis due to the lack of a group effect of NZBC or PLA, thus, this approach was adopted as it allowed examination across the multiple time points and the multiple time points of interest (PRE, POST, 24 h and 48 h), between SNP genotypes. Future research should look to implement a 'stress the genotype' approach and recruit a large number of participants and screen them for their respective genotypes before allocating them to intervention groups as has been done previously (Montgomery et al. 2002). Utilising such an approach would allow researchers to appropriately explore gene-environment interactions and establish whether specific genotypes increase an individual's susceptibility to EIMD following strenuous exercise.

4.6 Conclusion

A key challenge facing sports medicine professionals is to determine the high inter-individual variability in the response to exercise and nutrition interventions for the development of inexpensive individualised health management programmes to prevent injuries and lengthy recovery periods (Burke, Jones, Jeukendrup and Mooses, 2019; Buford et al. 2013). The data presented in this Chapter suggests that common polymorphisms in the *ACTN3* and *ACE* gene

may explain part of the individual variation observed within the CMJ neuromuscular variables eccentric peak force and net impulse and concentric average power, respectively, during recovery following a half-marathon event. However, no associations could be observed for the SNP within the *TTN* gene. Despite it being frequently discussed that an individual's genotype likely plays a role in their susceptibility to EIMD (Clarkson et al. 2005; Baumert et al. 2016), future research should look to i) increase the sample size as much is logistically possible to ensure adequate representation of both major and minor allele frequencies for candidate SNPs, ii) use a more extensive selection of candidate SNPs to assess for any potential associations with susceptibility to EIMD.

The individual statistical approaches used in the present Chapter simply characterized whether or not individuals experienced positive meaningful responses and are not designed to determine the cause of each individual's response. It is important to note that the statistical approaches used in the second part of this Chapter represent an application of magnitude-based inferences (MBI). Unlike traditional null-hypothesis testing, MBI appraises effect sizes relative to pre-determined thresholds to gauge whether a given treatment should be implemented (Hopkins and Batterham, 2016). Although the statistical principles underlying MBI have been heavily debated (Hopkins and Batterham, 2018; Sainani, 2018), this debate has focused on performing MBI for group-level analysis. At present, statistical approaches for classification of individual response that do not rely on MBI are lacking and this represents an important area for future research. Despite the aforementioned limitations, application of the statistical approaches used within this Chapter based on Swinton et al. (2018) and Bonafiglia et al. (2018), have the potential to improve statistical inference and evidence-informed exercise and nutrition prescription decision-making compared to threshold-based dichotomous classifications of 'responder' and 'non-responder' that assumes responses to interventions are binary, when they are in fact likely to be more graded in nature (Sisson et al. 2009).

To further investigate whether NZBC supplementation has any potential recovery benefits following muscle damaging exercise, Chapter 5 will examine the effects of NZBC supplementation under repeated crossover, laboratory-controlled conditions in an attempt to reduce some of the variation between supplement and placebo groups observed in Chapters 3 and 4. Further, Chapter 5 will utilise a broader range of measures that can assess recovery from EIMD to build on the observations in the current Chapter.

5. The Effect of New Zealand Blackcurrant Extract on Recovery from Muscle Damage Induced by Drop Jumps

5.1 Abstract

Introduction. New Zealand blackcurrant (NZBC) is a rich source of anthocyanins. Limited evidence is available as to whether anthocyanin-rich supplements can aid recovery in the days following EIMD. The aim of this study was to examine if NZBC extract supplementation improves recovery following EIMD induced by drop jumps. **Methods.** Twelve recreationally active male volunteers (mean±SD: age 29±6 years, stature 1.80±0.07 m, body mass 78.0±10.7 kg) were pre-conditioned to eccentric exercise by performing a 100-drop jump protocol (100-DJP). Then, following a double-blind, repeated crossover design, the 12 participants ingested either 600 mg·day^{-1} capsules with a NZBC extract (CurraNZ™; containing a total of 210 mg anthocyanins) or a visually matched placebo (PLA) 7-days prior and 3-days after completing a 100-DJP. Measures of MVIC of knee extensors, electrically stimulated (ES) contractions, CMJ, perceived muscle soreness, serum interleukin-6 (IL-6) and prostaglandin-E2 (PGE_2) were made pre- (baseline), immediately-, 24-, 48- and 72 h-post the 100-DJP. MVIC, ES, CMJ, muscle soreness, serum IL-6 and PGE_2 variables were analysed using a two-way repeated measures ANOVA with significance set *a priori* at $P<0.05$. **Results.** The MVIC peak force was reduced immediately-post 100-DJP, compared to baseline (NZBC: 90±10; PLA: 93±11%; $P=0.001$), but returned to baseline at 24 h ($P>0.05$) with no difference between groups ($P=0.940$). The ES doublet peak force was reduced compared to baseline immediately post, 24, 48 and 72 h following 100-DJP ($P<0.05$) with no difference between groups ($P=0.798$). Perceived muscle soreness increased immediately-post 100-DJP for both NZBC (4±2) and PLA (3±1) ($P=0.001$) and returned to baseline by 72 h-post ($P>0.05$), with no difference between groups ($P=0.404$). All other ES contraction variables, CMJ, PGE_2 and IL-6 were unaffected by the 100-DJP or NZBC extract ($P>0.05$). **Conclusion.** NZBC extract did not affect the recovery of MVIC or ES doublet peak force, perceptions of muscle soreness or inflammation following EIMD induced by 100-DJP in recreationally active males and large inter-individual variation in responses were present.

5.2 Introduction

Chapter 3 demonstrated that at the group level there was no effect of NZBC on EIMD following a half-marathon event. However, Chapter 4 highlighted that there was individual variation between participants in the magnitude of the changes in the markers of EIMD. The half-marathon study reported in Chapter 3 and 4 used an independent groups design during a competitive running event. This may have contributed to the large variability observed within both the control and intervention groups and limited comparisons between groups at the individual level.

Whilst the use of a half-marathon race as part of the exercise protocol used in Chapters 3 and 4 had high ecological validity, it also meant that the level of experimental control was limited and only three measures (CMJ, self-perceptual muscle soreness and fatigue and urinary IL-6) were used as markers of EIMD. Thus, in an attempt to reduce the variability and increase the number and fidelity of measurements, the present Chapter sought to investigate the effects of NZBC extract supplementation in a laboratory setting using a standardised protocol previously shown to induce EIMD (100 drop-jumps) using a randomised placebo controlled cross-over design.

The magnitude of muscle damage is often less after individuals have been exposed to an initial bout of the same or similar exercise stimulus for up to six months afterwards and is termed the RBE (Hubal et al. 2008; McHugh et al. 2003; Nosaka and Clarkson, 1995; Chen et al. 2019). In order to eliminate the influence of the RBE on the randomised cross-over design, the experimental design in this Chapter required participants to complete a familiarisation/pre-conditioning session where they completed 100 drop-jumps at least 14 days before the 7-day intake of the supplement of placebo began.

As discussed in Sections 2.3.1 – 2.3.3, EIMD has been reported to present in a biphasic response. The primary damage is related to physical damage to the muscle ultrastructure and/or impairments in EC coupling, which occurs during the exercise stimulus and in the 12 h

following, are likely the main stimuli responsible for initiating the muscle damage process (Hyldahl and Hubal, 2014; Howatson and van Someren, 2008; Warren et al. 2002; Clarkson and Hubal, 2002). The secondary damage, which occurs around 12 h to up to 7-days following the exercise stimulus, is associated with transient increases in biomarkers of inflammation and oxidative stress (Peake et al. 2017; Close et al. 2004; Nikolaidis et al. 2008), muscle soreness (Warren et al. 1999; Clarkson and Hubal, 2002), impaired range of movement (Warren et al. 1999) and marked and prolonged declines in muscle function (Warren et al. 1999; Paillard, 2008). Whilst the inflammatory cascade during the secondary damage phase have been suggested to be part of the key adaptive/remodelling response (Butterfield et al. 2006; Chazaud, 2016), it may counterintuitively exacerbate the magnitude of damage via the release of cytotoxic RNOS and lysosomal proteases and elastases (Pizza et al. 2005; Toumi and Best, 2003) and cause additional physical damage to myofibrils (Lapointe et al. 2002; Pizza et al. 2005). Thus, the concept of utilising a nutritional intervention to accelerate recovery from EIMD, typically focus on the secondary cascade that targets the inflammatory response (Bowtell et al. 2011; Howatson et al. 2010; Sousa et al. 2013).

As reviewed in Section 1.1, NZBC extract is a rich source of anthocyanins and *in vitro,* anthocyanins have been observed to disrupt reactive oxygen species (ROS) formation through the donation of hydrogen atoms, interrupting the free radical chain reaction and subsequent formation (Nimse and Pal, 2015). The phenolic structure of anthocyanins allows ROS to donate a proton, thereby preventing ROS oxidation (Nimse and Pal, 2015). Additionally, anthocyanins inhibit the inflammatory enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), displaying similar anti-inflammatory properties to non-steroidal anti-inflammatory drugs (NSAID) (Seeram et al. 2001).

In order to accurately assess the efficacy of a polyphenol supplement as a recovery intervention following EIMD, it is important to utilise a range of measures that can capture information about the improvement of muscle function, both voluntary and involuntary, muscle soreness and the resolution of the inflammatory cascade. Thus, measures that are able to

assess changes in muscle function, muscle soreness and systemic markers of inflammation were utilised in the present Chapter; MVIC, CMJ, ES contractions, perceived muscle soreness, serum IL-6 and PGE_2, to ensure that a more comprehensive picture was obtained of the recovery process following EIMD induced by the 100-DJP.

The aim of this study was to examine whether intake of NZBC extract 7-days before and 3-days following 100-DJP affect the recovery of markers of EIMD.

The following hypothesis was tested:

- Given its high anthocyanin content, NZBC extract would help to mitigate the inflammatory cascade, thereby facilitating a faster recovery of muscle function and muscle soreness.

5.3 Methods

5.3.1 Participants

Twelve healthy non-resistance trained men volunteered to participate (age 29±6 years, height 1.80±0.07 m, body mass 78.0±10.7 kg, sum of four skinfold thickness 35.7±12.3 mm, baseline MVC 497±120 N). Using the findings of previous studies that examined group differences in isometric strength following drop-jumps (Clifford et al. 2015), it was calculated using G*Power that at 80% power, and an α of 0.05, at least 12 volunteers were required to detect a group difference of 10% (using change from MVIC peak force baseline data) (7.5% SD, based on percentage change from baseline data) at any time points post the muscle damaging exercise.

To assess study eligibility, participants completed a health screening questionnaire; those with any known food allergies, cardiovascular or gastrointestinal complaints, musculoskeletal injury or receiving prescribed anti-inflammatory medications were excluded from participating (n=0). The use of any putative recovery treatments (i.e., massage, cold water therapy, compression garments) and nutritional supplements, including anti-inflammatory medications were prohibited for the duration of the study. Participants were instructed to limit their physical

activity outside of the trial requirements in the 48 h prior to and throughout data collection. Lastly, participants were asked to refrain from consuming any food or drink (water *ab libitum*) for two-hours prior to data collection but were encouraged to consume a normal meal after the testing block to avoid pre or post-prandial performance effects on the consecutive testing days. In addition, participants were asked to avoid any caffeinated rich foods or beverages on the morning of the data collection as caffeine is known to reduce an individual's perception of fatigue (Burke, 2008). The study was approved by the University of Chichester Research Ethics Committee and conformed to the 2013 Declaration of Helsinki. All participants provided written informed consent after explanation of the experimental procedures.

5.3.2 Experimental design

This study employed a double-blind, placebo-controlled, repeated-measures crossover design. Figure 5.1 provides an overview of the experimental design where participants attended the laboratory on nine occasions in total. A familiarisation of the testing procedures took place no later than two-weeks before the first experimental session. During the familiarisation visit, participants were familiarised with all testing procedures (MVIC, CMJ and self-perceptual ratings of muscle soreness) except for the blood sampling. Furthermore, participants performed the 100-DJP in an attempt to pre-condition them to the eccentric exercise. At least 14 days after the familiarisation visit, participants received either NZBC extract capsules or placebo (PLA) in a counterbalanced design for the 7-days before- and for 3-days after (24, 48 and 72 h post) completing the 100-DJP, separated by a two-week washout period. Visits 1-4 were a four-day block of the experimental sessions for the first condition (NZBC or PLA), followed by a two-week wash-out. Visits 5-8 were the second four-day block of the experimental sessions for the second condition. Each laboratory visit was in the morning between 0700 and 1100 and was preceded by a 2-hour fast (water *ab libitum*). All testing was completed in the laboratory in ambient conditions (22.4±3.3°C), in this order, pre-, immediately post-, and on 3-days after (24, 48 and 72 h) completing the 100-DJP, participants performed the voluntary and electrically stimulated isometric contractions of the knee extensors, rated

their muscle soreness, completed the CMJ and provided a venous blood sample.

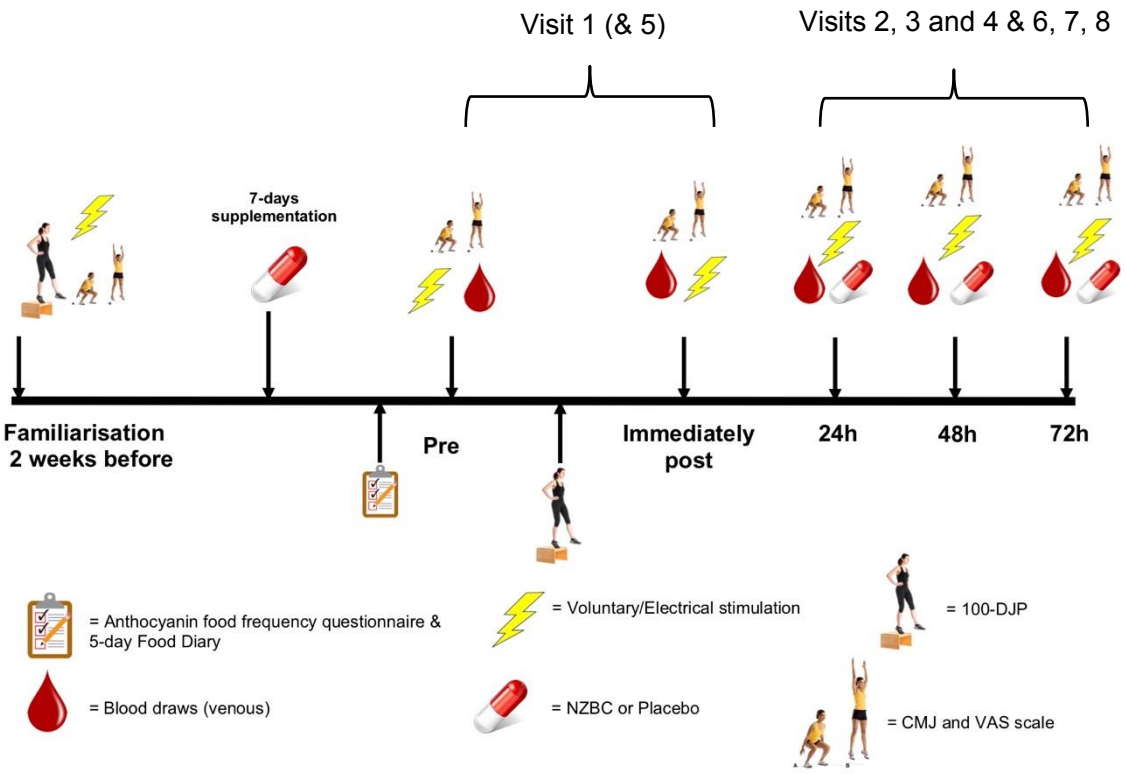


Figure 5.1 Double-blind, repeated crossover design. First 4-day block visits 1-4 and second four-day block visits 5-8, separated by a two-week washout.

5.3.3 Familiarisation

Each participant performed one familiarisation trial no later than 14 days before the first four-day block of data collection to confirm that they were accustomed to all testing measures involved with the experimental design, as it has previously been recommended to ensure reliability of muscle force characteristics (Maffiuletti et al. 2016). In the same session, each participants' twitch profile was established (described in 5.3.7) before they completed the electrically stimulated and voluntary isometric contractions of the knee extensors, CMJ and VAS for muscle soreness and body composition assessment (sum of four skinfolds). A test measure was repeated if the experimenter or participant thought that a maximal effort was not

given, or a learning effect was still apparent. At the end of this session, participants completed 100 drop-jumps to induce EIMD and attenuate the RBE in subsequent trials. It has been previously documented that the first eccentric exercise bout of the knee extensors confers the greatest adaptation, but further adaptation is induced when the exercise is repeated more than once (Chen et al. 2019; Black and McCully, 2008).

5.3.4 Muscle damaging exercise

The muscle damaging exercise was in the form of a high-force stretch-shortening cycle (SSC) protocol consisting of 100-DJP from a 0.6 m high plyometric training box. Each jump was separated by a 10 s interval and each 10 jumps by a 60 s rest period (10 sets of 10 repetitions) (Figure 5.2). This particular exercise protocol was chosen as it has previously been shown to cause EIMD (i.e., increased soreness, loss of function; Jakeman et al. 2017; Clifford et al. 2015). The protocol has also been suggested to cause very few changes in energy metabolism, thus minimal metabolic disturbance, and damage is as a result of mechanical disturbance (Bergström and Hultman, 1988; Kamandulis et al. 2010). Participants were instructed to drop off the box and land on two feet, immediately descending to a ~90^{o} knee angle followed by a maximal effort vertical jump. Participants were demonstrated this technique on several occasions before performing the drop-jumps and, if required, given corrective feedback throughout. Each participant received strong verbal encouragement to ensure maximal effort was maintained. Lastly, a custom-made metronome was used to ensure that the participants maintained the required pace and consistency in-between jumps.

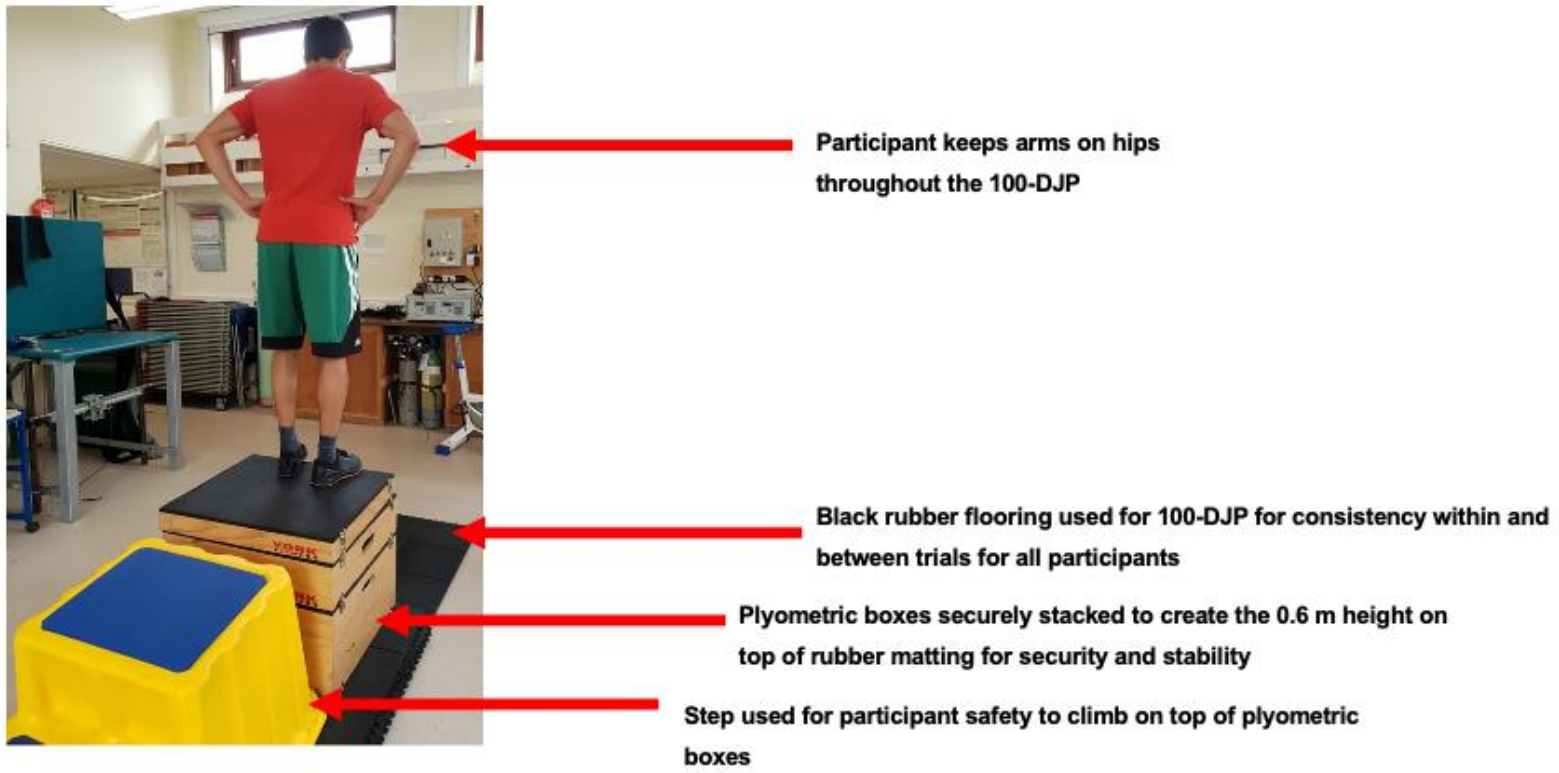


Figure 5.2 The 0.6 m high plyometric training box and steps used for drop-jumps

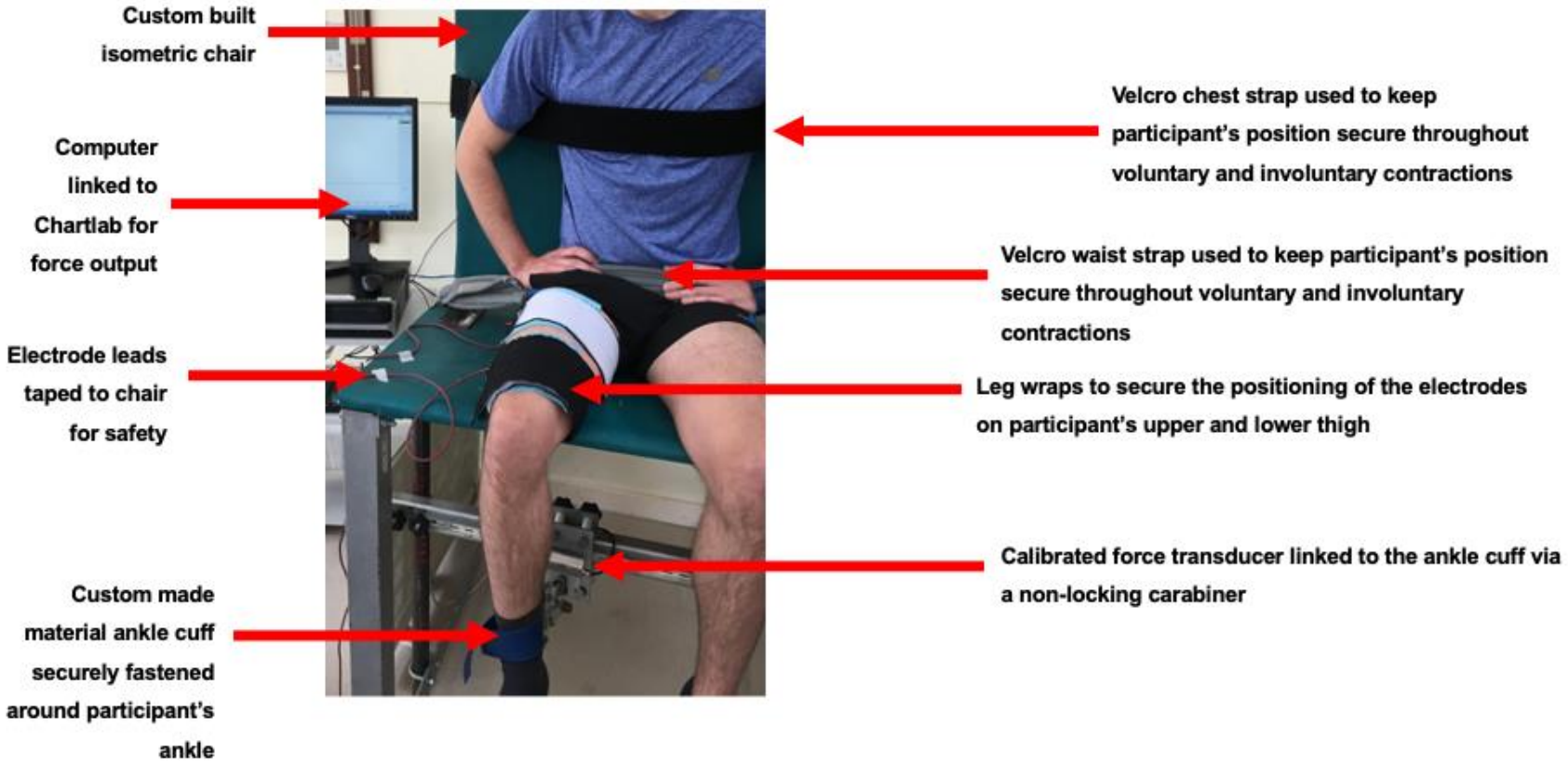


Figure 5.3 Participant positioned in the isometric chair with electrodes positioned for electrical stimulation.

5.3.5 Knee extensor force

Knee extensor force (*m. quadriceps femoris*) was measured on the right leg of all participants for consistency. Participants were seated at a 90^{o} hip angle up on a custom-built chair (University of Chichester, Chichester, UK), with arms crossed over their chest and Velcro straps were placed around the participants' chest and waist to restrict movement of the upper torso and hips (Figure 5.3). A cuff was placed around the participant's ankle (proximal to the fibular notch and medial malleolus) and attached to an s-beam load cell (RS 250 kg, Tedea Huntleigh, Cardiff, UK) via a steel chain at the base of the chair (previously calibrated with known weights). The force produced from the *m. quadriceps femoris* was recorded on a computer at 1000 Hz (Chartlab 4 V4.1.2, AD Instruments, Oxford, UK) (Figure 5.4) and raw force (N) traces analysed in a custom Microsoft Excel spreadsheet (Microsoft, Seattle, WA, USA). Two sponge covered rubber electrodes (13x10 cm) (EMS Physio LTD, Wantage, UK) soaked in saline were placed just above the patella and over the muscle belly of the *m. quadriceps femoris* in the proximal third part of the thigh of the non-dominant leg (Figure 5.5). The position of the electrodes was marked using permanent pen to ensure accurate placement on subsequent test days. For all electricity evoked test procedures, stimulation was provided through an electrical muscle stimulator (Model DS7A, Digitimer Limited, Welwyn Garden City, UK) and multiple pulses were controlled by a NeuroLog pulse generator (Digitimer Limited, Welwyn Garden City, UK) (Figure 5.6). When participants were first seated in the chair, they conducted a warm-up of three 5 second submaximal contractions (~150 N) to become accustomed to the experimental protocol. For each contraction described below, the onset threshold was calculated as 5 SD of slack force to account for noise associated with the period before muscle contraction (Dos Santos et al. 2017). The order of the tests is shown in Figure 5.7 and each measure described below.

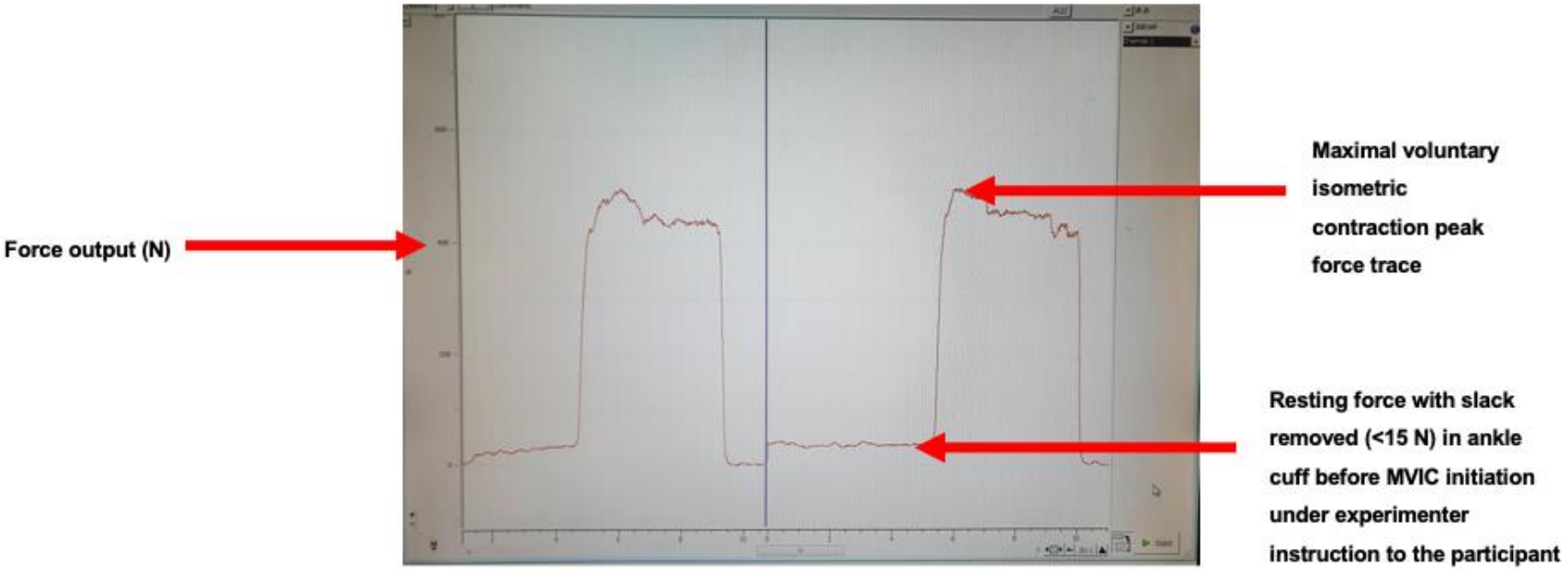


Figure 5.4 Raw force trace output from MVIC

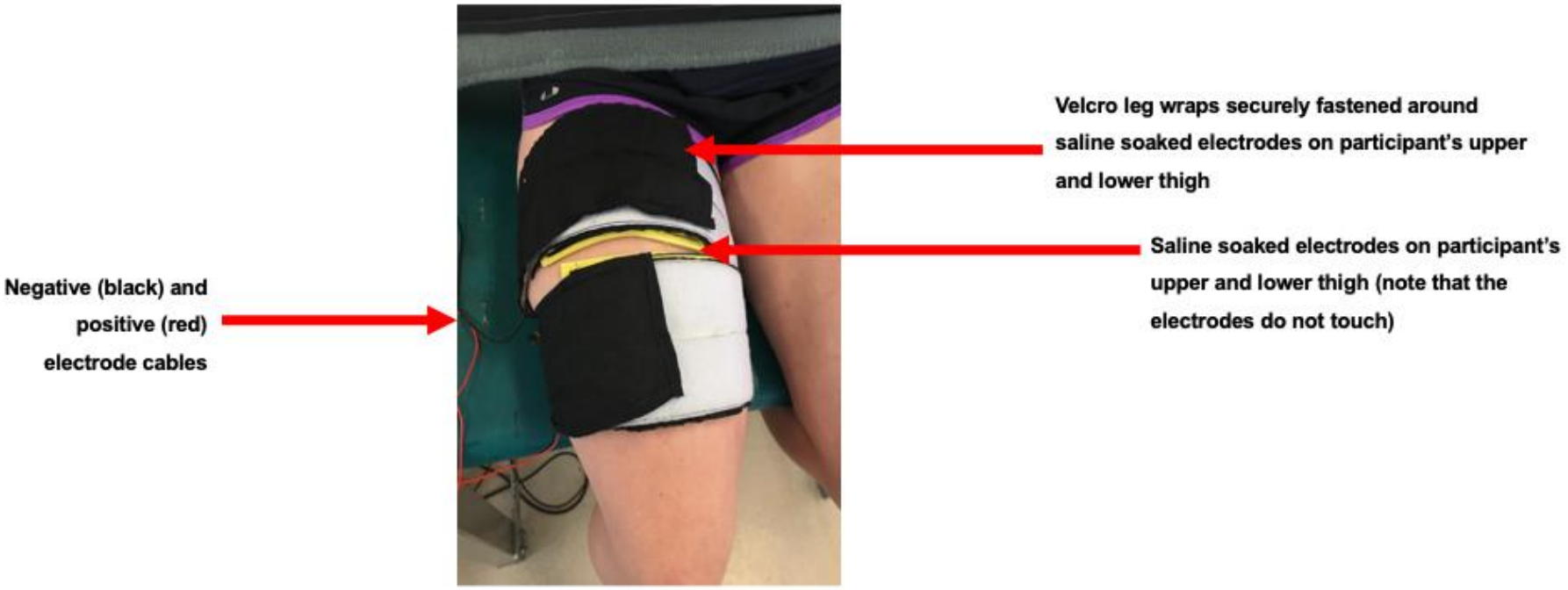


Figure 5.5 Standardised positioning of electrodes over belly of m. quadriceps femoris

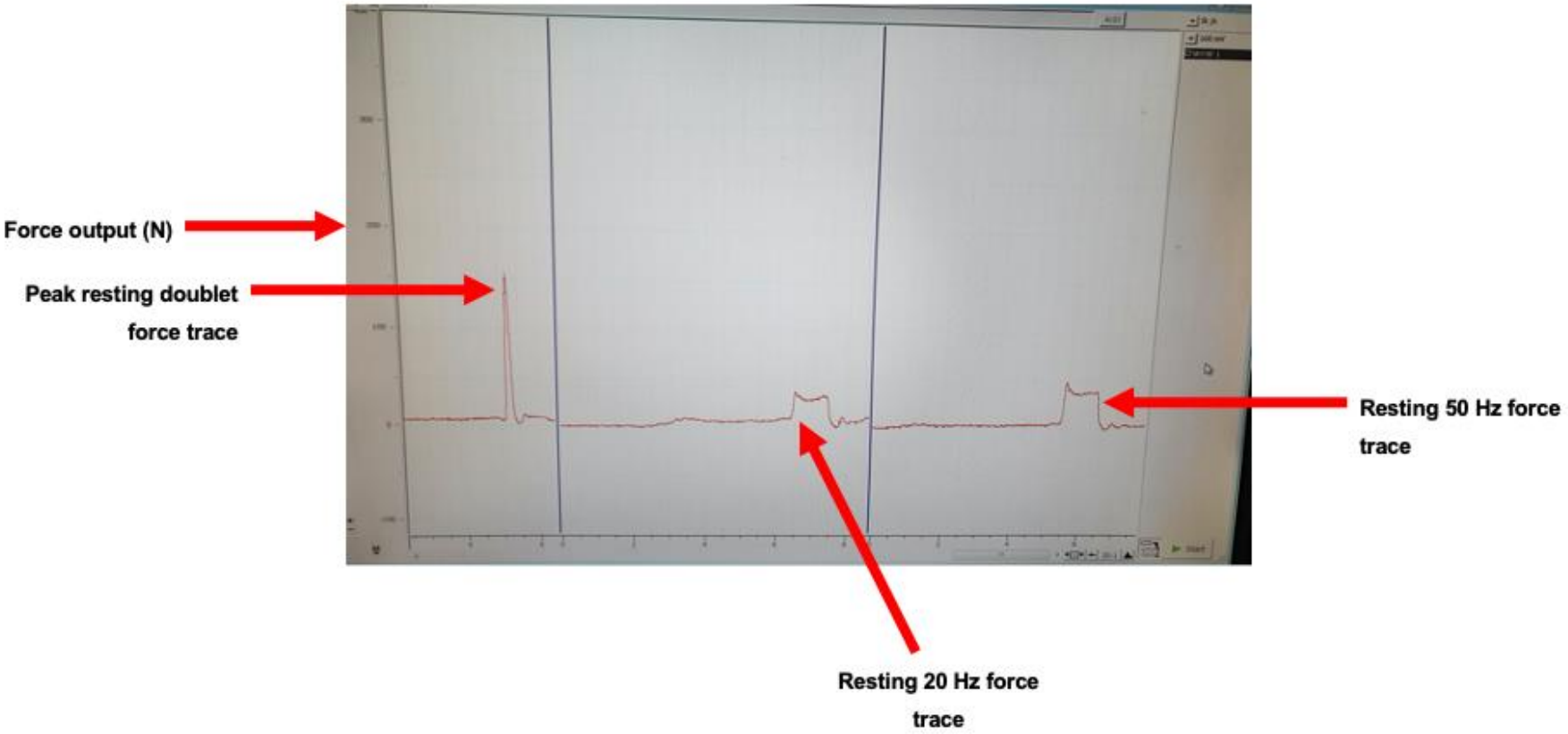


Figure 5.6 Raw force trace output from doublet and 20 and 50 Hz involuntary contractions

5.3.6 Maximal voluntary isometric contraction

Participants were instructed to relax and then take up slight tension in the connecting chain by exerting a minimal force on the strain gauge (<15 N) to negate countermovement and pre-tension with this baseline value being removed from the peak force. Participants then performed a MVIC of the knee extensors by exerting maximal force for 3-5 seconds. All efforts were performed at a joint angle equivalent to 90^{o} knee flexion, as assessed by a goniometer, and were recorded in Newtons (N). Each participant performed three MVIC separated by 60 seconds of passive (seated) recovery, with the average peak value of the best two MVIC used for analysis. This procedure had been employed in several previous investigations to quantify MVIC (Howatson et al. 2009; Clifford et al. 2015). A reliability trial conducted (n=11) before data collection start revealed that the inter-day CV, ICC and TE for MVIC peak force (N) was 5%, 0.99 and 23.7 N, respectively.

5.3.7 Twitch profile establishment

During the familiarisation session, the current for each participant's maximal twitch stimulation was quantified by progressively applying 50 mA pulses until the isometric twitch force reached a plateau. Once the maximal stimulation intensity was determined, the stimuli producing force equivalent to 5% of each participants MVIC force was calculated and termed the submaximal twitch force. Both submaximal and maximal stimulation pulses were recorded and used in all subsequent data collection visits.

5.3.8 Doublet stimulation

Participants were instructed to relax and then take up slight tension in the connecting chain by exerting a minimal force on the strain gauge (<15 N) to negate countermovement and pre-tension, with this baseline value being removed from the peak force, peak rate of force development (pRFD), contraction time and half-relaxation time calculation for each doublet. Three doublet pulses (two maximal single twitches separated by a 10 ms gap) were applied

to the *m. quadriceps femoris*, with 30 s rest between each doublet stimulation. Previous inter-day reliability trials have reported that resting peak doublet force (N), pRFD ($N \cdot s^{-1}$), half relaxation time (s) and contraction time (s) are reliable measures to quantify the contractile properties of *m. quadriceps femoris,* with 95% limits of agreement (LoA) of 13.9, 27.7, 11.8 and 4.9, respectively, for doublet stimulations (Blacker et al. 2013).

5.3.9 20:50 Hz stimulation

A 20 Hz and 50 Hz stimulation (each 0.5 s duration), with 30 s rest between stimulations, were applied to the *m. quadriceps femoris* using the sub-maximal twitch current. A sub-maximal current was used as it has been shown to give reliable estimate of contractile properties whilst being more tolerable for participants (Edwards et al. 1977). A ratio of the forces at 20 Hz and 50 Hz was calculated, a reduction in the ratio indicates the presence of low frequency fatigue (LFF) and an increase, high frequency fatigue (HFF) (Jones, 1996). An intra- and inter-day repeatability trial conducted previously in our laboratory revealed that the 95% limits of agreement (LoA) were 9.2% when assessing the 20:50 Hz ratio, that is one can have 95% confidence that LFF is present if an individual's 20:50 Hz ratio force has decreased by more than 9.2% (Blacker et al. 2013).

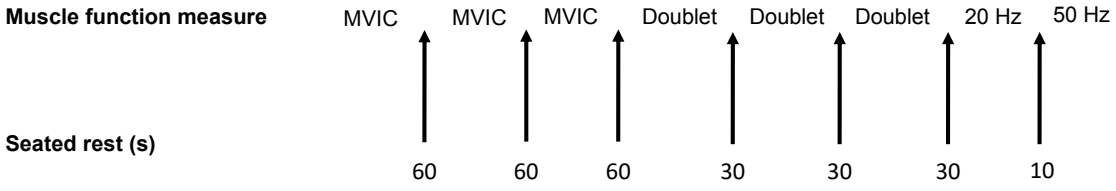


Figure 5.7 Test order for muscle function measures of the *m. quadriceps femoris* muscles by voluntary activation and surface electrical stimulation. Tests were conducted in an isometric chair (knee and hip angle 90°), in the same order at all data collection visits.

5.3.10 Countermovement jump

Please refer to CMJ in Section 3.3.6 for details on CMJ measurement.

5.3.11 Muscle soreness

Please refer to Section 3.3.7 for details on the VAS used for measurement of muscle soreness.

5.3.12 Blood collection and biochemical analysis

Blood samples were obtained following a 2-hour fast from a branch of the basilica vein at the antecubital fossa using a venepuncture (BD Vacutainer® Safety-Lok™ blood collection set, BD, UK) after the participant had a 10-minute supine rest. Blood was collected into 2 x 10 mL serum tubes (Sarstedt, Germany). Serum samples were centrifuged at 1000x*g* (4^{o}C) for 10 minutes following a 30 min clotting period. Lastly, serum supernatant was aspirated into a series of aliquots and stored at -80^{o}C for later analysis. Hematocrit and haemoglobin were measured in whole blood using hematocrit centrifuge (Centurion Scientific Ltd, C2012, Chichester, West Sussex, UK) and HemoCue® Hb 201+ System (HemoCue AB, Ängelholm, Sweden), respectively, to calculate pre-post exercise changes in plasma volume according to methods of Dill and Costill (1974).

5.3.13 Serum Interleukin-6 (IL-6) concentration

Serum samples were assayed for IL-6 concentration using commercially available human IL-6 enzyme linked immunosorbent assay (ELISA) kits (Quantikine®, R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, the serum samples were thawed and aliquots (200 µL) of each diluted sample, positive control or standard, with known concentrations of human IL-6 to establish standard values, were plated on a coated (monoclonal antibody specific for human IL-6) 96-well microlitre plate for 2 h. After washing, human IL-6 conjugate (200 µL) was added to each well and incubated for 2 h at room temperature (20^{o}C). After the wells were washed, substrate solution (200 µL) was added to each well at room temperature (20^{o}C) and protected from light. After adding 50 µL of stop

solution to each well, the intensity of the colour produced after 20 min was measured at 450 nm, with a 540 and 570 nm correction (Spectrostar Nano, BMG Labtech Ltd, Aylesbury, UK) and values were calculated with Excel 365 (Microsoft, v. 365, USA) by generating a four-parameter logistic curve fit. The minimum detectable dose of human IL-6 was 0.09 pg·mL (range: 0.2-10 pg·mL). The serum intra- and inter-assay CV for IL-6 analysis was determined as 7%. Samples for individual participants were analysed on the same plate to avoid inter-plate variation and all samples were analysed in duplicate with the mean being using for analysis.

5.3.14 Serum prostaglandin-E2 (PGE_2) concentration

Serum samples were assayed for PGE_2 concentration using commercially available human PGE_2 ELISA kits (High Sensitivity Prostaglandin E_2 EIA kit, Enzo Life Sciences, Lausen, Switzerland) according to the manufacturer's instructions. Briefly, the serum samples were thawed and aliquots (200 µL) of each diluted sample, positive control or standard, with known concentrations of human PGE_2 to establish standard values, were plated on a coated (monoclonal antibody specific for human PGE_2) 96-well microlitre plate and incubated overnight at 4^{o}C for 18 h. After washing, human PGE_2 conjugate (200 µL) was added to each well and incubated at 37^{o}C for 1 h with a plate cover and protected from light. After adding 50 µL of stop solution to each well, the intensity of the colour produced was measured immediately at 405 nm, with a 570 and 590 nm correction (Spectrostar Nano, BMG Labtech Ltd, Aylesbury, UK) and values were calculated with Excel 365 (Microsoft, v. 365, USA) by generating a four-parameter logistic curve fit. The minimum detectable dose of human PGE_2 was 8.26 pg·mL (range: 7.8–1000 pg·mL). The serum intra- and inter-assay CV for PGE_2 analysis was determined as 10%. Samples for individual participants were analysed on the same plate to avoid inter-plate variation and all samples were analysed in duplicate with the mean being using for analysis.

5.3.15 Supplementation protocol

Participants were provided with the same NZBC extract capsules and placebo outlined in Chapter 3 but instead supplemented with 600 mg·day^{-1} (two capsules) for 7-days before to 72 h after completing the 100-DJP. For information regarding the timing of supplement intake and blinding procedures, please refer to Section 3.3.4. Lastly, once the study was concluded, participants were asked to rank whether they believed they had received NZBC or PLA and why they thought that was the case, with only 53% of participants guessing correctly suggesting that the study blinding was effective.

5.3.16 Dietary and exercise control

Details of the dietary assessment and exercise control are described in Section 3.3.5. The only exceptions were that participants completed two 5-day food diaries (prior to each of the 4-day blocks), supplemented up to 72 h post the muscle-damaging exercise and were required to refrain from exercising in the 48 h before the muscle-damaging exercise and for the 3-days following to try and reduce any cofounding effects on their recovery.

5.3.17 Body composition assessment

Stature (Harpenden Wall Mounted Stadiometer, UK) and body mass (Seca, Seca 875, Birmingham, UK) was recorded and skinfold thickness (mm) and girths (cm) measured by the same International Society for the Advancement of Kinanthropometry (ISAK) certified female researcher. A four site (bicep, tricep, subscapular and iliac crest) skinfold measurement system using Harpenden Skinfold Callipers (Body Care, Southam, UK), with the total sum recorded (Durnin and Womersley, 1974), and two girth sites (mid-thigh and medial calf) in cm. Two measures were taken at each site and if the values differed by >5%, a third measure was recorded.

5.3.18 Data analysis

Statistical analyses were completed using SPSS 23.0 (SPSS, Chicago, Illinois). Differences in group dietary intake were analysed with paired samples *t*-tests. In accordance with many previous studies examining the effects of a nutrition intervention on EIMD (Clifford et al. 2015; Howatson et al. 2010; Trombold et al. 2010; 2011) and for consistency with Chapter 3, data analysis for MVIC, CMJ, ES contractions and VAS was conducted on data corrected to percentage change from baseline. Dependent variables (MVIC, ES contractions, CMJ, muscle soreness, serum IL-6 and PGE_2 analyses) were analysed using a mixed model ANOVA with two independent group levels (NZBC vs. PLA) and five repeated measures time points (pre-exercise, post-exercise, 24, 48 and 72 h post-exercise). Mauchly's test of sphericity was used to check homogeneity of variance for all variables; where necessary, any violations of the assumption were corrected using the Greenhouse-Geisser adjustment. Significant main and interaction effects (group*time) were followed up using Bonferroni post-hoc analysis. To ensure no order effects were present, paired samples *t*-tests were used comparing the pre 100-DJP trials for both supplementation groups (e.g. received NZBC 1st vs. received PLA 1st) for each dependent variable. Pearson (r) correlation coefficients were calculated for the relationship between muscle soreness and serum PGE_2. The alpha level for statistical significance was set at 0.05 *a priori* and Partial-eta^2 (ηp^2) effect sizes (ANOVA) are reported to indicate the magnitude of observed effects where applicable (Lakens, 2013) with respective 90%CI. Partial-eta^2 (ηp^2) effect sizes of 0.01-0.06, 0.06-0.14 or ≥0.14 are considered small, medium and large changes, respectively (Cohen, 1988). Graphically presented MVIC and doublet peak force data were corrected to percentage change from baseline to account for the large inter-individual variability as has been recommended previously (Bowtell et al. 2011; Howatson et al. 2009; Clifford et al. 2015). All data are reported as mean±SD for n=12, unless otherwise stated.

5.4 Results

5.4.1 Dietary intake

There were no differences in total energy (*P*=0.899), absolute carbohydrate (*P*=0.695), fat (*P*=0.930) or protein intake (*P*=0.167) or relative carbohydrate (*P*=0.895), fat (*P*=0.785) or protein intake (*P*=0.186) between the experimental visits (Table 5.1). Analysis of the food diaries indicated participants reported 100% adherence to the dietary requirements and supplementation regime.

Table 5.1 Absolute and relative average daily intake macronutrient intake prior to and for the 3-days following the muscle-damaging exercise.

	NZBC	Placebo
Total energy intake (kJ)	8222±2644	8149±2432
(kJ·body mass^{-1})	108±38	107±32
Carbohydrate (g)	211±89	224±73
(g·kg body mass^{-1})	2.9±1.6	2.9±0.6
Protein (g)	108±44	89±24
(g·kg body mass^{-1})	1.4±0.5	1.2±0.4
Fat (g)	75±37	76±26
(g·kg body mass^{-1})	1.0±0.4	1.0±0.3
Habitual anthocyanin intake (mg·day^{-1})	232±93	

Values are mean±SD, n=12. NZBC, New Zealand blackcurrant.

5.4.2 Isometric contractions (voluntary and electrically stimulated)

There was no difference in MVIC peak force at baseline between NZBC and PLA (P =0.621). Figure 5.8 shows the change in MVIC peak force following the 100-DJP. There was a main effect for time ($F_{(3,68)}$=10.37, P=0.001, ηp^2=0.320) but no effect for group (P=0.940, ηp^2=0.000) or interaction effects (P=0.661, ηp^2=0.024). The MVIC peak force decreased immediately after the 100-DJP in both groups (P=0.005) and was not different to baseline 24, 48 or 72 h post (P>0.05).

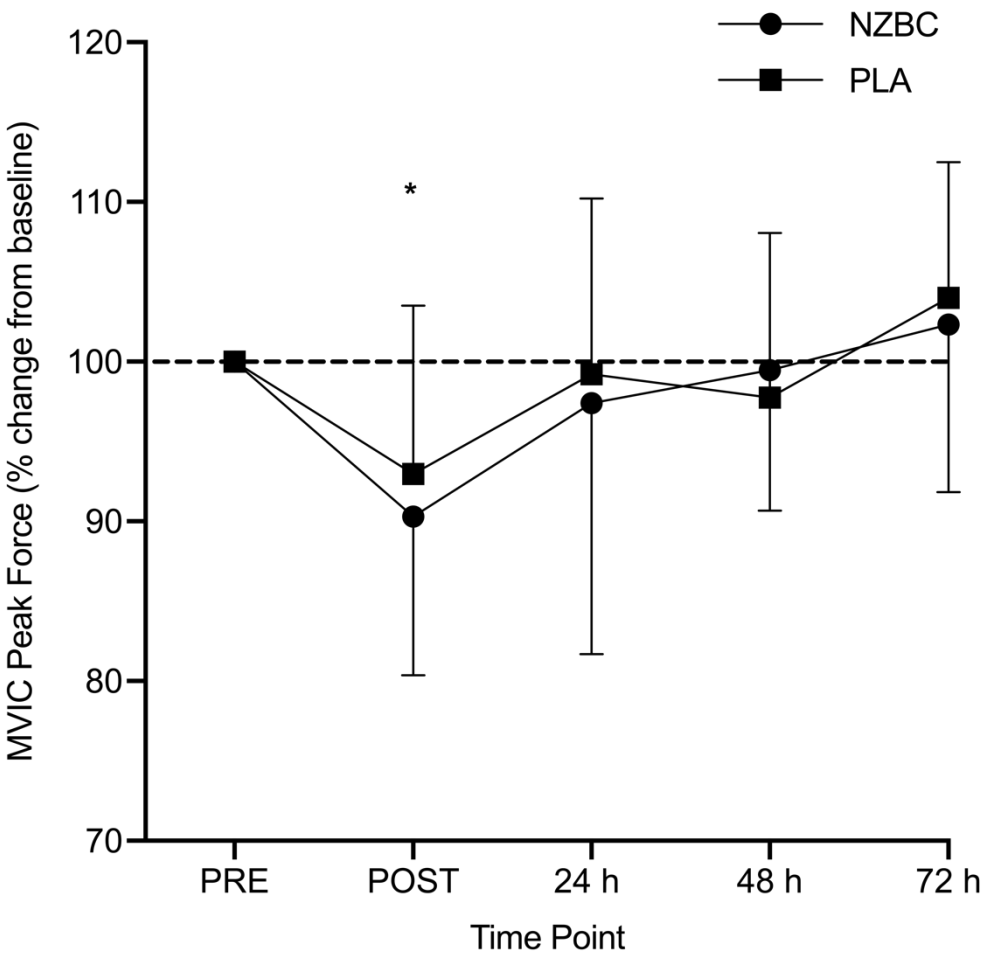


Figure 5.8 Percentage change in maximal voluntary isometric contraction (MVIC) peak force before and after 100-DJP. *Represents Bonferroni post-hoc comparison immediately post 100-DJP (*P*=0.005). Values are means±SD (n=12 per group).

There was no difference in doublet peak force at baseline between NZBC and PLA (*P* =0.970). Figure 5.9 shows the change in doublet peak force following the 100-DJP. There was a main effect for time ($F_{(1.8,\ 39.7)}$=8.48, *P*=0.001, ηp^2 =0.278) but no effect for group (*P*=0.798, ηp^2=0.003) or interaction effects (*P*=0.390, ηp^2 =0.041). Doublet peak force decreased immediately post, 24, 48 and 72 h following the 100-DJP compared to baseline (*P*=0.004, *P*=0.001, *P*=0.002, *P*=0.003, respectively).

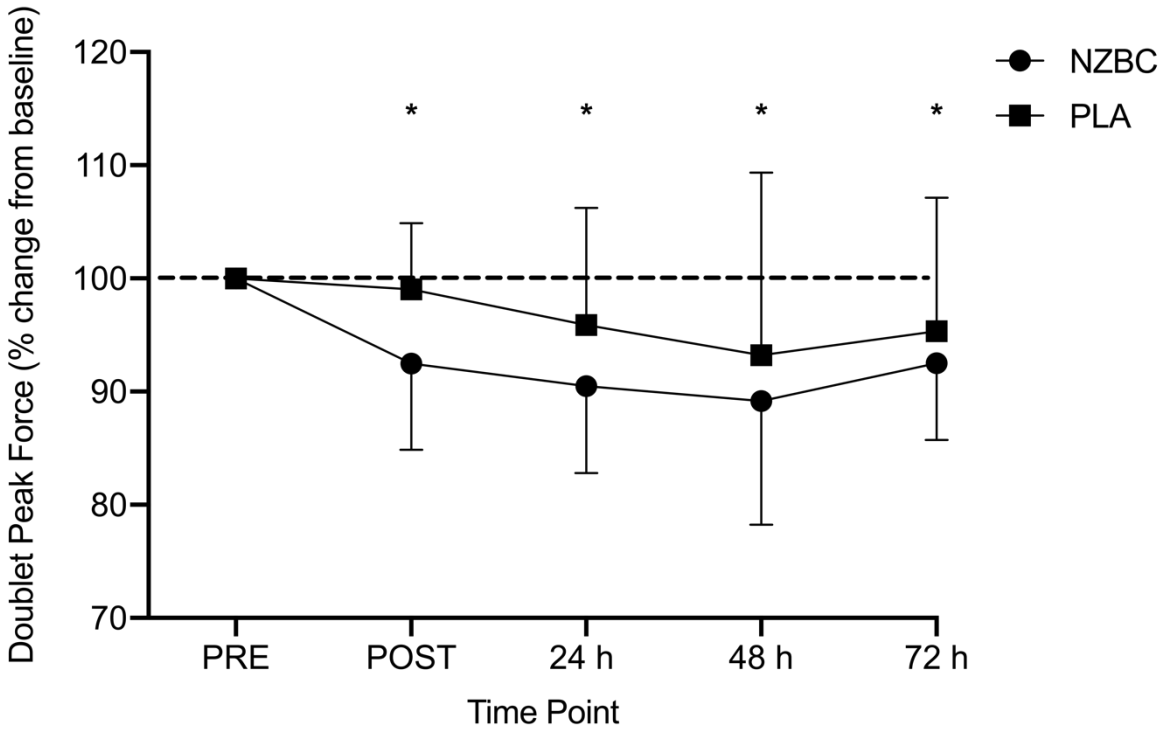


Figure 5.9 Percentage change in doublet peak force before and after 100-DJP. *Represents Bonferroni post-hoc comparison immediately post, 24, 48 and 72 h following 100-DJP (*P*<0.05). Values are means±SD (n=12 per group).

In both NZBC and PLA groups, doublet pRFD, contraction time and half-relaxation time and 20:50 Hz ratio were unaffected by the exercise protocol, showing no time, group or interaction effects (*P*>0.05) (Table 5.2). No order effects were observed for any of the isometric voluntary or involuntary variables, irrespective of supplementation group (*P*>0.05).

Table 5.2 MVIC, doublet characteristics and 20:50 Hz ratio pre- and post-muscle damaging exercise (100-DJP).

Isometric contraction variable	Pre 100-DJP	Post 100-DJP	24 h post 100-DJP	48 h post 100-DJP	72 h post 100-DJP
MVIC (N)*					
NZBC	500±151	452±151	487±152	493±138	504±131
PLA	491±152	454±146	485±158	479±154	512±175
Doublet pRFD (N/s^{-1})					
NZBC	5921±1565	5616±1160	5593±1485	5845±1724	6097±1961
PLA	5763±1533	5241±1005	6278±1687	5986±1905	6420±1835
Doublet contraction time (s)					
NZBC	0.368±0.254	0.256±0.299	0.202±0.355	0.323±0.381	0.449±0.320
PLA	0.271±0.379	0.323±0.374	0.310±0.400	0.293±0.374	0.283±0.342
Doublet half-relaxation time (s)					
NZBC	0.060±0.096	0.059±0.098	0.032±0.109	0.053±0.068	0.059±0.064
PLA	0.094±0.021	0.088±0.017	0.077±0.026	0.062±0.051	0.057±0.048
20:50 Hz ratio					
NZBC	0.76±0.15	0.74±0.18	0.78±0.13	0.84±0.17	0.78±0.13
PLA	0.74±0.16	0.69±0.19	0.73±0.17	0.80±0.07	0.80±0.07

Values are mean±SD (n=12 per group). *Main effect of time immediately post 100-DJP; $P<0.05$. NZBC, New Zealand blackcurrant; PLA, placebo; 100-DJP, 100-drop jump protocol; MVIC, maximal voluntary isometric contraction; pRFD, peak rate of force development.

5.4.3 Countermovement jump

There was no difference in CMJ eccentric phase duration at baseline between NZBC and PLA (*P*=0.142). Table 5.3 shows the change in CMJ eccentric phase duration following the 100-DJP. There was a main effect of time ($F_{(2.7, 53.9)}$=2.99, *P*=0.044, ηp^2=0.130) but no effect for group (*P*=0.566, ηp^2=0.017) or any interaction effects (*P*=0.753, ηp^2 =0.018). Further, Bonferroni post-hoc comparisons revealed no differences between groups (*P*>0.05). There was no difference in CMJ eccentric breaking phase at baseline between NZBC and PLA (*P*=0.055). Table 5.3 shows the change in CMJ eccentric breaking phase following the 100-DJP. There was a supplement*time interaction ($F_{(2.7, 60.7)}$=1.06, *P*=0.048, ηp^2=0.115) but no main effect of time (*P*=0.369, ηp^2=0.046) or group effects (*P*=0.931, ηp^2=0.000). However, no significant group differences were detected with Bonferroni post-hoc comparisons (*P*>0.05). There were no other time, group or interaction effects for the CMJ variables (*P*>0.05) (Table 5.3). No order effects were observed for any of the CMJ variables, irrespective of supplementation group (*P*>0.05).

Table 5.3 CMJ variables pre- and post-the 100-DJP.

CMJ variable	Pre 100-DJP	Post 100-DJP	24 h post 100-DJP	48 h post 100-DJP	72 h post 100-DJP
Jump height (cm)					
NZBC	0.25±0.08	0.23±0.07	0.24±0.08	0.25±0.09	0.24±0.09
PLA	0.23±0.09	0.22±0.09	0.22±0.08	0.23±0.09	0.25±0.07
RSImod (index)					
NZBC	0.23±0.08	0.22±0.08	0.22±0.07	0.24±0.08	0.22±0.10
PLA	0.23±0.10	0.23±0.09	0.22±0.08	0.21±0.07	0.24±0.09
Time to take off (s)					
NZBC	1.22±0.15	1.05±0.14	1.12±0.19	1.05±0.11	1.07±0.15
PLA	1.04±0.16	1.01±0.15	1.01±0.16	1.07±0.18	1.07±0.19
Concentric phase average peak force (N/kg)					
NZBC	16.22±1.36	16.21±1.53	16.31±1.63	16.58±1.71	16.23±1.47
PLA	16.53±1.89	16.38±1.91	16.23±1.65	16.21±1.28	16.57±1.80
Concentric phase net impulse (Ns/kg)					
NZBC	2.17±0.40	2.08±0.37	2.15±0.35	2.23±0.40	2.09±0.48
PLA	2.18±0.39	2.09±0.42	2.06±0.36	2.09±0.39	2.21±0.35

CMJ variable	Pre 100-DJP	Post 100-DJP	24 h post 100-DJP	48 h post 100-DJP	72 h post 100-DJP
Concentric phase average power (W/kg)					
NZBC	19.34±4.31	18.85±4.16	19.47±3.69	20.52±4.42	18.62±4.85
PLA	19.90±4.59	19.50±4.95	18.57±4.10	18.79±3.79	20.30±4.48
Concentric phase average duration (s)					
NZBC	0.36±0.05	0.35±0.07	0.36±0.06	0.35±0.06	0.34±0.06
PLA	0.35±0.05	0.35±0.06	0.35±0.06	0.35±0.06	0.35±0.05
Eccentric phase average peak force (N/kg)					
NZBC	18.17±2.40	18.15±2.18	17.71±2.64	18.21±2.81	18.14±2.55
PLA	18.69±2.89	18.06±2.31	17.56±2.30	17.47±2.01	18.18±2.36
Eccentric phase net impulse (Ns/kg)					
NZBC	1.03±0.23	1.01±0.19	1.01±0.22	1.06±0.21	1.07±0.22
PLA	1.08±0.22	1.07±0.18	1.02±0.19	1.01±0.19	1.06±0.20
Eccentric phase displacement (braking phase) (m)#					
NZBC	0.74±0.15	0.67±0.14	0.74±0.18	0.68±0.10	0.71±0.13
PLA	0.69±0.12	0.69±0.11	0.68±0.10	0.74±0.14	0.75±0.16

CMJ variable	Pre 100-DJP	Post 100-DJP	24 h post 100-DJP	48 h post 100-DJP	72 h post 100-DJP
Eccentric phase average duration (s)*					
NZBC	0.29±0.07	0.25±0.05	0.29±0.08	0.29±0.06	0.29±0.07
PLA	0.26±0.07	0.25±0.04	0.27±0.05	0.27±0.05	0.28±0.07

Values are mean±SD, n=12 per group. #Supplement*time interaction (P =0.048, ηp^2=0.115). *Decreased below baseline immediately post 100-DJP (time effect, P=0.044, ηp^2=0.130); NZBC, New Zealand blackcurrant; PLA, placebo; CMJ, countermovement jump; 100-DJP, 100-drop jump protocol.

5.4.4 Muscle soreness

There was no difference in muscle soreness VAS ratings at baseline between NZBC and PLA (*P* =0.107). Figure 5.10 shows the change in muscle soreness VAS ratings following the 100-DJP. There was a main effect of time ($F_{(2.2,\ 49)}$=15.75, *P*=0.001, ηp^2=0.417) and a supplement*time interaction effect ($F_{(2.2,\ 49)}$=3.07, *P*=0.050, ηp^2=0.122) but no effect of group (*P*=0.397, ηp^2=0.033). Post hoc comparisons indicated that muscle soreness recovered quicker in the PLA group vs. NZBC at immediately post (*P*=0.001), 24 h (*P*=0.001) and 48 h (*P*=0.011) post 100-DJP (Figure 5.10) compared to baseline. No order effects were observed for muscle soreness, irrespective of supplementation group (*P*>0.05). No significant relationships were observed between muscle soreness and serum PGE_2 levels (Table 5.4) for either NZBC or PLA at any time points (*P*>0.05).

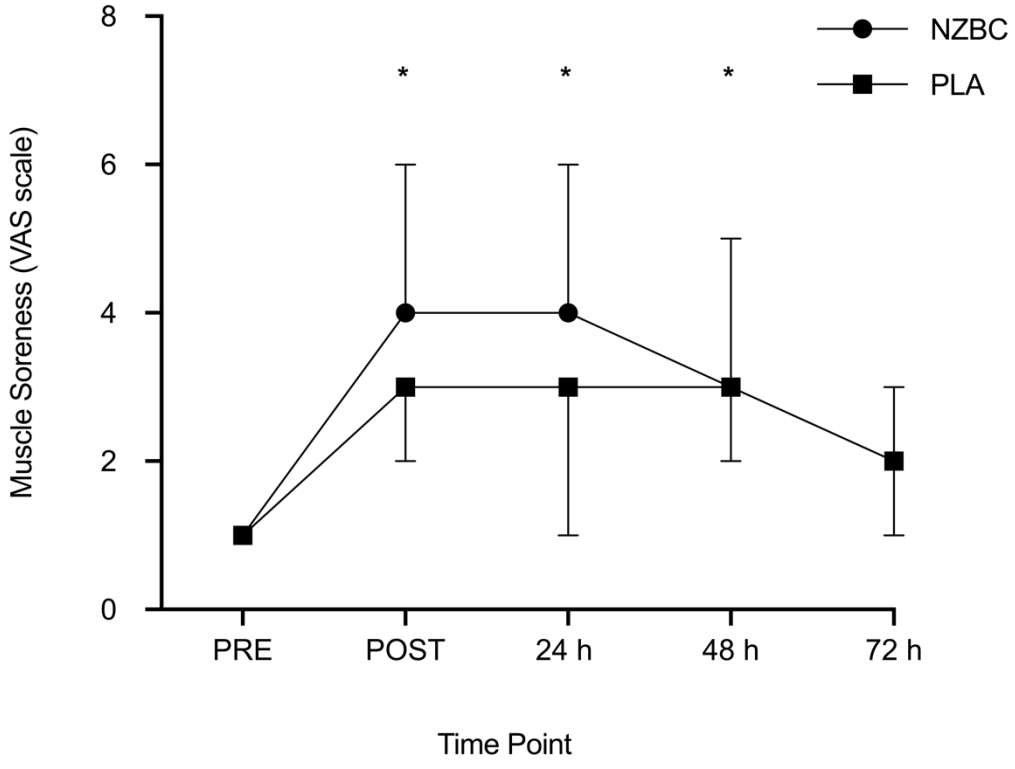


Figure 5.10 Absolute changes in self-perceptions of muscle soreness on a visual analogue scale (VAS) before and after muscle damaging exercise. *Represents main effects of time for New Zealand blackcurrant (NZBC) vs. placebo (PLA); *P*<0.001. Values are means±SD (n=12 per group).

5.4.5 Systemic inflammatory response

There was no difference in serum IL-6 at baseline between NZBC and PLA (*P* =0.769). Table 5.4 highlights the lack of change in serum IL-6 following the 100-DJP. There were no time, group or interaction effects for serum IL-6 (*P*>0.05) (Table 5.4).

Prostaglandin-E_2 (PGE_2) results are based on nine participants, due to their values falling above the LOD during ELISA quantification. There was a difference in serum PGE_2 at baseline between NZBC and PLA (*P*=0.037) with greater serum PGE_2 concentrations in NZBC compared to PLA. However, table 5.4 highlights the lack of change in serum PGE_2 following the 100-DJP. There were no time, group or interaction effects for serum PGE_2 (*P*>0.05) (Table 5.4). No order effects were observed for serum PGE_2 or IL-6, irrespective of supplementation group (*P*>0.05)

Table 5.4 Serum IL-6 and PGE_2 values pre- and post-muscle damaging exercise (100-DJP).

Inflammation variable	Pre 100-DJP	Post 100-DJP	24 h post 100-DJP	48 h post 100-DJP	72 h post 100-DJP
IL-6 ($pg \cdot mL^{-1}$)					
NZBC	1.10±0.77	1.15±0.65	0.86±0.33	0.70±0.45	0.72±0.32
PLA	1.01±0.57	0.96±0.58	0.79±0.49	0.73±0.36	0.86±0.47
PGE_2 ($pg \cdot mL^{-1}$)					
NZBC	241±114	214±113	218±131	197±62	203±59
PLA	167±57	197±71	308±268	243±149	194±84

Values are mean±SD (n=12 per group for IL-6; n=9 per group for PGE_2). IL-6, interleukin-6; PGE_2, prostaglandin-E_2; 100-DJP, 100-drop jump protocol; NZBC, New Zealand blackcurrant; PLA, placebo.

5.5 Discussion

The primary finding of this Chapter was that the 100-DJP caused EIMD as shown by a reduction in MVIC and doublet force and increased soreness, but that NZBC did not improve recovery. This is surprising as it was hypothesised that NZBC extract would accelerate recovery of muscle function and attenuate muscle soreness following EIMD, possibly by attenuating the acute inflammatory response. However, the inflammatory markers of serum IL-6 and serum PGE_2 did not show an attenuation in the acute inflammatory response.

The decrease in MVIC peak force immediately post the drop-jump protocol indicates that EIMD was present (Warren et al. 1999; Warren at al. 2002). However, there was no difference in the change between groups over time. This finding is similar to Rowland (2018) who observed that 12-days intake of NZBC extract was unable to attenuate declines in MVIC peak force following EIMD caused by 4 sets of 15 maximal concentric and eccentric elbow flexor contractions on an isokinetic dynamometer. However, Rowland et al. (2018) did show that NZBC extract reduced CK concentration 96 h after EIMD and attenuated muscle soreness in the 96 h following EIMD compared to PLA. It is important to highlight that despite the frequent use of CK as a marker of EIMD, it is now widely accepted that CK is not a reliable standalone indicator of the presence or magnitude of EIMD due to the inherent inter-individual variability with CK appearance systemically following an EIMD stimulus and the inconsistent relationship between more direct markers of EIMD such as muscle force producibility (Baird et al. 2012; Clarkson and Hubal, 2002; Warren et al. 1999; Paulsen et al. 2012).

In contrast to the observation that NZBC extract did not attenuate functional impairments following EIMD, recovery of isometric peak force has been shown to be enhanced with other polyphenol-rich 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). However, only Bowtell et al. (2011) used the contralateral leg to complete the second bout of muscle damaging exercise, to mitigate the effect of the

RBE (Howatson and van Someren, 2008). All of the other aforementioned studies used one bout of exercise to induce EIMD and study the effectiveness of the supplementation intervention. Whilst this classical approach allows for proof of concept by maximising the possibility for muscle damage to be induced in unaccustomed individuals, it does lack ecological validity as most athletes partake in training regimes that they are accustomed too (Currell and Jeukendrup, 2008; Hopkins, 2000). Furthermore, it is pertinent to highlight that in all of the aforementioned studies where a recovery enhancement is evident following supplementation with a polyphenol-rich beverage, polyphenol restricted diets have been followed prior to and during them. In contrast, the present study did not restrict polyphenols from the diet, which likely increases the ecological validity but may have contributed to the lack of observed differences between NZBC extract and placebo. In addition, although cherries, pomegranate and blueberry juice are abundant in polyphenols (Pérez-Jiménez et al. 2010), they all have varying anthocyanin profiles, which could be a contributing factor to the divergent observations reported in the literature following polyphenol supplementation alongside a muscle damaging exercise bout (Bowtell and Kelly, 2019). As discussed in Section 2.16, anthocyanins have been previously shown to improve flow mediated dilation (FMD) in a dose-dependent manner (Rodriguez-Mateos et al. 2016), which may help offset a reduction in blood flow and subsequently, muscular fatigue (Cook et al. 2017) and possible injury (Jones et al. 2017). Thus, it remains to be established whether there is an optimal amount of anthocyanin or blend of anthocyanin-rich fruits required to mitigate declines in muscle function following EIMD.

Electrical stimulation, such as a single twitch, doublet or tetani, can be used to assess the contractile properties of the muscle as changes in these parameters indicate sites of failure in the EC coupling process (Allen et al. 1995; Jones, 1996). Use of these measures in conjunction with other functional measures such as MVIC and CMJ, are important as it has been suggested that approximately 75% of the strength loss following muscle damaging

exercise can be accounted for by the failure of the EC coupling process (Warren et al. 2002) and may partially explain the underlying mechanism for peripheral fatigue (Millet et al. 2003).

Both the NZBC and PLA groups demonstrated a decrease in doublet peak force immediately, 24, 48 and 72 h post the 100-DJP compared to baseline. This observation suggests that the 100-DJP impaired the EC coupling process due to physical sarcomere damage or an inhibition of Ca^{2+} release or sensitivity in the type II fibres. As type II fibres are situated nearer the surface of the muscle belly proximal to where the surface electrodes would have been situated (Millet et al. 2011). Given that muscle damage is heterogeneously distributed in the muscle, it must be noted that changes in electrical stimulation parameters from the quadriceps may only be indicative of the motor units nearest the surface of the muscle belly and not representative of the whole tissue (Millet et al. 2011).

Expressing the peak force of an electrical stimulation of human skeletal muscle at low (10-20 Hz) and high (50-100 Hz) frequencies over (0.5-2.0 s duration) as a ratio was first proposed by Edwards et al. (1977) and has been used to assess the presence of low frequency fatigue (LFF). A decrease in the 20:50 Hz ratio is indicative of LFF, an effect which can last for hours or days after the initial muscle damaging bout (Edwards et al. 1977; Warren et al. 2002). Whereas a decrease in the peak force of a stimulation indicates neuromuscular impairment and may be caused by a combination of damage to the muscle structure, reduced myofibrillar Ca^{2+} sensitivity and reduced Ca^{2+} release (Allen et al. 1995). In the present Chapter, no presence of LFF was observed following the drop-jump protocol in either group. This observation is in line with previous research which did not observe any LFF following a maximal SSC exercise in the form of a customized sledge apparatus (Strojnik and Komi, 1998) and is possibly due to an effect with the RBE (Kamandulis et al. 2010).

The only CMJ outcome and neuromuscular variable that was affected by the 100-DJP was the eccentric phase duration and the other parameters remained unchanged. Eccentric phase duration decreased following the 100-DJP in both NZBC and PLA groups with no group or

interaction effects. It is probable that the reason for this observation is due to a post-activation potentiation effect within the muscle of the drop-jump exercise, which caused the eccentric phase of the CMJ to shorten immediately following the exercise task as opposed to an effect of the NZBC extract *per se* (Suchomel et al. 2015). With regards to the other CMJ variables it is likely that a meaningful change could not be detected due to the similarity of the kinematic characteristics of the CMJ test and the mode in which the muscle damage was induced (drop-jumps including a 90^{o} countermovement upon landing), which are both dynamic exercises and utilise the SSC (Komi, 2000; Nicol et al. 2006). This observation is similar to a previous study, which found that a muscle damaging protocol using drop-jumps did not cause any impairment in drop-jump height (Kamandulis et al. 2010). The precise mechanism by which the potential protective effect occurs is unclear, but it has been suggested to be related to the storage and re-utilisation of elastic energy, potentiation of the contractile machinery and altered reflexes within the CMJ (Jan van Ingen Schenau and de Haan, 1997; Byrne and Eston, 2002). Thus, this draws into question whether use of the CMJ as a tool to detect impairments of muscle function is appropriate following a damaging stimulus that shares kinematic characteristics as the CMJ movement pattern and perhaps use of a vertical jump such as the squat jump, which has no SSC component, is more appropriate (Byrne and Eston, 2002).

There was a substantial increase in self-perceptual ratings of muscle soreness in the 48 h following the 100-DJP, indicating that it effectively induced muscle soreness. These findings are in accordance with previous research that also showed an increase in ratings of muscle soreness in the 24 h following an acute 100-DJP performed by 27 physically active men (Jakeman et al. 2017). However, in the present Chapter, NZBC supplementation was unable to attenuate the increase in muscle soreness in the 72 h following the 100-DJP compared to PLA. These findings are in contrast to Rowland (2018) who observed an attenuation in ratings of self-perceptual muscle soreness in the 96 h following an acute elbow flexor muscle damaging protocol with NZBC supplementation compared to PLA in 23 healthy males and females. However, there are notable differences in study design that mean direct comparisons

are problematic. Firstly, Rowland (2018) utilised an EIMD protocol that targeted a smaller muscle group, the biceps brachii whereas in the present Chapter, the leg flexors and extensors were the EIMD focus via the 100-DJP. Furthermore, in Rowland (2018), participants were only exposed to the muscle damaging protocol acutely, whereas in the present Chapter, participants were preconditioned to the 100-DJP during the familiarisation visit. It has been previously shown that the magnitude of muscle damage is greater and the recovery of muscle function slower in the elbow flexors than the leg extensors following the same relative intensity muscle damaging exercise (Jamurtas et al. 2005). In addition, it has been previously shown that the magnitude in ratings of muscle soreness is greatest following the first muscle damaging exercise bout and then decrease in subsequent bouts in the elbow flexors (Chen et al. 2006). Thus, it is probable that the participants in Rowland (2018) experienced a greater magnitude of muscle soreness than the participants within the current Chapter. Therefore, it remains to be clarified in future research whether NZBC extract supplementation is able to mitigate the increase in perceptions of muscle soreness following EIMD.

Despite the precise mechanisms causing muscle soreness still remaining unclear, the most recent evidence suggests that intramuscular generation of noxious stimuli such as nerve growth factor (NGF), bradykinin, and PGE_2, are likely to play important roles (Hyldahl and Hubal, 2014; Murase et al. 2010; Mizumura and Taguchi, 2016; Sonkodi, Berkes and Koltai, 2020). Prostaglandins (PGE) are synthesised in skeletal muscle and have been shown to have profound effects on skeletal muscle protein turnover (Palmer, 1990; Rodemann and Goldberg, 1982; Vandenburgh et al. 1995), modulation of inflammation and pain (Ferreira, 1973) and muscle-specific stem cell (MuSCs) proliferation moderator (Ho et al. 2017). Prostaglandin synthesis is regulated at two levels: 1) by controlling the activity of the pH precursor arachidonic acid (AA) from membrane phospholipids, and 2) controlling the activity of the COX enzyme. Cyclooxygenase enzymes COX-1 and COX-2 catalyse the conversion of arachidonic acid (AA) to generate chemical mediators of inflammation (Reddy et al. 2005) and pain sensitivity (Hyldahl and Hubal, 2014; Mizumura and Taguchi, 2016; Cheung et al. 2003) such

as prostaglandin-E2 (PGE_2). The evidence to date suggests that the COX-2 isoform in skeletal muscle appears to be more responsive to injury-related stimuli (Trappe and Liu, 2013; Paulsen et al. 2010). Previously, a selective COX-2 inhibitor, celecoxib, was observed to attenuate symptoms of muscle soreness in recreationally active males and females following a muscle damaging protocol of the elbow flexors (Paulsen et al. 2010). However, the authors did not observe any benefit of the COX-2 inhibitor on muscle function recovery. Unlike Paulsen et al. (2010), we did not observe any attenuation of muscle soreness in the NZBC group compared to PLA, despite serum PGE_2 being reduced following the 100-DJP in NZBC group. Further, we did not observe any relationship between muscle soreness and serum PGE_2 secretion. Taken together, these observations suggest that the mechanism of muscle soreness and pain are multifaceted and that systemic PGE_2 secretion in isolation is not necessarily indicative of the whole process. Thus, future research should consider assessing a broader range of markers known to excite and sensitise local muscle nociceptors such as nerve growth factor (NGF), histamine and bradykinin (Hyldahl and Hubal, 2014; Mizumura and Taguchi, 2016; Murase et al. 2010).

Resting levels of PGE_2 are typically low, and in response to stimulation, tissue production can be increased greatly (Trappe and Liu, 2013). Baseline levels presented within the current Chapter (NZBC, 241±114 $pg \cdot mL^{-1}$; PLA, 167±57 $pg \cdot mL^{-1}$) were similar to what has been reported previously within the literature of between ~100 and 300 $pg \cdot mL^{-1}$ (Meneghel et al. 2014). Further, prostaglandins have a relatively short half-life of only a few seconds to minutes where 90% of PGE_2 is removed from systemic circulation in one pass through the pulmonary circulation (Piper et al. 1970). Previously, cyanidin-3-O-glucoside, a key parent anthocyanin in NZBC extract was found to inhibit COX-2 enzyme by 59% *in vitro* compared to non-COX specific controls (Reddy et al. 2005). Similarly, Seeram et al. (2001) observed that anthocyanins found in blackberries, which share similar anthocyanin profiles to NZBC, demonstrated a 46% inhibition of COX-2 enzyme. Thus, it appears that anthocyanins display similar anti-inflammatory properties to NSAIDs (Seeram et al. 2001). Although purely

speculative due to not being statistically significant, it appeared that there was an increase in serum PGE_2 following the drop-jump protocol in the PLA group and not in the NZBC group, which suggests that the 100-DJP may have caused an increase in this inflammatory signalling pathway and that the NZBC extract may have mitigated this increase. However, it must be noted that rodent models have demonstrated how inhibition of PGE_2 production through NSAIDs administration immediately following myofibril damage, hinders MuSC expansion and compromises muscle strength (Ho et al. 2017). Mechanistically, Ho et al. (2017) demonstrated that once PGE_2 engages the EP4 receptor within MuSCs, it activates cyclic adenosine monophosphate (cAMP) and the downstream proliferation-inducing transcription factor nuclear receptor related-1 protein (Nurr1), leading to accelerated MuSC proliferation. Thus, if a reduction of serum PGE_2 was present in the NZBC group it could indicate that supplementation protocol may have attenuated MuSC proliferation following the drop-jump damaging stimulus. However, this is purely speculation due to the existing evidence being from rodent models with NSAID supplementation but does highlight the importance of the hormesis caveat with nutrition interventions where desirable adaptations may be undesirably blunted (Owens et al. 2018; Peake et al. 2015).

As described previously, the participants in the present study were pre-conditioned to the 100-DJP during the familiarisation session in an attempt to mitigate the effects of the RBE in the randomised crossover design. The order effects analysis of all dependent variables (isometric contractions both voluntary and involuntary, CMJ outcome and neuromuscular, muscle soreness and serum PGE_2 and IL-6), revealed no differences between the first and second 100-DJP trials irrespective of condition, implying that no additional RBE was present. Interestingly, serum IL-6 remained unchanged following the 100-DJ protocol. This would suggest that the RBE effect had played a role in blunting the increase in systemic IL-6 response given that is has been previously shown following an acute 100-DJP with either high or low beetroot juice supplementation increases in plasma IL-6 concentrations were observed (Clifford et al. 2015). However, a notable difference between the observations in the current

Chapter and that of Clifford et al. (2015) is that the baseline IL-6 concentrations herein, were between ~0.33 and 1.87 pg·mL^{-1}, whereas in Clifford et al. (2015), baseline IL-6 concentrations were between ~0.20 and 0.72 pg·mL^{-1}. The exact reason for this difference is not abundantly clear but could be due to differences in participant training status (Fischer, 2006) and the time of day during which the sample was collected (Abedelmalek et al. 2013). Nonetheless, the baseline levels reported in this present Chapter are within the normal range of ~1 pg·mL^{-1} (Fischer, 2006). With regards to the RBE, it has been suggested that repeated bouts of eccentric exercise result in alterations in the inflammatory response and may be responsible, at least in part, for the lower indexes of damage and/or faster recovery from a repeated bout of eccentric exercise (Hirose et al. 2004; McHugh, 2003). Smith et al (2007) found a decrease in serum IL-6 from 12±1 pg·mL^{-1} to 6±1 pg·mL^{-1} (mean±SE) following repeated downhill treadmill running (-13.5% gradient at 75% VO_{2peak} for 60 mins) on two occasions in six untrained males. However, three previous studies showed no differences in plasma IL-6 between repeated bouts of eccentric exercise suggesting that IL-6 does not display or play a role in the RBE (Croisier et al. 1999; Willoughby et al. 2003). Aside from the RBE, another plausible explanation for the lack of change in serum IL-6 following the 100-DJP in the present Chapter, is that the participants had sufficient carbohydrate availability (Pedersen et al. 2004; Febbraio and Pedersen, 2002). It has been previously suggested that IL-6 may act as a sensor of carbohydrate availability and as muscle stores of glycogen deplete, plasma IL-6 increases in an exponential fashion and peaks at the end of exercise when the muscle glycogen stores are low (Gleeson and Bishop, 2000). The participants within the present Chapter, were asked to fast two hours prior to visiting the laboratory and from 5-day food diaries reported consuming ~2.9±1.6 and 2.9±0.6 g·kg body mass^{-1} of carbohydrate for NZBC and PLA groups, respectively, which is in line with the acute carbohydrate fuelling strategy recommendations of 1 – 4 g·kg body mass^{-1} 1 to 4 h prior to exercise for optimal carbohydrate availability (Burke et al. 2011). Thus, it is plausible that the lack of change in serum IL-6 was due to sufficient carbohydrate availability, blunting an upregulation of the sensory response of IL-6.

Notwithstanding, there was a strong rationale for examining serum IL-6, since it has been previously demonstrated that NZBC extract was able to accelerate recovery by mitigating the rise in IL-6 following a 30-minute indoor row at 80% VO_{2max} compared to PLA (Lyall et al. 2009). Therefore, IL-6 may represent a potential mechanism by which NZBC extract mediates anti-inflammatory effects. Furthermore, the present study is the first to assess the effect of an anthocyanin-rich supplement on the inflammatory responses following repeated bouts of eccentric exercise, in the form of 100-DJP. Perhaps having a larger number of participants to increase the statistical power to detect subtle changes in these inflammatory markers and measuring the level of inflammation with a greater array of systemic inflammatory markers would have elucidated greater insight into the exercise-induced inflammatory response in the present Chapter.

It is important to acknowledge the limitations of the research presented in this Chapter that might affect the applicability of the findings. Firstly, the magnitude of the RBE was not measured (i.e., collect data from the participants during the familiarisation session immediately pre-, post-, 24 h, 48 h and 72 h following the first bout of drop-jumps) and instead based on the evidence available to date that suggests that the magnitude of muscle damage is often less after individuals have been exposed to an initial bout of the same or similar exercise stimulus (Hubal et al. 2008; McHugh et al. 2003; Nosaka and Clarkson, 1995). However, no order effects were observed between the first and second 100-DJP trial, irrespective of supplementation group, for any of the isometric contraction variables (voluntary and involuntary), CMJ outcome and neuromuscular variables or muscle soreness suggesting that no additional RBE was present. Furthermore, in a pilot study (n=4) conducted before this experimental trial, it was observed that the drop-jump protocol did reduce muscle function (MVIC) by 26% relative to baseline immediately post, which is comparative to similar previous research (Clifford et al. 2015; Nicol et al. 2006). Furthermore, although this study used an ecologically valid design by allowing participants to maintain their habitual diets (i.e., not restricting polyphenol intake), it remains unclear whether a recovery benefit with NZBC extract

supplementation would have been apparent if participants were following polyphenol deplete diets. However, although classically studies assessing the effectiveness of polyphenol supplementation on recovery from EIMD restrict habitual polyphenol intake, substitution of dietary polyphenols for supplemental ones arguably artificially induces desired outcomes (Bowtell and Kelly, 2019). Thus, if a polyphenol supplement can be deemed an effective recovery strategy from EIMD, it needs to be able to exert any recovery benefit on top of habitual diets to be of practical use in a population.

5.6 Conclusion

The primary finding of this Chapter was that the 100-DJP induced EIMD as shown by a reduction in muscle force producing capability (MVIC and peak doublet force) and increased muscle soreness. However, the NZBC extract supplement did not improve recovery as indicated by measures of MVIC, electrically stimulated isometric contractions, CMJ outcome and neuromuscular variables, muscle soreness and serum PGE_2 and IL-6. The overall aim of this thesis was to examine the efficacy of NZBC extract as a recovery aid following strenuous exercise. This Chapter addressed the third question posed in the introduction of this thesis:

Does intake of NZBC extract 7-days before and 3-days following a strenuous bout of eccentric-heavy exercise in the form of 100 drop-jumps accelerate recovery compared to PLA?

The findings from this Chapter question the benefit of NZBC extract as a recovery aid following EIMD.

However, similarly to Chapter 3, inter-individual variation was apparent with both NZBC and PLA groups for CMJ and MVIC responses following the 100-DJP. It is possible that this variation may have impacted the ability to detect a meaningful change for CMJ outcome and neuromuscular variables and with the MVIC peak force between NZBC and PLA groups. Previously it has been observed that individuals homozygotic for the RR allele in the *ACTN3* gene, are more susceptible to reductions in MVIC force and demonstrate slower recovery profiles compared to XX allele homozygotes (Venckunas et al. 2012). Further, the authors

observed no influence of *ACTN3* genotype and the RBE following a drop-jump protocol separated by 14-days. Thus, the next Chapter will; (1) investigate the inter-and inter-individual variability in response to EIMD following the 100-DJP with NZBC extract or PLA intake using the SWC and response CI's. (2) Examine the association between the SNPs ACTN3, ACE and TTN and the CMJ outcome and neuromuscular variables and MVIC peak force responses to EIMD induced by an eccentric-heavy drop-jump protocol with NZBC extract or PLA intake.

6. Intra- and Inter-Individual Variability in Countermovement Jump and Maximal Voluntary Isometric Contraction Response to Drop Jump Exercise with NZBC Extract Supplementation: Statistical and Genotype Insights

6.1 Abstract

Introduction. At the group level, New Zealand blackcurrant (NZBC) extract did not improve recovery from EIMD following a 100 drop-jump protocol (100-DJP). However, large inter-individual variability was apparent and likely intra-individual variability. This aim of this Chapter was to examine some of the potential sources for the observed variability using the smallest worthwhile change (SWC) and targeted genotype analysis. **Method.** Using data collected in Chapter 5, mean individual observed scores with true response 95%CI and SWC for group (NZBC vs PLA) intra- and inter-individual variation were graphically constructed to determine individual responses and participants were retrospectively genotyped for candidate SNPs, α-actinin-3 (*ACTN3*), angiotensin-I converting enzyme (*ACE*) and titin (*TTN*) and associations with CMJ variables were examined pre- (baseline), immediately-, and at 24 h, 48 h and 72 h-post 100-DJP using a two-way repeated measures ANCOVA, with significance set *a priori* at $P<0.05$. **Results.** The SWC and response CI's demonstrated that no effects on recovery were observed for CMJ outcome (jump height, RSImod, time to take off), neuromuscular variables (concentric average peak force, net impulse, average power, duration, eccentric average peak force, net impulse, displacement and duration) or MVIC peak force with NZBC compared with placebo. Secondly, *ACTN3* and *TTN* genotypes appeared to influence the recovery of jump height and RSImod and eccentric average peak force, respectively, but no differences could be detected between NZBC and PLA groups. **Conclusion**. Analysis of both the intra- and inter-individual variability with the SWC and response CI's revealed that NZBC supplementation was unable to accelerate recovery for individuals following 100-DJP. *ACTN3* and *TTN* may explain some of the variability observed within CMJ outcome and neuromuscular variables.

6.2 Introduction

In the previous Chapter, when examining the group responses of NZBC extract intake for 7-days prior to- and 3-days following a 100-DJP, the supplementation intervention did not affect recovery as indicated by indices of muscle function, muscle soreness and inflammation. However, despite use of a repeated crossover design in an attempt to reduce some of the individual variability, large inter-individual variability in response was apparent for the CMJ outcome (jump height, RSImod, time to take off), neuromuscular variables (concentric average peak force, net impulse, average power, duration, eccentric average peak force, net impulse, displacement and duration) and MVIC peak force. It is probable, that even though the *a priori* power analysis indicated sufficient power was obtained to detect group differences of 10% (see Section 5.3.1), with 12 participants per group, the large variability in response may have masked potential effects in recovery with NZBC extract supplementation, particularly at the individual level.

Observations of inter-individual variability and 'responders and 'non-responders' to exercise training and nutrition interventions have been discussed previously (Mann et al. 2014; Bouchard and Rankinen, 2001; Hopkins, 2015). Attempts to quantify, predict and explain observed inter-individual variability in response to interventions has resulted in many discussions about the most appropriate method (Atkinson et al. 2019; Voisin et al. 2018; Sparks et al. 2017; Hecksteden et al. 2015) with no general consensus yet being agreed. To date, researchers have utilised a variety of approaches including the application of genotyping (Williams et al. 2017; Bouchard et al. 2015), replicated crossover designs (Goltz et al. 2019; Goltz et al. 2018; Senn et al. 2011) and statistical methods such as the SWC and response CI's (Swinton et al. 2018) and MBI (Atkinson and Batterham, 2015; Atkinson, Williamson and Batterham, 2019; Batterham and Hopkins, 2006). In Chapter 4, we utilised both a genotyping and statistical (SWC and response CI's) approach to assess the inter-individual response in an attempt to explain the individual variability observed in Chapter 3 However, due to the independent groups experimental design of Chapters 3 and 4, it was not possible to

confidently determine whether individuals who demonstrated positive, unchanged or negative meaningful responses to the intervention would present the same responses if the trial was repeated again (Senn, 2011). Further, it must be acknowledged that a source of inter-individual variability is founded in intra-individual variation (i.e., within-subject variation) (Chrzanowski-Smith et al. 2019). Thus, use of a repeated crossover design whereby the same participants are studied under similarly standardised testing conditions and procedures would allow for analysis of intra- and inter-individual variability in response to an intervention (Chrzanowski-Smith et al. 2019; Voisin et al. 2018; Solomon, 2018).

A large proportion of the evidence on *ACTN3* genotype and responses to EIMD has suggested that X-allele carriers are more susceptible to EIMD (Del Coso et al. 2018; Baumert et al. 2016). However, Venckunas et al. (2012) observed greater reductions in MVIC force and slower recovery profiles following drop-jumps in RR homozygotes compared to XX homozygotes. Further, the authors observed no influence of *ACTN3* genotype on the magnitude of the protective effect, from the RBE, when participants completed the drop-jump protocol a second time, 2-weeks after the initial bout. The authors concluded that *ACTN3* genotype can modulate the response of muscle function to plyometric jumping-exercise (i.e., drop-jumps) and that XX homozygotes recovery of baseline MVIC force was faster than RR homozygotes. Despite the lack of a clear consensus on which *ACTN3* genotype is more susceptible to EIMD, clearly it is possible that an individual's genotype may influence their rate of recovery from EIMD.

Therefore, this present Chapter utilised the same genotyping and statistical approaches as in Chapter 4 but with a repeated crossover design to allow for more accurate inference of the intra- and inter-individual responses to NZBC extract supplementation following 100-DJP.

Thus, the aims of this Chapter were; (1) to examine the intra- and inter-individual variability in response to EIMD induced by 100-DJP with NZBC or placebo supplementation using the SWC and response CI's. (2) To examine the association between the SNPs ACTN3, ACE and TTN

and the responses to EIMD induced by 100-DJP with NZBC extract or placebo supplementation.

The following hypothesis were tested:

- As no group benefit was observed in Chapter 4 with NZBC extract supplementation on recovery from EIMD following 100-DJP, that in utilising the SWC and response CI's, this would elucidate that some participants did experience positive responses on recovery from EIMD as indicated by muscle function measures with NZBC supplementation.
- Participants with the RR genotype for ACTN3, T allele for TTN and D (deletion) for ACE would show greater losses in muscle function when compared to the homozygous RX (XX), CC and II counterparts, respectively.

6.3 Methods

6.3.1 Participants

Twelve healthy non-resistance trained men completed all study procedures. All participants were recreationally active (see Section 5.3.1 (mean±SD: age 29±6 years, stature 1.80±0.07 m, body mass 78.0±10.7 kg, Σ of 4 skinfolds 35.7±12.3 mm, maximal voluntary isometric contraction (MVIC) baseline 497±120 N) (see Chapter 5, Table 5.1 for full participant characteristics).

6.3.2 Experimental design, familiarisation, muscle damaging exercise, knee extensor force, maximal voluntary isometric contraction, countermovement jump, saliva collection, DNA isolation, DNA genotyping, α-actinin-3 (*ACTN3*), angiotensin-I converting enzyme (*ACE*), Titin (*TTN*), supplementation protocol, dietary and exercise control and body composition assessment

Please refer to the Sections 5.3.2 – 5.3.6 for details on the experimental design, familiarisation, muscle damaging exercise, knee extensor force, maximal voluntary isometric contraction, Section 3.3.6 for CMJ, Sections 4.3.2 – 4.3.7 for the saliva collection, DNA isolation, DNA

genotyping, α-actinin-3 (*ACTN3*), angiotensin-I converting enzyme (*ACE*), Titin (*TTN*) and Sections 5.3.15 – 5.3.17 for the supplementation protocol, dietary and exercise control and body composition assessment. Participant genotypes for the retrospective SNP (*ACTN3, ACE* and *TTN*) analysis can be located in Table 6.1).

Table 6.1 Summary of participant genotypes.

Participant	*ACTN3*	*ACE*	*TTN*
1	RX	ID	CC
2	RR	II	CT
3	XX	ID	CC
4	RX	II	CC
5	RX	II	CT
6	RR	DD	CT
7	RX	DD	CC
8	RX	DD	CC
9	RX	ID	CC
10	RX	II	CC
11	RX	ID	CT
12	RX	ID	CC

Total for each SNP, alpha-actinin-3 (*ACTN3*) RR = 2, RX = 9, XX = 1; angiotensin-I converting enzyme (*ACE*) II = 4, ID = 5, DD = 3; titin (*TTN)* CC = 8, CT = 4, TT = 0. RR II, CC, homozygous dominant; RX, ID, CT, heterozygous dominant; XX, DD, TT, homozygous recessive.

6.3.3 Data analysis

To address the first aim of the present Chapter, data for each parameter were assessed for normal distribution with the Shapiro-Wilk test and the homogeneity of variance of each genotype was assessed using Levene's statistic. Hardy-Weinberg equilibrium was determined

for the *ACTN3, ACE* and *TTN* SNPs using a Chi-Square (X^2) test. As no group differences were apparent for NZBC vs. PLA from the repeated measures ANOVA in Chapter 5, supplementation groups were treated as the covariate to allow for independent assessment of any potential associations of genotype with the independent variables across time. Two-way mixed ANCOVAs [within-subjects factor: time (pre-exercise, post-exercise, 24, 48 and 72 h post exercise; between-subjects factor: genotype (RR, RX; II, ID; CC, CT, respectively); covariate: group (NZBC or PLA)] with Bonferroni post-hoc tests were used to detect associations between the *ACTN3*, *ACE* and *TTN* SNPs and CMJ outcome and neuromuscular variables and MVIC response before and following the 100-DJP. Differences in participant group and genotype characteristics were analysed with paired samples *t*-tests. Statistical analyses were completed using Statistical Package for Social Sciences 23.0 (SPSS, Chicago, Illinois). The alpha level for statistical significance was set at 0.05 *a priori*. Partial-eta^2 (ηp^2) effect sizes (ANOVA) with respective 90%CI are reported to indicate the magnitude of observed effects (Lakens, 2013). Partial-eta^2 (ηp^2) effect sizes of 0.01 – 0.06, 0.06 – 0.14 or ≥0.14 are considered small, medium and large changes, respectively (Lakens, 2013).

To address the second aim of this present Chapter, a reliability trial conducted before data collection enabled quantification of the absolute test re-test reliability of each CMJ outcome and neuromuscular variable and MVIC peak force (see Chapter 4, Table 4.2 for CMJ and Section 5.3.6 for MVIC). The typical error of measurements (TE) was determined using the methods recommend by Swinton et al. (2018), and true score 95% confidence intervals (CI) were then constructed using the TE corrected by sample size (Swinton et al. 2018). To aid inference of the graphical representations, a descriptive interpretation is provided (Bonafiglia et al. 2018). The smallest worthwhile change (SWC) was calculated as 0.2 x SD, representing a ‘small’ effect size and the smallest meaningful decrement in performance (Swinton et al. 2018; Copay et al. 2007), plus the SEM (see Chapter 4, Table 4.2 for CMJ and Section 5.3.6 for MVIC).

Data in text and tables are reported as mean (95%CI) and data in figures as mean with individual data points (Weissgerber et al. 2015) for group and genotype effects and mean individual observed score with true 95%CI and SWC for group inter-individual variation (Swinton et al. 2018; Bonafiglia et al. 2018), unless otherwise stated.

6.4 Results

Data relating to group characteristics and dietary intake can be located in Chapter 4, Table 4.1 and Table 4.2, respectively.

6.4.1 Countermovement jump and maximal voluntary isometric contraction intra- and inter-individual responses to 100-DJP

6.4.2 Inter-individual CMJ height responses

Figure 6.1, panel A shows that immediately post the 100-DJP, 2 participants in both the PLA and NZBC group demonstrated negative meaningful responses for CMJ height. In addition, 1 participant in the PLA group demonstrated a positive meaningful response immediately post 100-DJP for CMJ height.

Figure 6.1, panel B shows that at 24 h post the 100-DJP, 3 participants in both the PLA and NZBC group demonstrated negative meaningful responses for CMJ jump height. In addition, 2 participants in the PLA and NZBC group demonstrated positive meaningful responses 24 h post 100-DJP for CMJ height.

Figure 6.1, panel C shows that at 48 h post the 100-DJP, 4 and 2 participants in PLA and NZBC groups, respectively, demonstrated negative meaningful responses. Further, 4 participants and 1 participant in PLA and NZBC groups, respectively, demonstrated positive meaningful responses for CMJ height.

Figure 6.1, panel D shows that at 72 h post the 100-DJP, 3 participants in NZBC group demonstrated a negative meaningful response for CMJ height. In addition, 3 and 2 participants

in the PLA and NZBC groups, respectively, demonstrated positive meaningful responses for CMJ height.

6.4.3 Intra-individual CMJ height responses

Figure 6.1, panel A demonstrates that for CMJ height, participants 3, 4, 5, 7 and 12 had differing responses for NZBC and PLA supplementation immediately post 100-DJP. Figure 6.1, panel B demonstrates that participants 6, 7, 9, 10, 11 and 12 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.1, panel C demonstrates that participants 1, 5, 7, 9, 10 and 12 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Lastly, figure 6.1, panel D demonstrates that participants 3, 4, 5, 6, 10 and 12 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP.

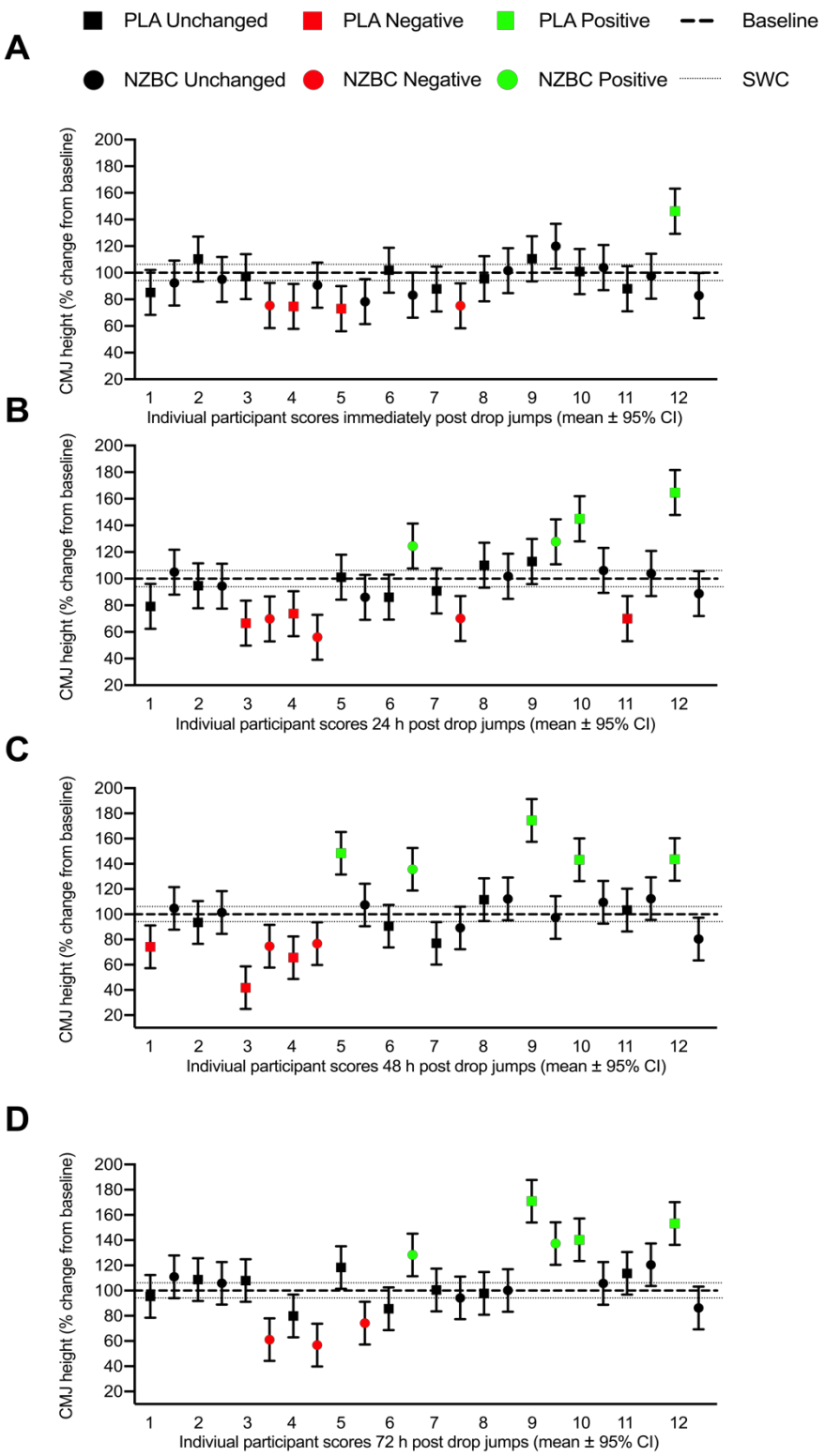


Figure 6.1 Individual observed values for CMJ height (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 6% for CMJ height. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.4 Inter-individual CMJ TTT responses

Figure 6.2, panels A and B demonstrates that immediately and 24 h post the 100-DJP, 1 participant in NZBC group demonstrated a positive meaningful response for CMJ TTT.

Figure 6.2, panel C demonstrates that at 48 h post 100-DJP, 2 participants in NZBC group demonstrated positive meaningful response for CMJ TTT. In addition, 1 participant in PLA group demonstrated a negative meaningful response for CMJ TTT.

Figure 6.2, panel D demonstrates that at 72 h post 100-DJP, 1 participant in both NZBC and PLA groups demonstrated a negative meaningful response and 1 participant in the NZBC group only, demonstrated a positive meaningful response for CMJ TTT. All other responses for CMJ height and TTT, were unchanged in both NZBC and PLA groups.

6.4.5 Intra-individual CMJ TTT responses

Figure 6.2, panel A demonstrates that for CMJ TTT, participant 11 had differing responses for NZBC and PLA supplementation immediately post 100-DJP. Figure 6.2, panel B demonstrates that participant 4 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.2, panel C demonstrates that participants 1, 4 and 10 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Figure 6.2, panel D demonstrates that participant 4 had a differing response for NZBC and PLA supplementation, 72 h post 100-DJP. All other participant responses for CMJ height and TTT remained the same in both NZBC and PLA conditions across all time points.

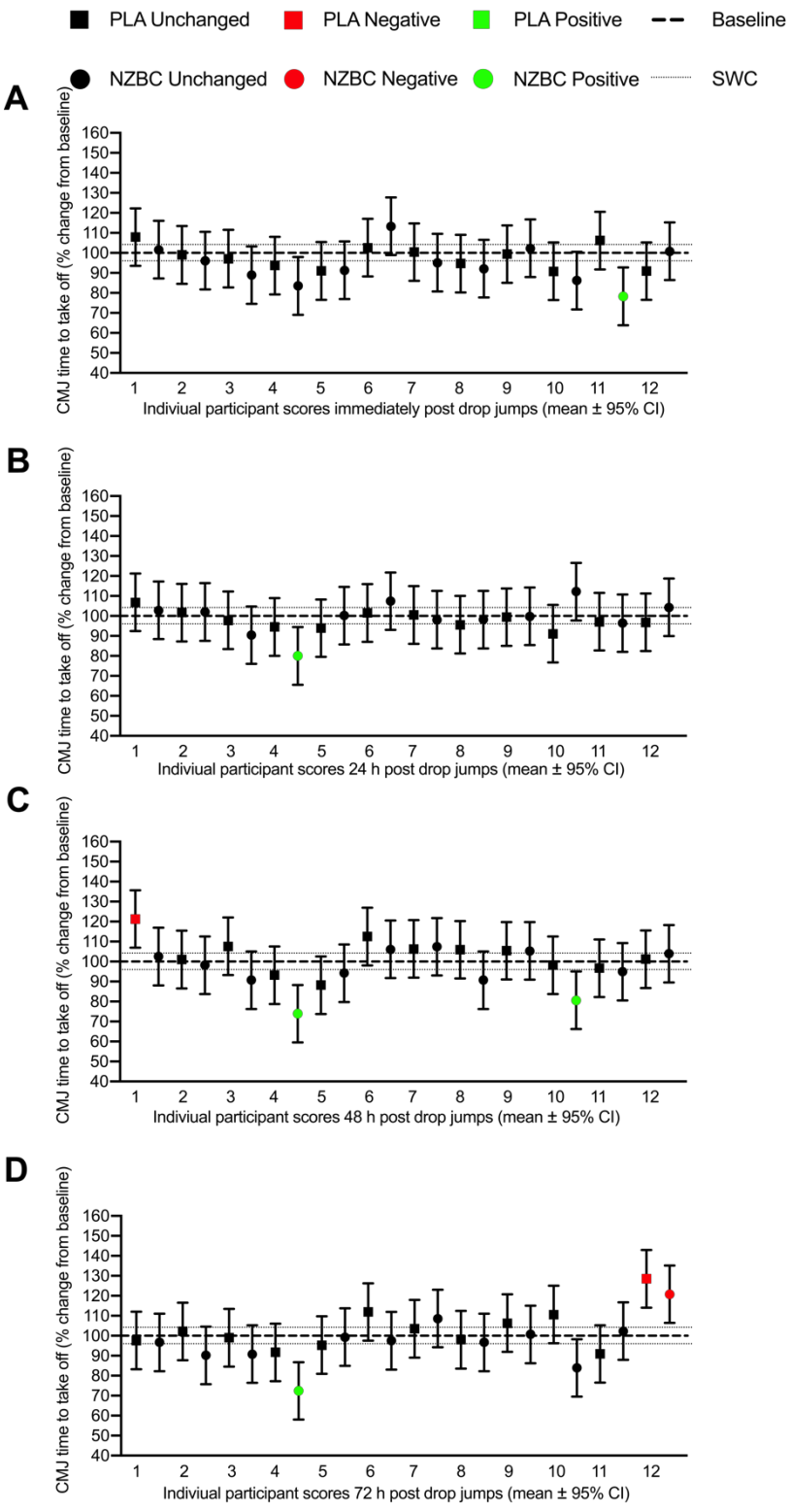


Figure 6.2 Individual observed values for CMJ TTT (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 4% for CMJ TTT. **Red**, **black** and **green** circles represent **negative**, **unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.6 Inter-individual CMJ concentric average power responses

Figure 6.3, panel A demonstrates that 1 participant in PLA and NZBC group demonstrated negative meaningful responses immediately following 100-DJP for CMJ concentric average power.

Figure 6.3, panel B demonstrates that 3 participants and 1 participant in the PLA and NZBC group, respectively, demonstrated negative meaningful responses 24 h post 100-DJP. Further 1 participant in the PLA group demonstrated positive meaningful responses 24 h post 100-DJP for CMJ concentric average power.

Figure 6.3, panel C demonstrates that 4 participants in PLA group demonstrated negative meaningful responses 48 h following 100-DJP for CMJ concentric average power. Furthermore, 2 participants and 1 participant in the PLA and NZBC groups, respectively, demonstrated positive meaningful responses 48 h following 100-DJP for CMJ concentric average power.

Figure 6.3, panel D demonstrates that 3 participants in the NZBC group demonstrated negative meaningful responses 72 h following 100-DJP for CMJ concentric average power. In addition, 1 participant and 2 participants in the PLA and NZBC group, respectively, demonstrated positive meaningful responses 72 h following 100-DJP for CMJ concentric average power. All other participant responses for CMJ concentric average power remained the same in both NZBC and PLA conditions across all time points.

6.4.7 Intra-individual CMJ concentric average power responses

Figure 6.3, panel B demonstrates that for CMJ concentric power, participant 5, 11 and 12 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.3, panel C demonstrates that participants 1, 4, 5, 7, 8 and 11 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Lastly, figure 6.3, panel D shows participants 3, 4, 6, 7, 9 and 11 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP. All other participant responses for CMJ concentric power remained the same in both NZBC and PLA conditions across all time points.

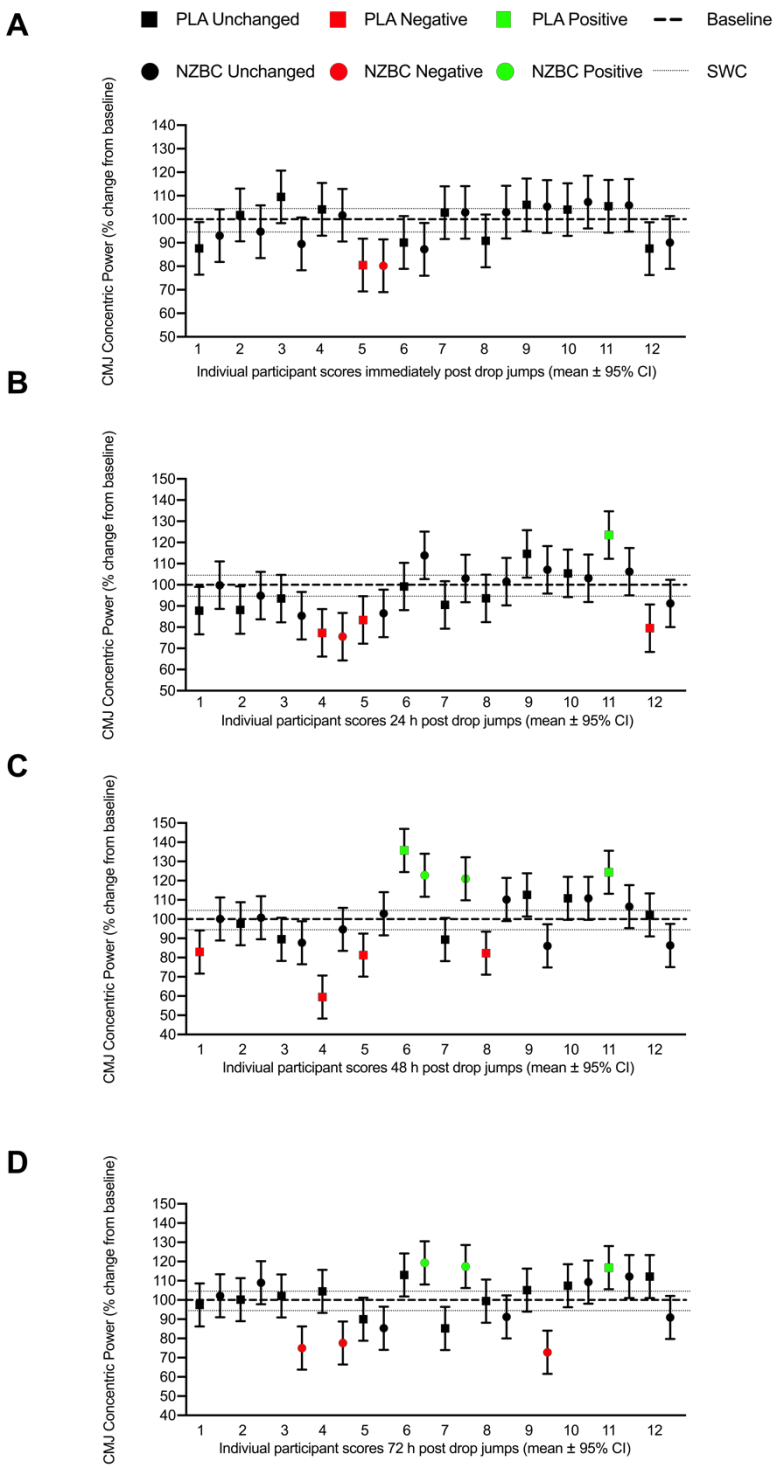


Figure 6.3 Individual observed values for CMJ concentric average power (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 5% for CMJ concentric average power. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.8 Inter-individual CMJ concentric net impulse responses

Figure 6.4, panel A demonstrates that all responses for CMJ concentric net impulse remained unchanged immediately post 100-DJP for both PLA and NZBC groups.

Figure 6.4, panel B demonstrates that at 24 h post 100-DJP, 3 and 2 participants in PLA and NZBC, respectively, demonstrated negative meaningful responses for CMJ concentric net impulse and 1 participant in PLA group demonstrated positive meaningful responses.

Figure 6.4, panel C demonstrates that at 48 h post 100-DJP, 3 and 2 participants in PLA demonstrated negative and positive meaningful responses, respectively, for CMJ concentric net impulse.

Lastly, Figure 6.4, panel D demonstrates that at 72 h post 100-DJP, 3 participants in NZBC and 1 participant in PLA demonstrated negative and positive meaningful responses, respectively, for CMJ concentric net impulse. All other responses for CMJ concentric average power and net impulse, were unchanged in both PLA and NZBC groups.

6.4.9 Intra-individual CMJ concentric net impulse responses

Figure 6.4, panel B shows that for CMJ concentric net impulse, participants 11 and 12 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.4, panel C demonstrates that participants 1, 4, 5, 6 and 11 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Lastly, figure 6.4, panel D shows that participants 3, 4, 5, 9 and 11 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP. All other participant responses for CMJ concentric power and net impulse remained the same in both NZBC and PLA conditions across all time points.

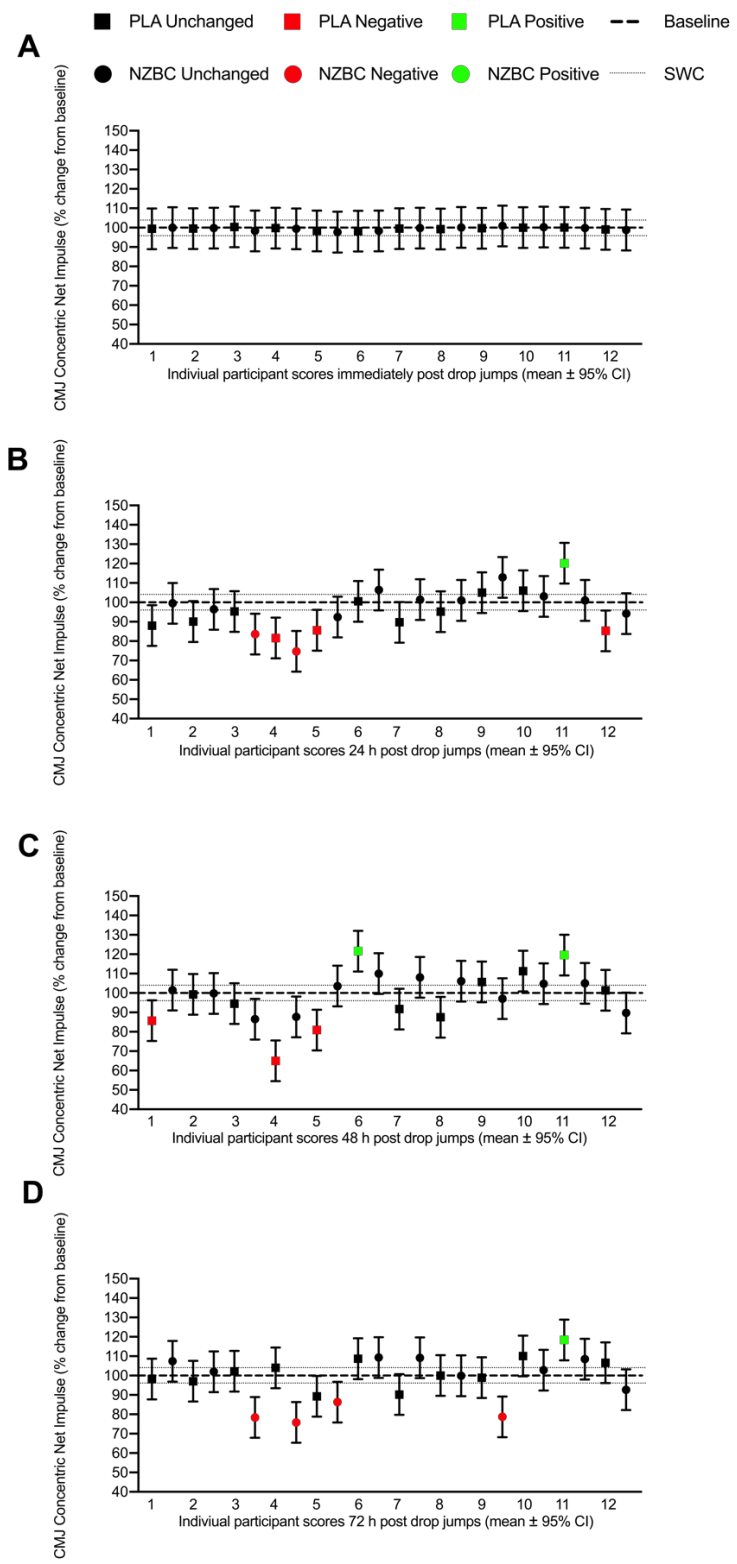


Figure 6.4 Individual observed values for CMJ concentric net impulse (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 4% for CMJ concentric net impulse. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.10 Inter-individual CMJ concentric peak force responses

Figure 6.5, panel A demonstrates that immediately post 100-DJP, 2 participants in NZBC group demonstrated negative meaningful responses for CMJ concentric peak force.

Figure 6.5, panel B demonstrates that at 24 h post 100-DJP, 3 and 2 participants in NZBC group demonstrated negative and positive meaningful responses, respectively, for CMJ concentric peak force.

Figure 6.5, panel C demonstrates that at 48 h post 100-DJP, 1 participant and 3 participants in PLA and NZBC, respectively, demonstrated negative meaningful responses for CMJ concentric peak force. Further, 1 participant in NZBC group demonstrated positive meaningful responses for CMJ concentric peak 48 h post 100-DJP.

Figure 6.5, panel D demonstrates that at 72 h post 100-DJP, 4 and 3 participants in NZBC group demonstrated negative and positive meaningful responses, respectively, for CMJ concentric peak force. All other responses for CMJ concentric peak force, were unchanged in both NZBC and PLA groups.

6.4.11 Intra-individual CMJ concentric peak force responses

Figure 6.5, panel A demonstrates that for CMJ concentric peak force, participants 3, 5 and 6 had differing responses for NZBC and PLA supplementation immediately post 100-DJP. Figure 6.5, panel B demonstrates that participants 3, 4, 5, 6 and 7 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.5, panel C shows that participants 3, 4, 6, 9, 10 and 12 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Lastly, figure 6.5, panel D shows that participants 3, 4, 5, 6, 7, 9 and 11 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP.

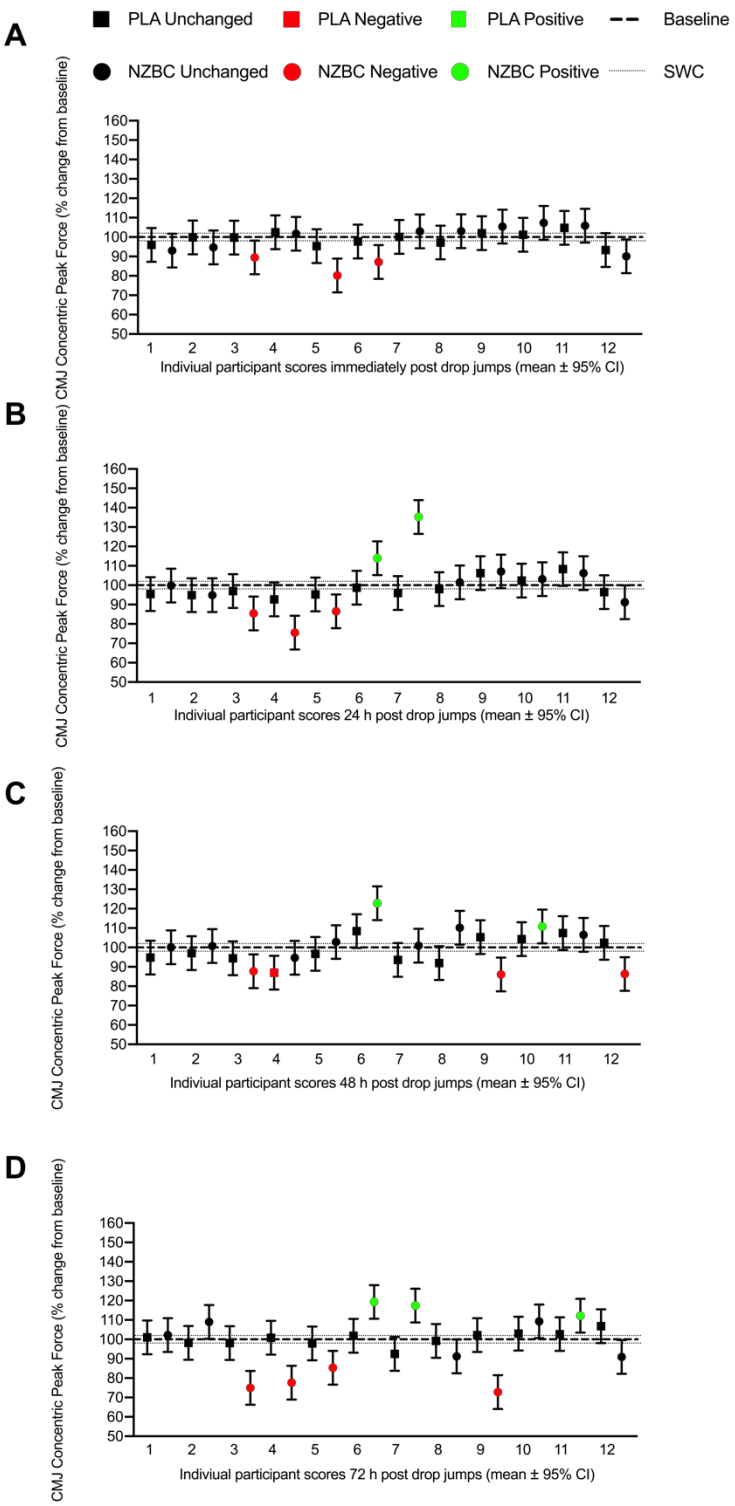


Figure 6.5 Individual observed values for CMJ concentric average peak force (panels A, B, C, D) and immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 2% for CMJ concentric average peak force. **Red**, **black** and **green** circles represent **negative**, **unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.12 Inter-individual CMJ eccentric peak force responses

Figure 6.6, panel A demonstrates that immediately post 100-DJP, 1 participant in PLA demonstrated negative meaningful responses for CMJ eccentric peak force.

Figure 6.6, panel B and C demonstrates that at both 24 h and 48 h post 100-DJP, 1 participant in both PLA and NZBC groups demonstrated negative meaningful responses for CMJ eccentric peak force. All other responses for CMJ eccentric peak force, were unchanged in both NZBC and PLA groups.

6.4.13 Intra-individual CMJ eccentric peak force responses

Figure 6.6, panel A demonstrates that for CMJ eccentric peak force, participant 7 had differing responses for NZBC and PLA supplementation immediately post 100-DJP. All other participant responses for CMJ concentric and eccentric peak force remained the same in both NZBC and PLA conditions across all time points.

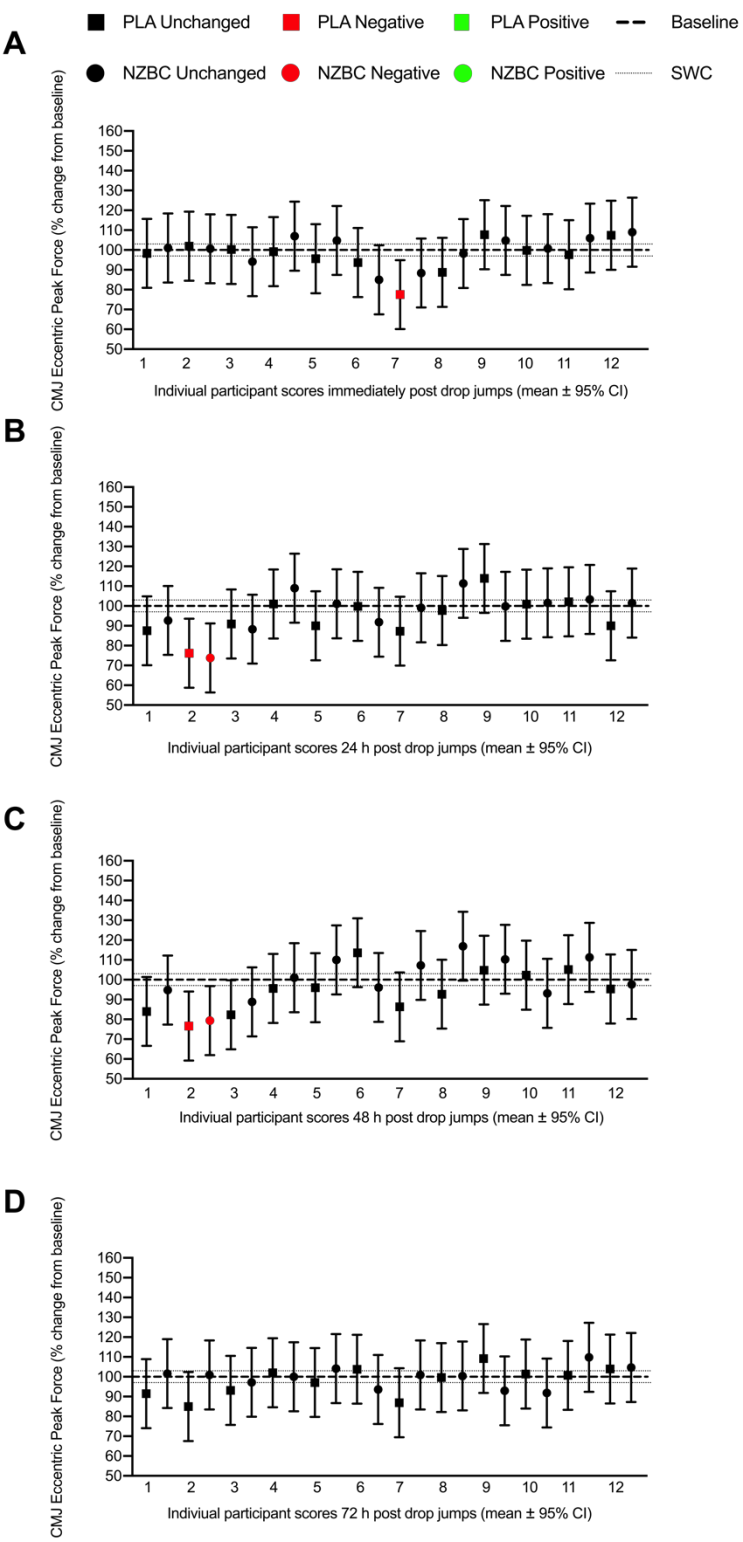


Figure 6.6 Individual observed values for CMJ eccentric average peak force (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) is calculated as 3% for CMJ eccentric average peak force. **Red, black** and **green** circles represent **negative, unchanged,** and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.14 Inter-individual CMJ eccentric net impulse responses

Figure 6.7, panel A demonstrates that all responses for CMJ eccentric net impulse remained unchanged immediately post 100-DJP for both PLA and NZBC groups.

Figure 6.7, panel B demonstrates that at 24 h post 100-DJP, 1 participant in both PLA and NZBC groups demonstrated negative meaningful responses for CMJ eccentric net impulse.

Figure 6.7, panel C demonstrates that at 48 h post 100-DJP, 2 participants and 1 of participant in PLA and NZBC groups, respectively, demonstrated negative meaningful responses and 1 participant in both PLA and NZBC groups demonstrated positive meaningful responses for CMJ eccentric net impulse.

Figure 6.7, panel D demonstrates that at 72 h post 100-DJP, 1 participant in PLA group demonstrated positive meaningful responses for CMJ eccentric net impulse. All other responses for CMJ eccentric net impulse, were unchanged in both NZBC and PLA groups.

6.4.15 Intra-individual CMJ eccentric net impulse responses

Figure 6.7, panel C demonstrates that for CMJ eccentric net impulse, participants 1, 6 and 11 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Figure 6.7, panel D demonstrates that participant 6 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP. All other participant responses for CMJ eccentric net impulse remained the same in both NZBC and PLA conditions across all time points.

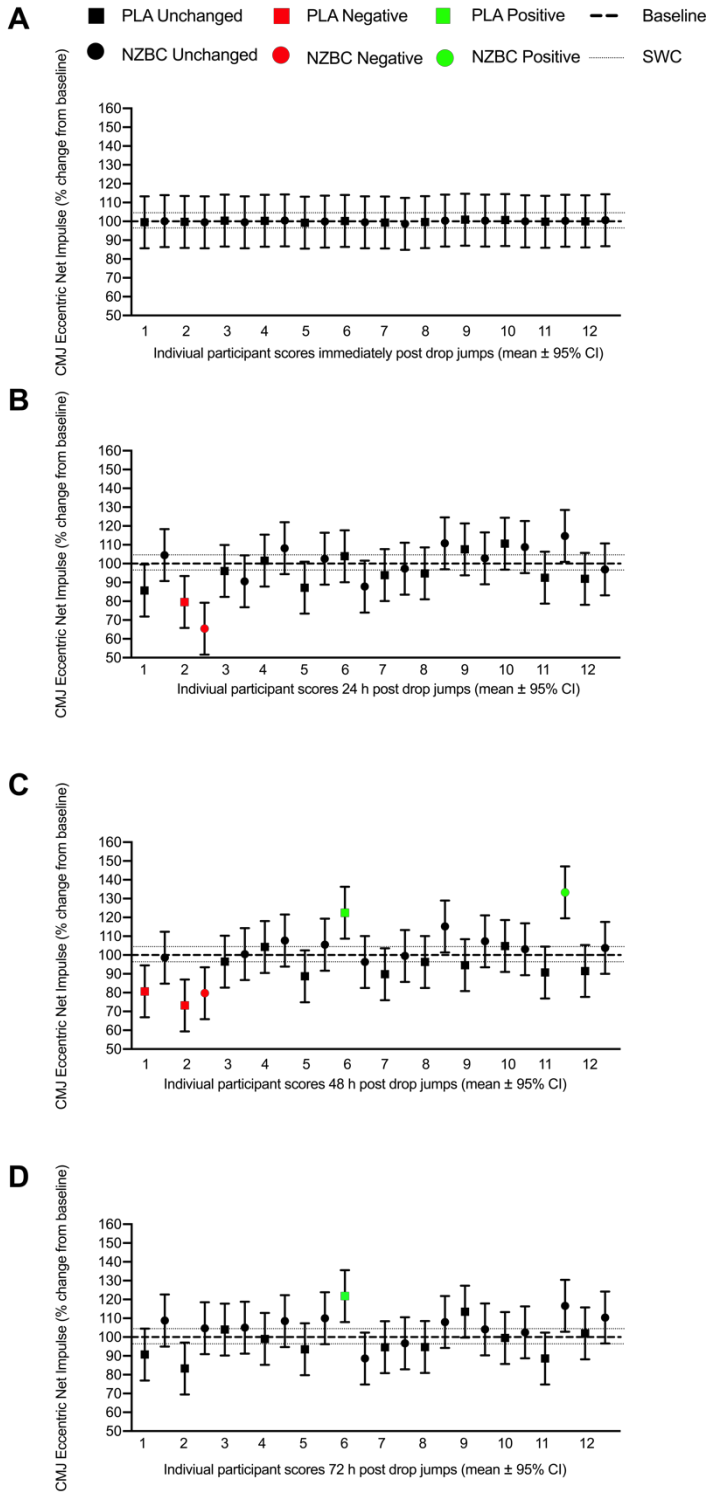


Figure 6.7 Individual observed values for CMJ eccentric net impulse (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) was calculated as 5% for CMJ eccentric net impulse. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.16 Inter-individual CMJ eccentric phase duration responses

Figure 6.8, panel A demonstrates that all responses for CMJ eccentric phase duration remained unchanged immediately post 100-DJP for both PLA and NZBC groups.

Figure 6.8, panel B demonstrates that at 24 h post 100-DJP, 4 and 5 participants in PLA and NZBC groups, respectively, demonstrated negative meaningful responses and 2 participants in both PLA and NZBC groups demonstrated positive meaningful responses for CMJ eccentric phase duration.

Figure 6.8, panel C demonstrates that at 48 h post 100-DJP, 3 and 2 participants in PLA and NZBC groups, respectively, demonstrated negative meaningful responses. Further, 1 participant and 2 participants in PLA and NZBC, respectively, demonstrated positive meaningful responses for CMJ eccentric phase duration at 48 h post 100-DJP.

Lastly, Figure 6.8, panel D demonstrates that at 72 h post 100-DJP, 2 participants in both PLA and NZBC groups demonstrated negative meaningful responses and 2 participants in NZBC group only, demonstrated positive meaningful responses for CMJ eccentric phase duration. All other responses for CMJ eccentric phase duration, were unchanged in both NZBC and PLA groups.

6.4.17 Intra-individual CMJ eccentric phase duration responses

Figure 6.8, panel B shows that for CMJ eccentric phase duration, participants 1, 4, 5, 8 and 10 had differing responses for NZBC and PLA supplementation 24 h post 100-DJP. Figure 6.8, panel C shows that participants 2, 4, 6, 9, 10 and 12 had differing responses for NZBC and PLA supplementation 48 h post 100-DJP. Figure 6.8, panel D shows that participants 2, 4, 6, 9 and 10 had differing responses for NZBC and PLA supplementation 72 h post 100-DJP. All other participant responses for CMJ eccentric phase duration remained the same in both NZBC and PLA conditions across all time points.

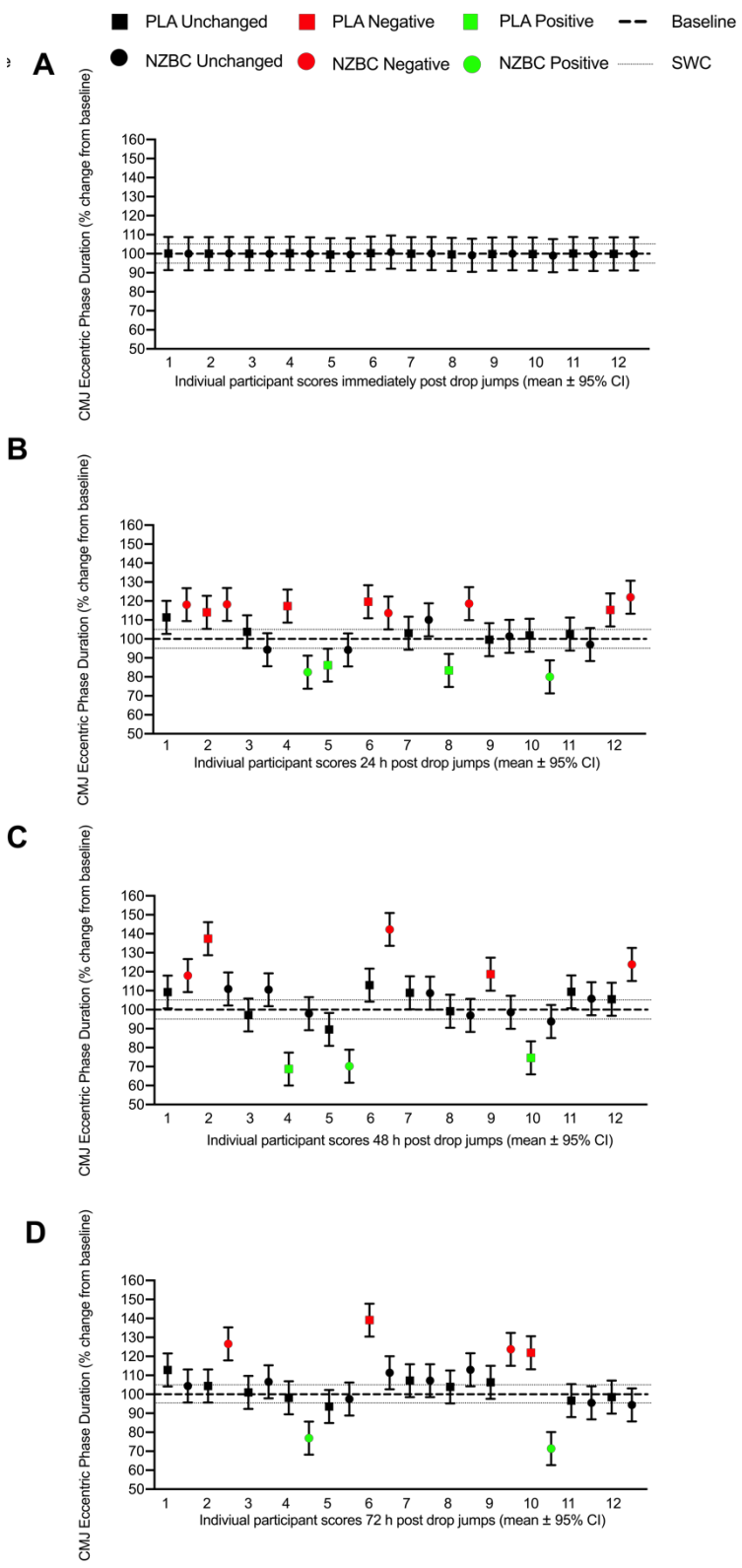


Figure 6.8 Individual observed values for CMJ eccentric phase duration (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) was calculated as 5% for CMJ eccentric phase duration. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.18 Inter-individual CMJ concentric phase duration responses

Figure 6.9, panels A and B demonstrates that all responses for CMJ concentric phase duration remained unchanged immediately and 24 h post 100-DJP for both NZBC and PLA groups.

Figure 6.9, panel C and D demonstrates that at both 48 h and 72 h post 100-DJP, 1 participant in NZBC group demonstrated positive meaningful responses for CMJ concentric phase duration. All other responses for CMJ concentric phase duration, were unchanged in both NZBC and PLA groups.

6.4.19 Intra-individual CMJ concentric phase duration responses

Figure 6.9, panels C and D demonstrate that for CMJ concentric phase duration, participant 4 had differing responses for NZBC and PLA supplementation 48 h and 72 h post 100-DJP. All other participant responses for CMJ concentric phase duration remained the same in both NZBC and PLA conditions across all time points.

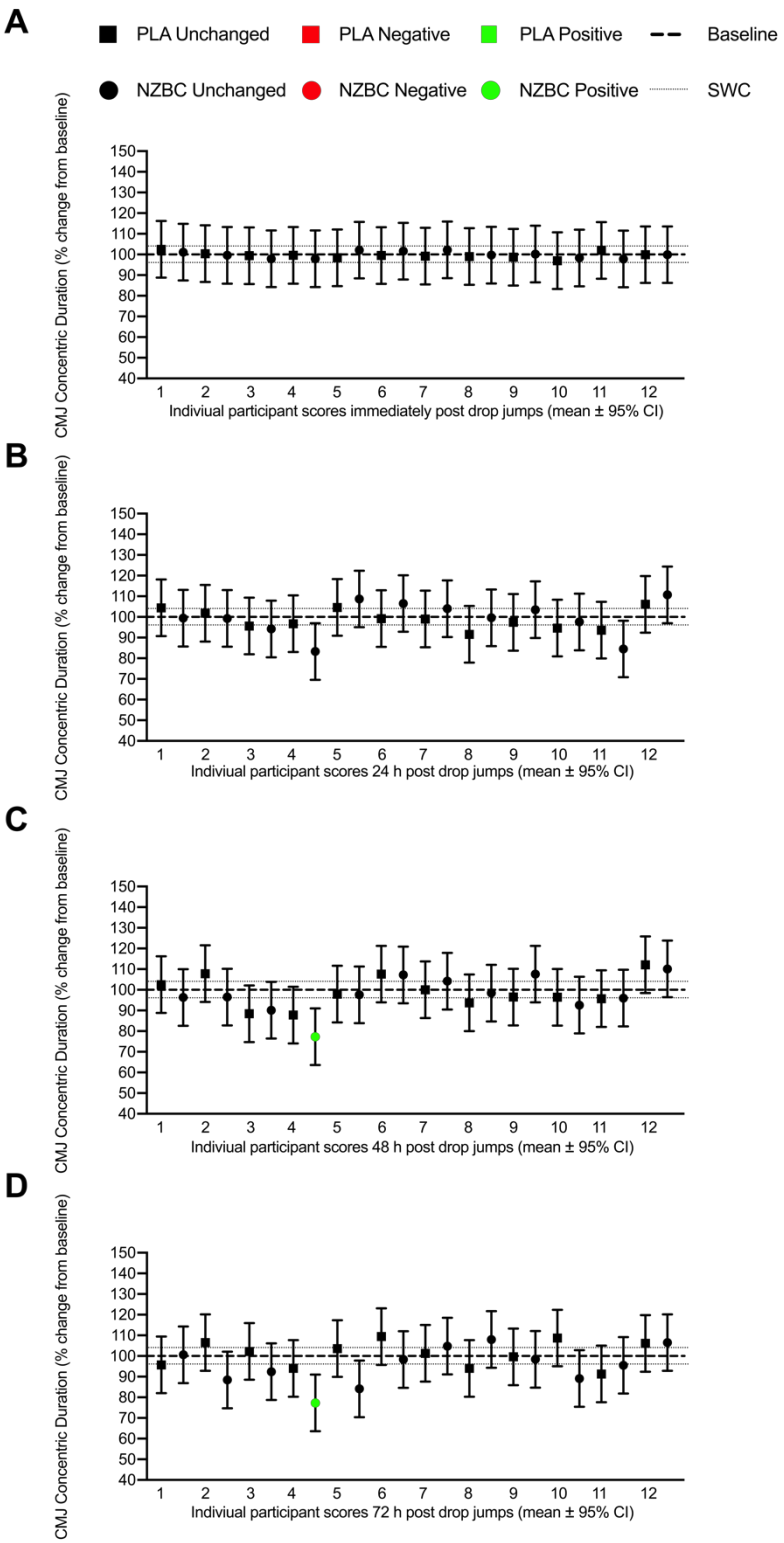


Figure 6.9 Individual observed values for CMJ concentric phase duration (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) was calculated as 3% for concentric phase duration. **Red**, **black** and **green** circles represent **negative**, **unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.20 Inter-individual MVIC peak force responses

Figure 6.10, panels A, B, C and D shows that all responses for MVIC peak force for both NZBC and PLA groups remained unchanged at all time points following 100-DJP.

6.4.21 Intra-individual MVIC peak force responses

Figure 6.10, panels A, B, C and D shows that all responses for MVIC peak force for both NZBC and PLA groups remained the similarly unaffected at all time points following 100-DJP.

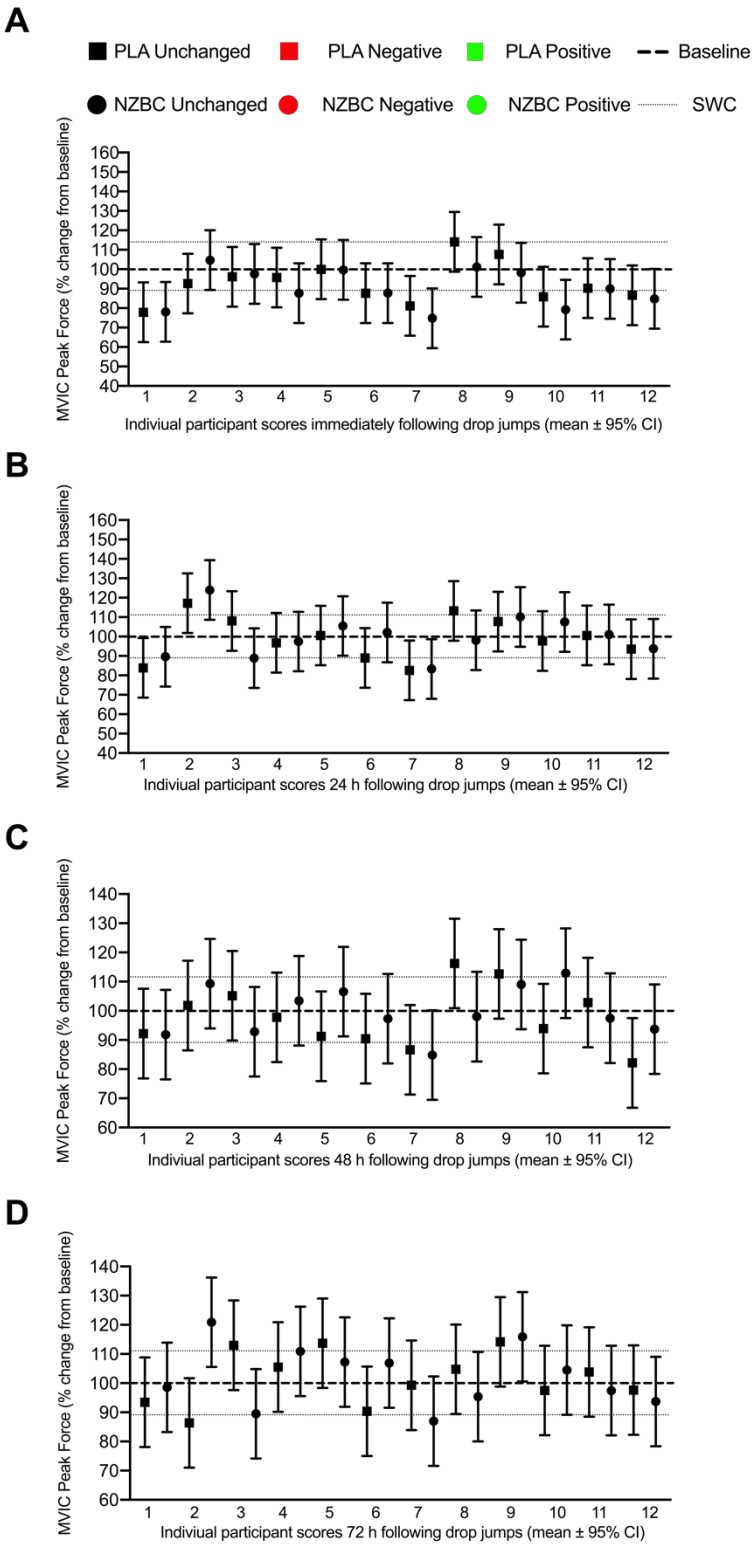


Figure 6.10 Individual observed values for MVIC average peak force (panels A, B, C, D) immediately post, 24, 48 and 72 h after 100-DJP with 95% true score CI's. Smallest worthwhile change (SWC) was calculated as 11% for MVIC average peak force. **Red, black** and **green** circles represent **negative, unchanged**, and **positive** meaningful response, respectively. Participant numbers are matched within and across panels. Data are n=12 per group.

6.4.22 SNP associations with markers of EIMD following 100-DJP

Genotype frequency distribution for the *ACTN3* (rs1815739, X^2=3.750, *P*=0.153), *ACE* (rs4341, X^2=2.000, *P*=0.368) and *TTN* (rs10497520, X^2=2.178, *P*=0.140) SNPs were in Hardy-Weinberg equilibrium. Due to the low numbers of *ACTN3* XX homozygotes (n=1), *ACE* DD homozygotes (n=3) and *TTN* TT homozygotes (n=0) present in the sample population, these data were combined with those heterozygotes (RX, ID and CT, respectively) as has been previously recommended (Stebbings et al. 2017; Del Coso et al. 2017a; Del Coso et al. 2017b).

No main effects of time were evident for *ACTN3, ACE* and *TTN* for CMJ jump height or RSImod. However, a between-subjects effect was observed for *ACTN3* genotype with CMJ jump height ($F_{(1, 19)}$=5.27, *P*=0.033, ηp^2=0.217) and RSImod ($F_{(1, 20)}$=4.64, *P*=0.044, ηp^2=0.188) (Table 6.2). A main effect of time was apparent for *ACE* genotype and CMJ TTT ($F_{(4, 76)}$=3.14, *P*=0.019, ηp^2=0.142) and an interaction effect was observed ($F_{(4, 76)}$=2.98, *P*=0.024, ηp^2=0.136). Post-hoc comparisons revealed that no significant differences occurred at any time points (*P*>0.05). No main effects of time or interaction effects were apparent for *ACTN3, ACE* and *TTN* for CMJ eccentric peak force. However, a between-subjects effect was observed for *TTN* genotype ($F_{(1, 21)}$=6.45, *P*=0.019, ηp^2=0.235) with CMJ eccentric peak force (Table 6.2). There were no other significant genotype main effects or associations with any CMJ outcome or neuromuscular variable for *ACTN3, ACE* or *TTN* (*P*>0.05) (Table 6.3; Table 6.4).

Table 6.2 *ACTN3* and *ACE* genotype significant between-group responses for CMJ performance and neuromuscular variables following 100-DJP.

SNP	rs-number	Between subjects-factor	Beneficial (*n*)	Detrimental (*n*)	*P*-Value	Effect Size (ηp^2) (90% CI)
ACTN3	1815739	Jump height	RR (2)	RX (XX) (10)	0.033	0.217 (0.01–0.435)
		RSImod			0.044	0.188 (0.01–0.404)
TTN	10497520	Eccentric average peak force	CC (8)	CT (TT) (4)	0.019	0.235 (0.02–0.442)

ACTN3, alpha-actinin-3; titin, *TTN*; RR, CC, homozygous dominant; RX, CT, heterozygous dominant; XX, TT, homozygous recessive; SNP, single nucleotide polymorphism; 100-DJP, 100-drop jump protocol; RSImod, reactive strength index modified.

Table 6.3 ACTN3 and TTN genotype group responses pre- and post-100-DJP with NZBC supplementation. Values are mean (95%CI). 95%CI have been adjusted for sample size. ACTN3 (n=2, RR; n=10, RX) and TTN (n=8, CC; n=4, CT).

SNP (rs-number)	CMJ Variable	Pre drop-jumps	Post drop-jumps	24 h post drop-jumps	48 h post drop-jumps	72 h post drop-jumps
ACTN3 (1815739)	Jump height (cm)					
	RR	0.30 (0.15–0.45)	0.27 (0.10–0.45)	0.32 (0.24–0.39)	0.34 (0.27–0.42)	0.34 (0.23–0.45)
	RX	0.22 (0.16–0.28)	0.21 (0.15–0.26)	0.22 (0.17–0.27)	0.23 (0.17–0.30)	0.20 (0.14–0.26)
	RSImod (index)					
	RR	0.31 (0.13–0.49)	0.28 (0.04–0.51)	0.31 (0.19–0.42)	0.34 (0.21–0.47)	0.37 (0.19–0.55)
	RX	0.20 (0.16-0.24)	0.20 (0.16–0.24)	0.21 (0.16–0.25)	0.23 (0.17–0.29)	0.18 (0.14–0.23)
TTN (10497520)	Eccentric average peak force (N/kg)					
	CC	18.80 (16.96–20.63)	18.79 (17.29–20.28)	18.84 (16.94–20.73)	19.03 (16.70–21.36)	18.60 (16.43–20.77)
	CT	16.92 (14.00–19.84)	16.87 (13.84–19.90)	15.45 (13.48–17.43)	16.57 (14.32–18.82)	17.21 (14.62–19.79)

ACTN3, alpha-actinin-3; titin, *TTN*; RR, CC, homozygous dominant; RX, CT, heterozygous dominant; XX, TT, homozygous recessive; SNP, single nucleotide polymorphism; 100-DJP, 100-drop jump protocol; RSImod, reactive strength index modified; NZBC, New Zealand blackcurrant.

Table 6.4 ACTN3 and TTN genotype group responses pre- and post-100-DJP with PLA supplementation. Values are mean (95%CI). 95%CI have been adjusted for sample size. ACTN3 (n=2, RR; n=10, RX) and TTN (n=8, CC; n=4, CT).

SNP (rs-number)	CMJ Variable	Pre drop-jumps	Post drop-jumps	24 h post drop-jumps	48 h post drop-jumps	72 h post drop-jumps
ACTN3 (1815739)	Jump height (cm)					
	RR	0.32 (0.26–0.38)	0.34 (0.25–0.43)	0.29 (0.21–0.37)	0.29 (0.23–0.36)	0.31 (0.18–0.44)
	RX	0.22 (0.16–0.28)	0.20 (0.15–0.26)	0.21 (0.15–0.26)	0.21 (0.15–0.27)	0.24 (0.19–0.29)
	RSImod (index)					
	RR	0.34 (0.29–0.39)	0.35 (0.26–0.44)	0.30 (0.23–0.37)	0.29 (0.21–0.37)	0.31 (0.16–0.45)
	RX	0.21 (0.15–0.28)	0.20 (0.14–0.26)	0.20 (0.15–0.26)	0.20 (0.15–0.25)	0.23 (0.17–0.29)
TTN (10497520)	Eccentric average peak force (N/kg)					
	CC	19.50 (17.21–21.79)	18.79 (17.29–20.28)	18.59 (16.95–20.23)	17.98 (16.33–19.63)	19.08 (17.31–20.85)
	CT	17.07 (14.05–20.08)	16.62 (13.25–19.99)	15.51 (14.07–16.96)	16.45 (14.50–18.39)	16.38 (14.41–18.34)

ACTN3, alpha-actinin-3; titin, *TTN*; RR, CC, homozygous dominant; RX, CT, heterozygous dominant; XX, TT, homozygous recessive; SNP, single nucleotide polymorphism; 100-DJP, 100-drop jump protocol; RSImod, reactive strength index modified; NZBC, New Zealand blackcurrant.

Figure 6.11, panel A highlights that there was a main effect of time for *ACE, ACTN3* and *TTN* for MVIC peak force. Post-hoc comparisons revealed that MVIC peak force was reduced immediately post 100-DJP compared to baseline for *ACE* (*P*=0.025) and *TTN* (*P*=0.017) only. There were no significant genotype main effects or associations with MVIC peak force for *ACTN3, ACE* o*r TTN* (*P*>0.05).

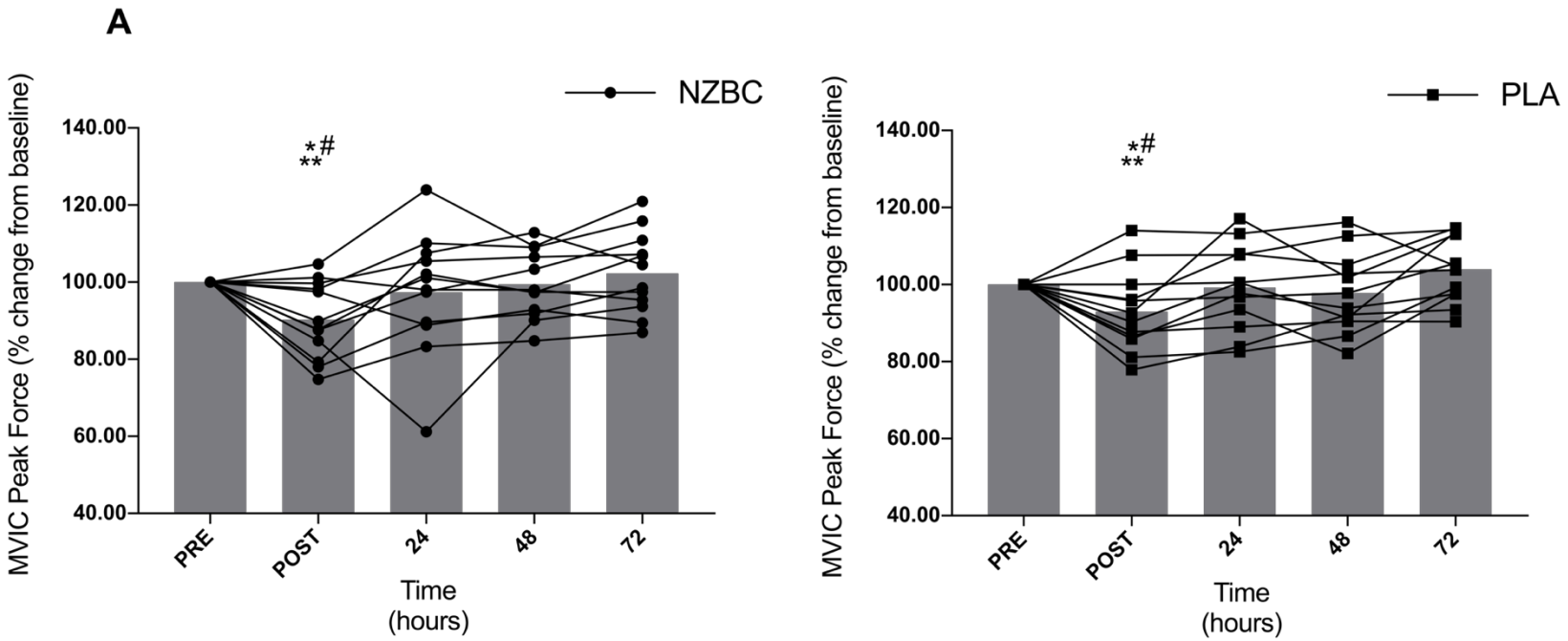


Figure 6.11 Percentage changes in MVIC average peak force (panel A), immediately pre-, post-, 24, 48 h and 72 h following 100-DJP. *, **, and # indicate a main effect of time for ACTN3, ACE and TTN genotype, respectively (P<0.05); data are presented as mean (bars) and individual data points (n=12 per group).

6.5 Discussion

The purpose of the present Chapter was to investigate the intra- and inter-individual variability in response to a 100-DJP with NZBC extract or placebo supplementation. Firstly, by examining the intra- and inter-individual variability in response to EIMD induced by 100-DJP with NZBC or placebo supplementation using the SWC and response CI's. Secondly, by examining the association between the candidate SNPs ACTN3, ACE and TTN and the responses to EIMD induced by 100-DJP with NZBC or placebo supplementation. The main findings from this study were that on the individual level, contrary to the first hypothesis, no effects on recovery were observed for CMJ outcome (jump height, RSImod, time to take off), neuromuscular variables (concentric average peak force, net impulse, average power, duration, eccentric average peak force, net impulse, displacement and duration) or MVIC peak force with NZBC than with placebo. Secondly, in partial support of the second hypothesis, *ACTN3* and *TTN* genotypes appeared to influence the recovery of jump height and RSImod and eccentric average peak force, respectively, but no differences could be detected between NZBC and PLA groups. However, this observation is a preliminary finding and requires further studies with a much larger sample size than in the present investigation, to enable definitive conclusions to be drawn. First, the intra- and inter-individual CMJ and MVIC recovery responses following 100-DJP are discussed and second, the associations of *ACTN3* and *TTN* genotype on muscle function recovery following 100-DJP.

6.5.1 Intra- and inter-individual responses on recovery following eccentric-heavy drop jump exercise with NZBC supplementation

Utilising the recommended approaches of Swinton et al. (2018) and Bonafiglia et al. (2018), it was observed that, contrary to our hypothesis, NZBC extract supplementation did not accelerate individual's muscle function recovery following 100-DJP. Further, when comparing individual participant responses across supplementation groups (i.e., intra-individual variation), at each time point for CMJ outcome and neuromuscular variables, only a few participants had differing responses above the SWC and TE thresholds. For example, for CMJ

height immediately post 100-DJP, participants 3 and 7 demonstrated negative meaningful responses when supplementing with NZBC extract, compared to unchanged meaningful responses when supplementing with placebo (Figure 6.1, panel A). In contrast, all responses for MVIC peak force at immediately-, 24, 48 and 72 h-post 100-DJP remained unchanged regardless of supplementation group (Figure 6.10, panels A, B, C and D, respectively). However, when considering the combined SWC and TE of the MVIC peak force measure, it is apparent by visual inspection of the 95%CI plots, that the majority of individuals in both NZBC and PLA groups did not exceed these thresholds. Arguably, the use of 95%CI and a SWC and TE of 11% was too conservative meaning that we were more likely to make a Type 2 error (false negative) when interpreting the individual plots. Further, the inter-day CV for the MVIC peak force measure was 5%, which is greater than the 1.1% (Clifford et al. 2015) and 3.7% (Bell et al. 2015) that has been previously reported for this technique. However, when considering the reduction in MVIC peak force of 26% relative to baseline in a pilot study (n=4) conducted before this main trial when participants were first exposed to the 100-DJP, it is probable that the lack of a positive or negative meaningful response in MVIC peak force for both supplementation groups following 100-DJP in this present study is due to the RBE (McHugh et al. 2003; Nosaka and Clarkson, 1995). Thus, it appears that if individuals are habituated to an eccentric-heavy exercise, such as 100-DJP, then the protective effect associated with the RBE renders a nutrition intervention such as NZBC extract ineffective. Whilst it has been suggested that researchers interested in nutrition interventions that can accelerate recovery from EIMD should avoid repeated crossover designs due to the known phenomenon of the RBE (Howatson and van Someren, 2008), athletes rarely partake in training sessions that are novel to them, instead carrying out sessions that they are habituated to and yet can still experience symptoms associated with EIMD such as DOMS (Gentle et al. 2011; Moreira et al. 2014; Souglis et al. 2015; Doma et al. 2017). Arguably, if a nutrition intervention can be deemed an effective recovery aid, it needs to be able to accelerate recovery even when the exercise task is not novel. Nonetheless, this Chapter documents for

the first time the intra- and inter-individual responses to EIMD induced by 100-DJP with NZBC or PLA supplementation utilising the SWC and 95% response CI.

Although it has been previously suggested that as the movements for 100-DJP and CMJ are similar, the CMJ may be a more sensitive and specific test for detecting subtle performance changes compared with MVIC, which requires no dynamic movement (Clifford et al. 2015; Baker et al. 1994), the lack of observable meaningful change with the CMJ outcome and neuromuscular variables, particularly immediately post 100-DJP, is likely due to a post-activation performance enhancement effect due to the 100-DJP and CMJ sharing similar kinematic characteristics (Tillin and Bishop, 2009; Blazevich and Babault, 2019). In Chapter 5, a main effect of time was observed immediately post the 100-DJP for eccentric phase duration where both groups appeared to take less time to complete this phase of the CMJ. In the present Chapter, it is clear that this response was uniform across both groups with all participants demonstrating unchanged meaningful responses immediately post the 100-DJP. However, at the recovery visits; 24, 48 and 72 h post 100-DJP where no prior postactivation performance enhancement effect is apparent, it is clear that a few individuals took longer to perform the eccentric phase of the CMJ, regardless of supplementation group (Figure 6.8, panel A, B, C and D). Interestingly, it appears that the same individuals took longer performing the eccentric phase of the CMJ in both supplementation groups (i.e., participants 2 and 6) at 24 h post 100-DJP (Figure 6.8, panel B). This observation is similar to that of previous findings where it has been suggested that following a *k*-cluster-based analysis and stratification into three groups (high-responders, moderate-responders and low-responders), it appears that some individuals are be more susceptible to EIMD than others (Damas et al. 2016).

6.5.2 Genotype associations with time

In Chapter 5, main effects of time were apparent for MVIC peak force and CMJ eccentric phase duration indicating that muscle damage was present but as no group or interaction effects were evident for NZBC extract or PLA, supplementation group was used as a covariate

herein, so that genotype association on recovery from 100-DJP could be independently assessed.

6.5.3 *ACTN3* genotype associations on recovery following eccentric-heavy drop jump exercise

In contrast to Venckunas et al. (2012), it was observed that *ACTN3* genotype may have played a role in the variability of individual's CMJ height, with RR homozygotes demonstrating greater CMJ heights at all time points pre- and post-drop-jumps compared to RX heterozygotes (Table 6.4 and 6.5). This observation is reflected in the CMJ RSImod index values being greater in RR homozygotes than RX heterozygotes at all time points pre- and post-drop-jumps (Table 6.4 and 6.5). Conversely, as no main effects of time were apparent for CMJ height and RSImod, indicating that the RBE may have occurred following the familiarisation trial, these findings are in accordance with Venckunas et al. (2012), whereby it does not appear that *ACTN3* genotype influences the RBE. However, this is a preliminary observation and future studies are warranted with larger sample sizes before any firm conclusions can be made.

It must be noted that *ACTN3* genotype for CMJ jump height and RSImod only account for 22% and 19%, respectively (Table 6.3), of the total variance observed, thus may only be a small part of a much bigger picture. What is more likely, is that the cumulative influence of several genetic polymorphisms, including *ACTN*3, are related to the magnitude of EIMD response following strenuous exercise (Del Coso et al. 2018; Deuster et al. 2013).

6.5.4 TTN genotype associations on recovery following eccentric-heavy drop jump exercise

To date, the evidence for an association between *TTN* genotype, EIMD and the potential influence on the RBE is limited. For the first time in the present Chapter, we observed a potential influence of *TTN* genotype on CMJ eccentric peak force variability, where CC homozygotes appeared to demonstrate greater eccentric peak force than CT heterozygotes at all time points (Table 6.4 and 6.5). In accordance with the available evidence to date, this

observation may be explained by the observation of Stebbings et al. (2017) where T-allele carriers were found to have shorter skeletal muscle fascicle lengths compared to CC homozygotes. As fascicle length has an influence on the force-velocity and force-length relationships (Timmins et al. 2016), during the eccentric phase of the CMJ, it is possible that the T-allele carriers are at a tenuous disadvantage for being able to produce as much force as CC homozygotes (Lieber and Ward, 2011; Lieber and Friden, 2000). However, this is purely speculation and further research with a larger sample size, particularly with more TT homozygotes, is needed. It has been suggested that structural proteins such as titin, are crucial regulators of eccentric force production (Herzog, 2017). As the eccentric phase of the SSC is an integral part of dynamic actions, such as CMJ performance (Cormie et al. 2009; Gathercole et al. 2015), knowing individuals genotype for structural proteins such as *TTN* may help individuals incorporate more eccentric loading work into their training in an attempt to attenuate the magnitude of EIMD experienced. It must be noted that *TTN* genotype for CMJ eccentric peak force only accounted for 24% (Table 6.3), of the total variance observed, thus may only be a small part of a much bigger picture. What is more likely, is that the cumulative influence of several structural protein genetic polymorphisms, including *TTN*, are related to the magnitude of EIMD response following strenuous exercise (Baumert et al. 2016).

6.6 Limitations

The present Chapter presents some limitations derived from the experimental design chosen that have to be discussed to improve the scope and applicability of the outcomes. Firstly, although we attempted to explore the inter- and intra-individual variability in response to EIMD induced by 100-DJP with NZBC extract or placebo supplementation using the SWC and response CI's in a repeated crossover design, it is acknowledged that in order to capture true intra-individual differences, researchers should look to utilise a replicated randomised controlled trial (RCT) design (Chrzanowski-Smith et al. 2019). The application of this study design requires researchers to provide an adequate washout period in between each of the RCTs, utilising the same participants to examine if individuals demonstrate a consistent

response or non-response to an intervention relative to control (Chrzanowski-Smith et al. 2019; Senn et al. 2011; Hecksteden et al. 2015; Atkinson et al. 2019). However, implementation of this design poses considerable logistical and practicality challenges for both the participants and researchers. Despite its challenges, a few studies have implemented this design in trying to establish true inter- and intra-individual variability with appetite (Goltz et al. 2018), training (Lindholm et al. 2016) and drug treatment (Senn et al. 2011) interventions. The appearance of such study designs demonstrates a move towards the importance of measuring intra-individual variation to determine whether true inter-individual response differences exist above the measurement and biological error. Despite a hesitancy to study intervention effectiveness with EIMD in a repeated crossover design, due to the known adaptive response with the RBE following an initial unaccustomed exercise bout, we have demonstrated for the first time that both intra- and inter-individual responses to EIMD may exist as indicated by CMJ outcome and neuromuscular variables and MVIC peak force.

Second, this study retrospectively studied the genotype of *ACTN3, ACE* and *TTN* for each of the participants in repeated measures (i.e., within-subject comparisons). Although repeated measures designs increase statistical power, the sample size in this present study is far from optimal for any firm conclusions to be drawn about genotype associations. Given the low frequency of the minor allele for *ACTN3, ACE* and *TTN* homozygotes (i.e., XX, DD, TT) in Caucasian populations, it is perhaps not surprising that we were unable to find many, if any, in our sample. For example, based on the X-allele frequency for the *ACTN3* genotype that was observed within the present study, future studies would require at least 312 participants to obtain approximately 50 XX homozygotes. However, this Chapter did not intend on evaluating the multitude of interactions that may occur between involved genes and their protein products (Noble, 2011), but instead, aimed to try and examine any possible associations between the candidate SNPs and markers of EIMD to help elucidate some of the individual variation observed within the data from Chapter 5. Conversely, using this approach meant using the supplementation groups as a covariate in the analysis due to the lack of a

group effect of NZBC extract or PLA, thus, this approach was adopted as it allowed examination across the multiple time points and the multiple time points of interest (PRE, POST, 24 h, 48 h and 72 h), between SNP genotypes. Future research should look to implement a 'stress the genotype' approach and recruit a large number of participants and screen them for their respective genotypes before allocating them to intervention groups as has been done previously (Montgomery et al. 2002). Utilising such an approach would allow researchers to appropriately explore gene-environment interactions and establish whether specific genotypes increase an individual's susceptibility to EIMD following strenuous exercise.

6.7 Conclusion

This Chapter highlights for the first time how intra-individual variation contributes to the inter-individual variability apparent with EIMD induced by 100-DJP and that NZBC extract supplementation was unable to accelerate recovery from 100-DJP. Further, that common polymorphisms in the *ACTN3* and *TTN* gene may explain part of the individual variation observed within the CMJ outcome measures jump height and RSImod and neuromuscular variable eccentric peak force, respectively, during recovery post 100-DJP. However, much larger sample sizes that provide representation of both major and minor alleles and a broader range of candidate SNPs that may elucidate more potential associations with susceptibility to EIMD are warranted before definitive conclusions can be made.

Although this Chapter was able to highlight that intra-individual variability is apparent and a likely contributor to the inter-individual variation that is a frequently observed with EIMD (Vincent et al. 2010; Clarkson et al. 2005), a key question that remains unanswered is whether or not the NZBC extract supplementation was able to increase circulating levels of key phenolic acids from anthocyanin when superimposed on top of a habitual diet so that they could exert desired biological effects, such as mitigate symptoms associated with EIMD (Bowtell and Kelly, 2019). To date, studies that have examined the plasma uptake and

bioavailability of phenolic acids following NZBC extract supplementation, have utilised polyphenol-restricted diets to ensure proof of concept (Matsumoto et al. 2001; Roehrig et al. 2019; McGhie et al. 2003) but lack in ecological validity. Thus, the aim of Chapter 7 is to examine the time course of selected key phenolic acids from anthocyanin (VA, GA and PCA) following acute ingestion of a single dose (300mg) of NZBC extract in individuals following a non-polyphenol restricted diet. Chapter 8 will then draw on the observations from Chapter 7, to examine what the plasma uptake of VA, GA and PCA was retrospectively in Chapter 5 and whether there were any relationships between the phenolic acid appearance of the measures of muscle function (MVIC and CMJ).

7. Plasma Uptake of Selected Phenolic Acids Following New Zealand Blackcurrant Extract Supplementation in Humans

7.1 Abstract

Introduction. New Zealand blackcurrant (NZBC) extract is a rich source of anthocyanins but in order to be able to exert health and/or physiological effects, the supplement needs to be bioavailable *in vivo*, even when superimposed on top of a habitual diet. This Chapter investigated the plasma uptake of selected phenolic acids following NZBC extract supplementation alongside a habitual diet. **Methods.** Twenty healthy volunteers (eleven men) (mean±SD: age 28±7 years, height 1.73±0.09 m, body mass 73±11 kg, body mass index 24±3 kg/m^2) consumed a 300 mg NZBC extract capsule (CurraNZ™; containing a total of 105 mg anthocyanins) following an overnight fast. Venous blood samples were taken pre (0) and 1, 1.5, 2, 3, 4, 5 and 6 h post-ingestion of the capsule. Plasma concentrations of vanillic acid (VA), gallic acid (GA), and protocatechuic acid (PCA) were analysed by reversed-phase high-performance liquid chromatography (HPLC). All dependent variables were analysed using a treatment x time one-way, repeated measures analysis of variance (ANOVA). Pearson (r) correlation coefficients were calculated for the relationship between habitual anthocyanin intake and each metabolite (VA, GA and PCA) and between each metabolite (VA, GA and PCA). The alpha level for statistical significance was set at 0.05 *a priori* and Partial-eta^2 (ηp^2) effect sizes are reported. **Results.** The HPLC analysis identified two dihydroxybenzoic (VA and PCA) and one trihydroxybenzoic acids (GA) in plasma following NZBC supplementation. Habitual anthocyanin intake was 168 (95%CI: 68–404) mg·day^{-1} and no associations were observed between this and VA, PCA and GA plasma uptake. Plasma time-concentration curves revealed that VA, GA and PCA were most abundant at 3, 4 and 1.5 h post-ingestion, respectively. **Conclusion.** This is the first study to demonstrate that a NZBC extract supplement increases the plasma uptake of phenolic acids VA, GA and PCA when consumed alongside non-polyphenol restricted habitual diet and inter-individual variability is apparent. This information could inform future *in vivo* work that examines the health and performance related benefits with acute NZBC extract supplementation by indicating the times at which

peak plasma concentrations are likely to be achieved and the overall plasma uptake of VA, GA and PCA.

7.2 Introduction

The results in Chapters 3, 4, 5 and 6 demonstrated that supplementation with NZBC extract alongside a non-polyphenol restricted diet resulted in individual variation in the recovery of markers of EIMD following strenuous exercise in the form of a half-marathon or drop-jumps. This raises the question as to whether the NZBC extract intake was able to increase systemic levels of anthocyanin phenolic acids in individuals to an extent where they would be able to exert a possible physiological effect (Bowtell and Kelly, 2019; Hurst et al. 2019).

Within the last decade, consuming functional foods such as whole berry fruits or concentrated berry extracts have grown in popularity likely due to their reported health and sport and exercise performance benefits (Bell et al. 2015; Braakhuis, Somerville and Hurst, 2020; Cook and Willems, 2018), which have been attributed to the berry fruits rich polyphenol content (Bowtell and Kelly, 2019). Flavonoids are one of the most abundant naturally occurring polyphenols, which have been the focus of much research interest and can be divided into six subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins and isoflavones (Martin and Appel, 2010).

Blackcurrant (*Ribes nigrum*) is a rich source of anthocyanin; however, the anthocyanin content can vary depending on the berry fruits specific cultivar, cultivation site, temperature exposure, processing, storage and ripeness (Cook and Willems, 2018; Mikulic-Petkovsek, Koron, Zorenc and Veberic, 2017; Del Rio, Borges and Crozier, 2010; Chalker-Scott, 1999). Blackcurrants have a distinct colour and are usually associated with high levels of anthocyanins, which are glycosides generated from anthocyanidins and are the pigments often responsible for the orange, red, and blue colours in fruits, vegetables, flowers and other storage tissues in plants (Blando, Gerardi and Nicoletti, 2004). Native cultivars of blackcurrants grown in New Zealand often have a higher anthocyanin content than European cultivars grown in North America (Schrage et al. 2010; Moyer, Hummer, Finn, Frei and Wrolstad, 2002). For example, Non-New Zealand cultivars, Ojebyn, contained 179 mg·100 mL anthocyanin, compared to New Zealand

cultivars, Ben Rua, containing 477 mg·100 mL^{-1} anthocyanin (Schrage et al. 2010; Moyer, Hummer, Finn, Frei and Wrolstad, 2002).

The high anthocyanin content within New Zealand blackcurrant (NZBC) has led to an increase in research interest in this particular cultivar (Braakhuis, Somerville and Hurst, 2020). Lyall et al. (2009) first reported acute use of NZBC extract capsules (240 mg anthocyanins in total) supplementation immediately before and following a 30-minute ergometer row. The authors concluded that the anthocyanins in NZBC were able to alleviate oxidative stress and complement the ability of exercise to enhance responsiveness to potential pathogens when presented with an *ex-vivo* lipopolysaccharide challenge. Subsequent NZBC extract supplementation studies have utilised a chronic dosing strategy whereby seven-days before the experimental visit, participants supplement with NZBC each day, and on day seven, perform the set exercise task/assessment. Supplementation with NZBC extract has been shown to have a range of potential health benefits, such as in increased fat oxidation (Cook et al. 2015; Cook et al. 2017a; Strauss et al. 2018), improved cardiovascular response (Willems et al. 2015; Cook et al. 2017b), improve glycaemic control (Willems et al. 2017) and enhance exercise performance (Willems et al. 2015; Cook et al. 2015; Cook et al. 2017a; Strauss et al. 2018; Perkins et al. 2015). These studies have used either commercially available NZBC extract in the form of capsules (CurraNZ™, Health Currancy Ltd, Surrey, UK) or NZBC powder dissolved in water (Sujon New Zealand blackcurrant, Gibb Holdings Ltd, New Zealand) at doses of 105 mg·day^{-1} (Willems et al. 2015; Cook et al. 2015; Cook et al. 2017a; Cook et al. 2017b), 138.6 mg·day^{-1} (Perkins et al. 2015; Willems et al. 2017), 210 mg·day^{-1} (Cook et al. 2017a; Cook et al. 2017b; Strauss et al. 2018; Willems et al. 2016; Murphy et al. 2017; Godwin, Cook and Willems, 2017) and 315 mg·day^{-1} (Cook, Myers, Gault, Edwards and Willems, 2017a; Cook, Myers, Gault, Edwards and Willems, 2017b). These doses are far higher than the reported estimations of habitual anthocyanin dietary intake which range from 19 to 65 mg·day^{-1} in European countries (Zamora-Ros et al. 2011).

For anthocyanins to be able to exert these health benefits *in vivo*, they would need to be bioavailable in sufficient quantities to produce systemic effects (Koli et al. 2010; Toutain and Bousquet-Melou, 2004). Of the anthocyanin pharmokinetics data available, it has been suggested that the phenolic acids, such as protocatechuic acid (PCA), often appear in systemic circulation in much higher concentrations than that of their parent compounds and that they could be responsible for the associated health benefits of anthocyanins (Fang, 2014). These phenolic acids provide unique taste, flavour and health-promoting properties and are found in many fruits and vegetables (Tomas-Barberan and Espin, 2001). Previously, Slimestad and Solheim (2002) identified ~15 different anthocyanins in Nordic-grown blackcurrant, of which the four which account for ~98% of the total anthocyanin content are; delphinidin 3-*O*-glucoside, delphinidin 3-*O*-rutinoside, cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside. The phenolic acids, PCA and gallic acid (GA) are the most abundant degradation products of cyanidin and delphinidin, respectively, the two major parent anthocyanins detected in NZBC whole berry (Slimestad and Solheim, 2002) and concentrate (Matsumoto et al. 2001). Whereas vanillic acid (VA) is a reported breakdown phenolic acid of peonidin 3-*O*-rutinoside, which has been identified as a minor compound in blackcurrant (Matsumoto et al. 2001).

To date, none of the aforementioned NZBC extract supplementation studies have provided data regarding the plasma uptake of the phenolic acids of anthocyanins. Understanding the plasma uptake of both acute and chronic intakes of NZBC extract would better inform optimal dosing strategies and intake guidelines and move dosing protocols towards a more rigorous pharmacokinetic approach. Furthermore, most plasma uptake studies incorporate dietary polyphenol restriction in their design in an attempt to reduce the background noise that may be introduced by variation in dietary polyphenol intake (Matsumoto et al. 2001; Keane et al. 2016). However, this approach may also maximise the effects produced by polyphenol supplementation (Bowtell and Kelly, 2019). Therefore, it has been suggested to ensure ecological validity (Bowtell and Kelly, 2019), superimposing polyphenol supplementation onto

a habitual diet is the most appropriate method to assess the plasma uptake of anthocyanin-rich berries and the potential applicability to exert a health or physiological effect *in vivo*.

Despite a number of reports indicating beneficial effects of NZBC extract supplementation, the plasma uptake of phenolic acids, VA, GA and PCA following NZBC extract ingestion when supplemented during a non-polyphenol restricted diet has yet to be investigated. Therefore, the aim of this investigation was to examine the time course of VA, GA and PCA following acute ingestion of a single dose of NZBC extract in individuals following a non-polyphenol restricted diet.

The following hypothesis was tested:

- The NZBC extract supplementation would increase plasma concentrations of VA, GA and PCA in the hours following acute ingestion.

7.3 Methods

7.3.1 Participants

Eleven healthy men and nine women (mean±SD: Age 28±7 years, height 1.73±0.09 m, body mass 73±11 kg, body mass index 24±3 kg/m^2) volunteered to participate in the study. All participants were non-smokers, in apparent good health, with no known food allergies and not currently using any nutritional supplementation. The study was approved by the University of Chichesters Research Ethics Committee and conducted in accordance with the Helsinki Declaration (2013). Participants gave their written informed consent after explanation of the experimental procedures.

7.3.2 Experimental design

Participants completed a self-report food diary and an anthocyanin food frequency questionnaire to quantify habitual anthocyanin intake (Cook et al. 2017) in the three days leading up to the laboratory visit and refrained from strenuous exercise in the 48 h before their

visit. On arrival to the laboratory after an overnight fast (≥12 h), participant's height and body mass was recorded, and a cannula (BD Venflon™ intravenous cannula with port, 18 G, Becton Dickinson, Wokingham, UK) inserted into an antecubital vein. Resting venous blood samples were drawn pre (0) and 1, 1.5, 2, 3, 4, 5 and 6 h after NZBC extract capsule ingestion (Figure 7.1). Participants remained seated and rested for the duration of the blood sample time points to control for compartmental fluid shifts associated with changes in body posture (Hagan, Diaz and Horvath, 1978). Participants were permitted to only consume water *ab libitum* during their laboratory visit.

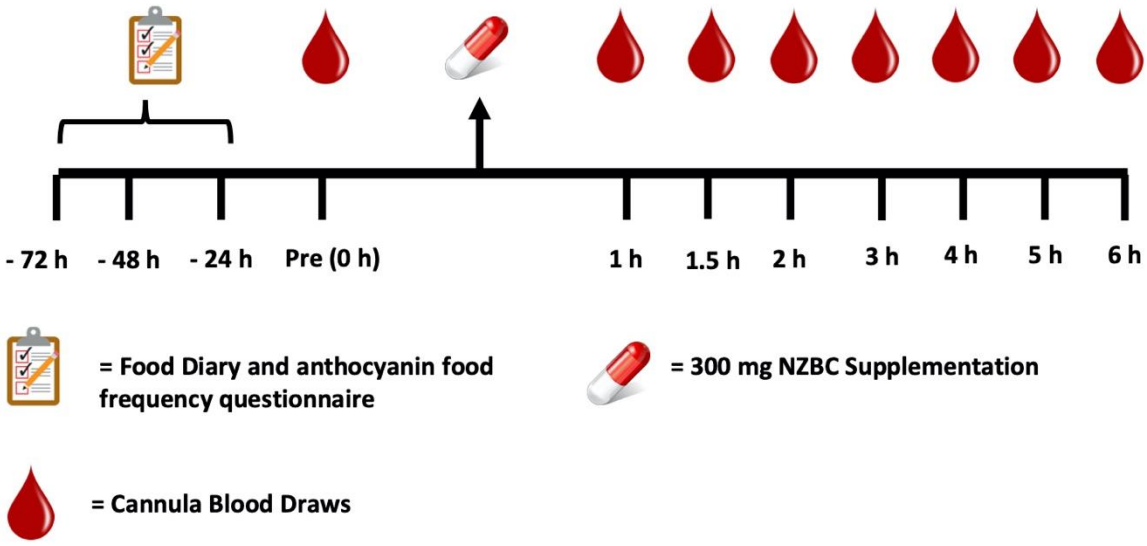


Figure 7.1 Study design

7.3.3 Supplementation, dietary intake and habitual anthocyanin intake

The NZBC extract capsules (CurraNZ™, Health Currancy Ltd, Surrey, UK) were stored at room temperature (~20°C) in an opaque container prior to use to avoid UV light degradation (Fossen, Luis and Andersen, 1998). Participants consumed one concentrated NZBC extract capsule (300 mg active cassis containing 105 mg of anthocyanins) after the resting blood draw

with water. The NZBC extract capsules manufacturer's HPLC analysis reported that each capsule contained 37–53 mg delphinidin-3-rutinoside, 5–12 mg delphinidin-3-glucoside, 32-47 mg cyanidin-3-rutinoside, 3-11 mg cyanidin-3-glucoside (New Zealand Pharmaceuticals Ltd.). Participants were instructed to maintain their normal diet prior their visit to maintain study ecological validity (Bowtell and Kelly, 2019). Participants recorded their three-day dietary intake in food diaries which were analysed (Nutritics LTD, Dublin, Ireland) for carbohydrate, fat and protein, and total energy intake (kJ). The habitual anthocyanin food frequency questionnaire detailed the amount and frequency of anthocyanin containing foods eaten within the last three months from the Phenol Explorer database (Neveu et al. 2010). The intake of anthocyanin was then calculated as the sum of the consumption frequency of each anthocyanin containing food, multiplied by the content of the anthocyanin content for the portion sizes.

7.3.4 Blood sampling

Fasting whole blood samples were collected into two chilled 5 mL K3 EDTA tubes (Sarstedt, AG and Co, Kommanditgesellschaft, Germany), inverted to mix the anticoagulant and immediately centrifuged at 3000xg for 10 min at 4^{o}C. Plasma was aspirated and pipetted into ~1 mL aliquots and then immediately stored at -80^{o}C for subsequent analysis.

7.3.5 Plasma Extraction

A method previously described (Keane at al. 2016) was used for the extraction of phenolic compounds from the plasma. Briefly, 1 mL of plasma and 0.5 mL of propyl gallate (internal standard, 50 µg, 100 µg·mL, Sigma Aldrich, UK) was mixed with 4 mL oxalic acid (10 nM, Sigma Aldrich, UK) and 0.1 mL hydrochloric acid (HCl; 12.6 M, Sigma Aldrich, UK) in 15 mL falcon tubes and centrifuged at 826xg for 5 min. The supernatant was absorbed on to a primed (washed with 5 mL methanol (MeOH, Sigma Aldrich, UK) with 0.2% trifluoroacetic acid (TFA, Fisher Scientific, UK) followed by 2×5 mL of water) solid phase extraction cartridge (Waters Sep-Pak c17, 360 mg sorbent per cartridge, 55-105 µm). The sample was eluted with 3 mL of

MeOH + 0.2% TFA and dried under N_2 at 45°C. Samples were then reconstituted in 400 µl of 0.1% formic acid (Sigma Aldrich, UK) in water: 2% HCl in MeOH (i.e., 1 mL of formic acid to 1000 mL water: 20 mL HCl in 1000 mL methanol) and filtered through a 0.2µm polytetrafluoroethylene filter (Sigma Aldrich, UK) prior to HPLC analysis. Samples were analysed on a batch basis, where each batch included standards (protocatechuic acid, gallic acid and vanillic acid) prepared in 0.1% formic acid in water: 2% HCl in MeOH, blank control, plasma samples, and fortified plasma samples at 1 (low), 10 (medium) and 25 (high) µg·mL. The recovery ranges of protocatechuic acid, gallic acid and vanillic acid were 89-95%, 89-102% and 90 - 103%, for low, medium and high fortified levels, respectively. The final results were collected for recovery at the low fortification level.

7.3.6 High Performance Liquid Chromatography (HPLC) analysis

Under the selected chromatographic conditions, calibration graphs were obtained by preparing standard samples of each compound in triplicate, with increasing concentration of each analyte. The limits of detection (LOD) were calculated mathematically by the relationship between the standard deviation (SD) of the calibration curve and its slope (S) using the multiplier suggested by the International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) standard. The LOD was calculated from the following equation: LOD = (3.3xSD/S). The analytical calibration curves (n=3) were linear over the concentration range from 0.1 to 50 µg·mL. The linearity was assessed through calculating the regression equation (y=mx+c) and the correlation coefficient (*r*) by the least squares method where, the y is the area of chromatographic peak, and x the concentration of standard solution in µg·mL (Table 7.1).

A high-pressure liquid chromatography-diode array detector (DAD) method for the detection and quantitation of selected phenolic compounds in the plasma samples (pre-supplementation through to 6 h post-supplementation) was carried out using a Dionex UltiMate 3000 HPLC System (Dionex, Camberly, UK) equipped with an UltiMate 3000 RS pump, an UltiMate 3000

autosampler and a 3000 RS UV/Vis Detector and a RS Fluorescence Detector (FLD). The filtered samples (20 µL) were injected on a Phenomenex Luna C_{18}(2) (250x2.0 mm, 5µm particle size) reverse-phase column thermostat controlled at 30°C. The mobile phase consisted of water with 1% acetic acid (solvent A, Fisher Scientific, UK), and acetonitrile (Fisher Scientific, UK) with 1% acetic acid (solvent B). After a 5-minute equilibration with 20% A, the elution programme was as follows: 0-15 min, 20-100% B, (0.2 mL·min) followed by a washing stage (100% B, 15-18 min, 1.0 mL·min) and return at the initial conditions within 2 minutes. Detection was performed at the following excitation/emission wavelengths: λex=278 nm and λem = 360 nm for PCA and propyl gallate (PG), λex=278n nm and λem=366 for GA and λex=260 nm and λem=422 nm for VA, respectively. The identification and quantitation of PCA, GA and VA content of plasma samples was based on a combination of retention time and spectral matching of reference standards. Final results are expressed as micrograms per millilitre (µg·mL).

Table 7.1 Selected UV-Vis wavelengths and retention times (mins) for quantification of phenolics by HPLC – UV/Vis.

Compound	UV/Vis wavelength (nm)	Retention time (mins)	LOD (µg·mL)	Range of linearity (µg·mL)
PCA	278/360	7.12	<0.04	0.1 – 50
VA	260/422	9.09	<0.04	0.1 – 50
GA	278/366	7.73	<0.05	0.1 – 50
PG	278/360	11.99		

LOD limits of detection, Protocatechuic acid (PCA), Vanillic acid (VA), Gallic acid (GA), Propyl gallate (PG)

7.3.7 Data analysis

Statistical analyses were completed using Statistical Package for Social Sciences 23.0 (SPSS, Chicago, Illinois). All dependent variables were analysed using a treatment (300 mg NZBC dose) by time (0, 1, 1.5, 2, 3, 4, 5 and 6 h) one-way, repeated measures analysis of variance (ANOVA). Mauchly's test of sphericity was used to check homogeneity of variance for all variables; where necessary, any violations of the assumption were corrected using the Greenhouse-Geisser adjustment. Main effects for time were followed up using Bonferroni *post hoc* analysis. Further analysis was conducted to identify maximum plasma concentrations (C_{max}) and times to achieve maximum plasma concentrations (t_{max}), which were directly obtained from the plasma concentration-time profiles (Toutain and Bousquet-Melou, 2004). As a measure of overall plasma uptake of individual phenolic acids, the area under the plasma concentration-time curve ($AUC_{0\text{-}6h}$) for each participant was estimated by using the linear trapezoidal rule, with the total sum and mean of each individual being reported. Pearson (r)

correlation coefficients were calculated for the relationship between habitual anthocyanin intake and each metabolite (VA, GA and PCA) and between each metabolite (VA, GA and PCA). The alpha level for statistical significance was set at 0.05 *a priori* and Partial-eta^2 (ηp^2) effect sizes (ANOVA) are reported to indicate the magnitude of observed effects (Lakens, 2013). Partial-eta^2 (ηp^2) effect sizes of 0.01–0.06, 0.06–0.14 or ≥0.14 are considered small, medium and large changes, respectively (Lakens, 2013). Data in text and tables are reported as mean (95% confidence intervals) and data in figures as mean with individual data points (Weissgerber et al. 2015).

7.4 Results

Vanillic acid results are based on 17 and PCA on 18 participants', due to their values falling below the LOD during HPLC analysis. Gallic acid results are based on all 20 participants.

7.4.1 Plasma time-concentration curves

Vanillic acid (VA)

There was no main effect of time on plasma VA plasma concentration (P=0.109, ηp^2=0.122; Figure 7.2a). The mean t_{max} was 3 h and C_{max} 0.49 (95%CI: 0.21-1.19) μg g·mL^{-1} for VA. The total sum $AUC_{0\text{-}6h}$ values for VA were 48.0 (95%CI: 44.8–51.2) μg h·mL^{-1}, and the mean of each individual $AUC_{0\text{-}6h}$ for VA was 2.8 (95%CI: 0.4–6.0) μg h·mL^{-1}.

Gallic acid (GA)

There was a main effect of time on plasma GA concentration ($F_{(3.9,\ 74.3)}$=25.3, P=0.001, ηp^2=0.571. Post-hoc comparisons revealed that GA concentrations were increased at 1, 1.5, 2, 3, 4, 5 and 6 h following supplementation when compared to baseline (all P<0.0001; Figure 7.2b). Furthermore, plasma GA concentrations were elevated at 2, 3, 4, 5 and 6 h compared to 1 h following supplementation (P<0.0001). GA levels in plasma were higher 4 and 5 h compared to 1.5 h following supplementation (P=0.004 and P=0.011, respectively). The mean t_{max} was 4 h and C_{max} 1.88 (95%CI: 1.24–2.52) μg g·mL^{-1} for GA. Total sum $AUC_{0\text{-}6h}$ values

for GA was 166.2 (95%CI: 161.9-170.4) μg h·mL^{-1}, and the mean of each individual $AUC_{0\text{-}6h}$ for GA was 8.3 (95%CI: 4.0-12.6) μg h·mL^{-1}.

Protocatechuic acid (PCA)

Following supplementation, there was a main effect of time on PCA plasma levels ($F_{(2.2, 40.9)}$=10.64, P=0.001, ηp^2=0.359). Post-hoc comparisons revealed that PCA concentrations were elevated at 1, 1.5, 2, 3, 4, 5 and 6 h following supplementation when compared to baseline (all P<0.0001; Figure 7.2c). The mean t_{max} was 1.5 h and C_{max} 1.66 (95%CI: 0.43-2.89) μg g·mL^{-1} for PCA. Total sum $AUC_{0\text{-}6h}$ values for PCA were 161.3 (95% CI: 158.0-164.6) μg h·mL^{-1}. The mean of each individual $AUC_{0\text{-}6h}$ for PCA was 8.5 (95%CI: 5.2-11.8) μg h·mL^{-1}.

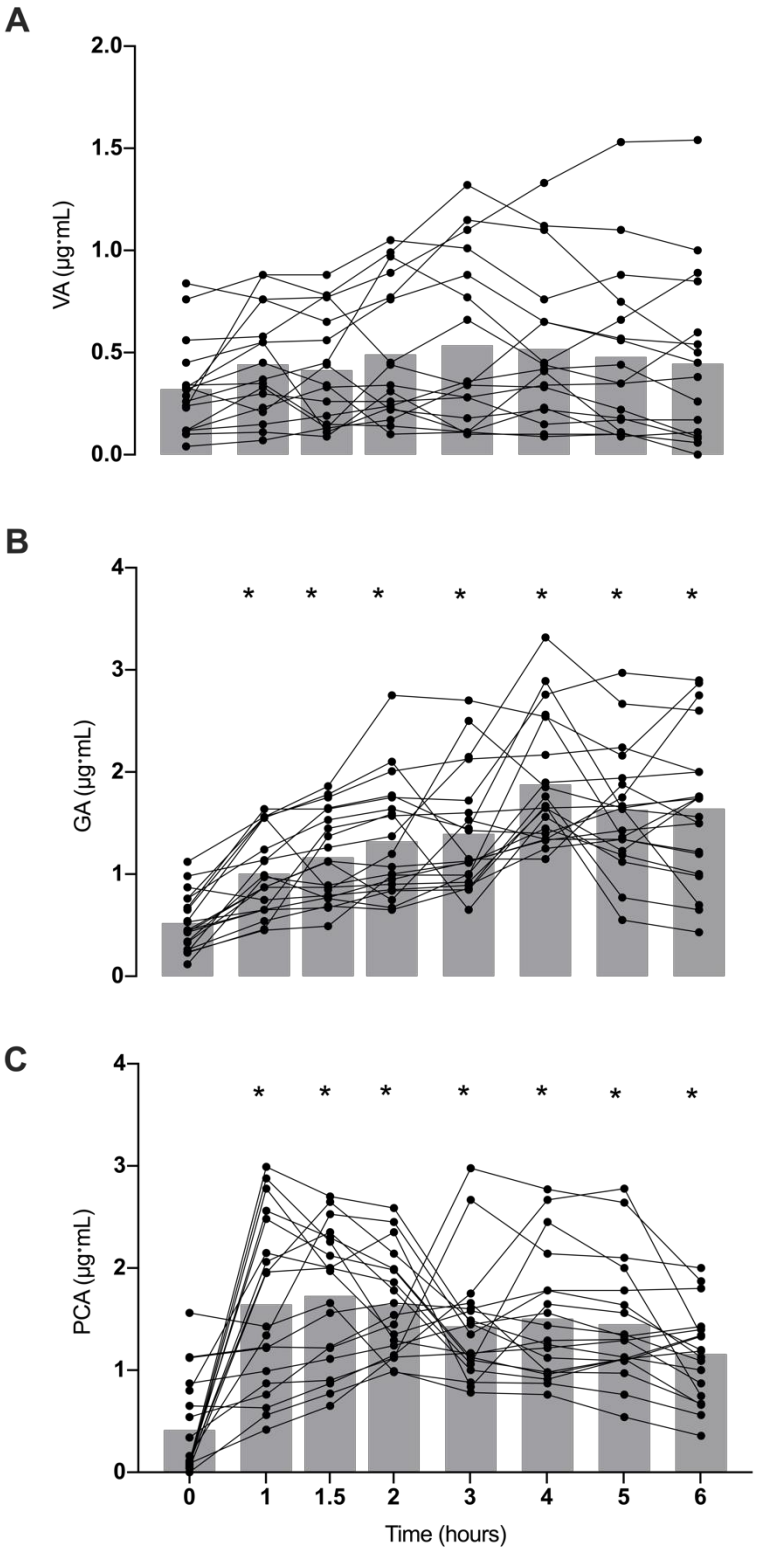


Figure 7.2 Vanillic acid (panel A, n=17), gallic acid (panel B, n=20) and protocatechuic acid (panel C, n=18) responses from baseline to supplementation with 300 mg NZBC extract capsule. Absolute baseline values were 0.27 (95%CI: 0.13-0.68) µg·mL^{-1}, 0.52 (95%CI: 0.12-0.92) µg·mL^{-1} and 0.39 (95%CI: 0.40-1.19) µg·mL^{-1} for VA, GA and PCA, respectively. Asterisk indicates a significant time effect compared to baseline (P<0.05); data are presented as mean (bars) and individual data points.

7.4.2 Relationships between phenolic compounds

A moderate positive significant relationship was observed between VA and PCA (r =0.59, P=0.014). However, no significant relationships were observed for GA and PCA (r = -0.24, P=0.322) and GA and VA (r=-0.39, P=0.129).

7.4.3 Dietary intake

Table 7.2 reports those data for the 72-h food diaries and anthocyanin food frequency questionnaires. There were no significant relationships between individual estimated habitual anthocyanin intake and the $AUC_{0\text{-}6h}$ for VA (r=-0.10, P=0.681), GA (r=-0.59, P=0.811), or PCA (r=0.32, P=0.189).

Table 7.2 Mean for each day (95%CI) absolute macronutrient intake 72 h prior to the experimental visit and habitual anthocyanin intake (n=20).

Variable	Value
Nutritional status	
Total energy intake (kJ)	8175 (6034-10317)
Carbohydrate (g)	205 (118-292)
Fat (g)	75 (49-102)
Protein (g)	100 (49-150)
Habitual anthocyanin intake ($mg \cdot day^{-1}$)	168 (68-404)

7.5 Discussion

This is the first study to demonstrate that, in support of the hypothesis, a NZBC extract supplement increases the plasma uptake of the phenolic acids VA, GA, and PCA when consumed alongside a habitual diet. The novel approach to this study was to investigate the

appearance and 6 h time course of these phenolic acids in plasma following acute consumption of a NZBC extract supplement.

There is a limited understanding about the metabolism and absorption of VA, GA and PCA following NZBC extract intake. However, the present study demonstrated that PCA and GA are most bioavailable in plasma 1.5 and 4 h post-NZBC extract ingestion, respectively. An independent HPLC analysis on a batch of the NZBC extract capsules confirmed no presence of peonidin-3-*O*-rutinoside. However, in our study the VA (a downstream metabolite of peonidin-3-*O*-rutinoside) was detectable in the plasma in 17 of the 20 participants, with transient increases from baseline observed in four out of 20 participants (Figure 7.2 panel A). The transient increases in VA response were not uniformly observed within the cohort. As such we cannot determine if any change in this metabolite was a result of NZBC extract supplementation *per se*, or an interaction between a dietary feature shared between these four participants, but absent from the others, or simply noise/measurement artefact. Furthermore, VA is the major degradation product of the parent compound, peonidin-3-*O*-rutinoside, and previously it has been shown that peonidin-3-*O*-rutinoside is a minor anthocyanin compound in blackcurrant (Frøytlog, Slimestad, and Andersen, 1998). The phenolic acid PCA can be extensively metabolized to numerous metabolites such as VA, hippuric acid, ferulic acid and 4-hydroxybenzaldehyde (Gao et al. 2006; de Ferrars et al. 2014). Therefore, it is possible that presence of VA in plasma over the 6 h was due to enterohepatic metabolism of PCA to VA and a reason as to why a positive relationship was observed in this present study between VA and PCA. However, it must be acknowledged that habitual dietary intake leading up to the experimental visit, could also be a contributing factor to the appearance of VA in plasma. High concentrations of VA *in vivo* have been linked to the abundance of anthocyanins in fruits and vegetables (Nurmi et al. 2009). Vanillic acid, GA and PCA were present at baseline in plasma for all participants before NZBC extract consumption, which is in accordance with previous research, where anthocyanin metabolites were still observable 48 h post- cyanidin 3-*O*-glucoside bolus ingestion (Czank et al. 2013). Thus, it is

plausible that foods rich in anthocyanins consumed by the participants prior to the 12 h overnight fast, resulted in bioaccumulation of phenolic acids, which were then detectable in plasma on the morning of the laboratory visit. Despite the equivocal results for this metabolite, future research would benefit from retaining VA within the panel of metabolites measured to shed further light on the interindividual variability.

The plasma concentrations of VA (C_{max} 0.49±0.06 μg g·mL^{-1}) and PCA (C_{max} 1.66±0.38 μg g·mL^{-1}) observed within this study are in accordance with previous research where Keane et al. (2016) observed that in in 12 healthy male participants who followed a low-polyphenol diet for 48 h prior to consuming either 30 or 60 mL of Montmorency tart cherry concentrate, that plasma VA plasma C_{max} was 0.30±0.01 μg·mL and 0.29±0.03 μg·mL for both groups, respectively. In the same study, PCA plasma C_{max} was 2.76±0.10 μg·mL and 2.75±0.13 μg·mL for the 30 and 60 mL Montmorency tart cherry concentrate groups, respectively. Within the current study, the NZBC extract supplement contained 105 mg of anthocyanin, whereas in Keane et al. (2016), the total anthocyanin content was 31.24±0.16 mg cyanidin-3-glucoside/L in the 30 mL Montmorency tart cherry concentrate and 62.47±0.31 mg cyanidin-3-glucoside/L in the 60 mL Montmorency tart cherry concentrate. Although the current study did not look to establish possible physiological mechanisms of action of the phenolic acids from NZBC extract supplementation, Keane et al. (2016) did show that the same plasma VA and PCA concentrations observed within the *in vivo* part of the study, when applied in an *in vitro* model were able to significantly increase the vascular smooth cell migration by 36±12% when combined, compared to the ethanol only control. A key question which remains is whether these phenolic acid amounts are capable of inducing beneficial physiological effects and by what mechanism/s. One study which partially answers this question is from Cimino et al. (2013) who previously showed in an *ex vivo* model how an anthocyanin-rich supplement composed of purified bilberry and blackcurrants (160mg anthocyanin in total) was able to significantly increase Nrf-2 nuclear accumulation activation in HUVECs under normoxic conditions. However, more mechanistic *in vitro* and *in vivo* research is warranted to establish

whether key phenolic acids, such as VA and PCA, from NZBC extract supplementation are able to upregulate endogenous antioxidant pathways such as Nrf-2 and lead to beneficial physiological effects.

Slimestad and Solheim (2002) previously highlighted that whole blackcurrant berries are composed of ~97% extractable anthocyanin pigment with the main anthocyanin pigments being delphinidin and cyanidin. Post-NZBC extract ingestion, GA increased in plasma concentration across the 6 h time frame, achieving t_{max} by 4 h, presented the greatest plasma uptake and had not returned to baseline at 6 h post-ingestion (Figure 7.2 panel B). This finding is in contrast to previous work, which reported GA t_{max} occurred at 1 h post-ingestion of a blackcurrant extract (Roehrig et al. 2019), however, that result was based on four participants due to one participant's falling below the limit of quantification (LOQ) at all time points. Gallic acid is a trihydroxybenzoic acid and the major stable phenolic acid derived from ring fission of the delphinidin skeleton, which is the most abundant anthocyanin in New Zealand blackcurrant. A study which investigated the enzymatic potential for *Bifiobacteria* and *Lactobacillus,* two predominant members of the intestinal microflora, to convert delphinidin and malvidin glycosides into their phenolic acid degradation compounds, observed that the *Lactobacillus* strain, *L. casei*, resulted in the highest concentrations of GA after 24 h of incubation (Ávila et al. 2009). Furthermore, the authors highlighted that as delphinidin 3-*O*-glucoside underwent chemical degradation to form mainly GA, and concentrations of this phenolic acid were detected at 1 h in samples, which increased throughout the time period concomitantly with delphinidin 3-*O*-glucoside clearance. Therefore, it is feasible that the appearance of GA in plasma over the 6 h in the present study was possibly a result of the gut microbiome profiles of the participants. Future research should consider studying faecal microflora samples before and following an anthocyanin-rich berry fruit supplementation to assess this relationship further (Tomas-Barberan, Selma and Espin, 2018).

Protocatechuic acid is the main degradation compound of cyanidin (Vitaglione et al. 2007). Previous research has reported observing a biphasic response in serum of PCA metabolite

kinetics, displaying an initial peak between 0 and 5 h and a second peak between 6 and 48 h following an isotopically labelled bolus of cyanidin 3-*O*-glucoside (de Ferrars et al. 2014). A similar response was apparent for several participants in the present study, where an initial peak occurred around 1 h, followed by a second peak at 3 h (Figure 7.2 panel C). The reason for these biphasic profiles has been suggested to be a result of metabolism occurring in multiple tissues, such as the liver and at different sites within the gastrointestinal tract (de Ferrars et al. 2014). Following blood orange consumption Vitaglione et al. (2007) observed a recovery of cyanidin 3-*O*-glucoside and PCA in the 24 h faecal samples, which they suggested indicated *in vivo* production of PCA by intestinal microflora, and that this slow and continuous release of antioxidant compounds into systemic circulation may have physiological relevance to maintain the concentration of blood antioxidants over 24 h. With regards to tissue accumulation of anthocyanin, Kirakosyan et al. (2015), studied the effect of 3-weeks of anthocyanin-rich cherry supplementation in rats on tissue bioavailability and found that some tissues preferentially stored phenolic acids. However, when humans supplemented over a 12-week period with anthocyanin-rich elderberry extract, no differences were observed in the concentration of metabolites post-prandial in urine prior to and following the 12-weeks of repeated anthocyanin dosing (de Ferrars, Cassidy, Curtis and Kay, 2013). Therefore, further research is warranted on whether anthocyanin metabolites can be preferentially stored in tissue or whether bioaccumulation is limited to a short window of opportunity.

To date only one study has demonstrated the effectiveness of an acute dose of NZBC extract on recovery; taken immediately before and after a rowing task, on mitigating oxidative stress (Lyall et al. 2009). However, the participants were required to adopt a polyphenol-restricted diet throughout the intervention period. In contrast, the majority of 7-day intake of NZBC extract supplementation and exercise performance studies (Cook et al. 2015; Cook et al. 2017a; Strauss et al. 2018; Willems et al. 2015; Cook et al. 2017b; Willems et al. 2017; Perkins et al. 2015; Willems et al. 2016; Murphy et al. 2017; Godwin et al. 2017) have adopted an ecologically valid approach by not restricting habitual polyphenol intake. These studies

provided the NZBC extract supplement between 2 to 3 h before data collection and found improvements in fat oxidation (Cook et al. 2015; Cook et al. 2017a; Strauss et al. 2018), time trial performance (Cook et al. 2015; Murphy et al. 2017), lactate clearance (Willems et al. 2015), cardiovascular responses (Cook et al. 2017b), repeated sprint performance (Cook et al. 2015; Cook et al. 2017a; Perkins et al. 2015) and insulin sensitivity (Willems et al. 2017). This ecologically valid study design approach is further supported by the findings of the present study, which suggest that plasma phenolic acid metabolite plasma uptake is still pronounced even when superimposed on top of a habitual non-polyphenol restricted diet.

The individual participant plasma uptake of VA, GA and PCA (Figure 7.2 panels A, B and C) in the present study supports previous observations by Keane et al. (2016) where large inter-individual variability was apparent following acute intake of 30 and 60 mL of Montmorency tart cherry concentrate. In an attempt to quantify the variation in individual participant plasma uptake in this present study, mean of each individual $AUC_{0\text{-}6h}$ for the plasma time-concentration curves was estimated (linear trapezoidal model). Comparing the mean of each individual $AUC_{0\text{-}6h}$ response to the mean total $AUC_{0\text{-}6h}$ response, it is apparent that some individuals present greater plasma uptake for each metabolite than others, which is independent of the relative NZBC extract dose given (i.e., mg·kg body mass). This could explain why in some NZBC extract supplementation studies, the magnitude of the response (i.e., fat oxidation) to the intervention varies between participants (Cook et al. 2015; Cook et al. 2017; Strauss et al. 2018). Large inter-individual variations in the plasma concentrations of phenolic compounds following an 8-week mixed berry diet intervention have been observed previously (Koli et al. 2010), with these variations being attributed to differences within participant's intestinal microflora, which can be affected by dietary intake and genetic differences. However, no relationships were observed in the present study between estimated habitual anthocyanin intake and overall VA, GA and PCA plasma uptake (quantified by total $AUC_{0\text{-}6h}$). Given the current focus on individualised sports nutrition (Betts and Gonzalez, 2016), it appears that when considering using an anthocyanin-rich supplement, such as NZBC

extract, practitioners should take into consideration the large inter-individual response that can occur. For example, if taking the mean response from this current study, it would seem plausible to suggest that most individuals will reach their peak plasma concentration for PCA at 1.5 h, however, as can be observed in Figure 7.2 (panel C), several participants did not achieve their peak plasma concentration until 3 h. Future research should consider quantifying the plasma uptake of anthocyanin metabolites before and following berry fruit extract supplementation, alongside physiological performance measures to assess the relationship between them as has been done previously with parent anthocyanins together with a typing task (Matsumoto et al. 2005).

7.6 Limitations

A limitation of the present study is that the analysis was not exhaustive and so not every plasma metabolite was analysed; instead, the focus was on the degradation products of two of the main anthocyanidins reported in NZBC extract and VA, the methylated degradation compound of PCA, that have purported health and physiological benefits. Given that anthocyanins degrade and are extensively metabolised *in vivo,* it is possible that accumulation of multiple phenolic metabolites may ultimately be responsible for the reported bioactivity of anthocyanins (Kirakosyan et al. 2015). Furthermore, the present study utilised an acute dose of NZBC extract (105 mg anthocyanin) and so it is not possible to forecast the impact of a longer supplementation period on plasma phenolic uptake. Although previous NZBC extract research has utilised longer (seven-days) supplementation periods, none of them have quantified the plasma uptake of anthocyanin phenolic aids such as VA, GA and PCA. For the first time, this study characterizes the appearance of VA, GA and PCA following an acute dose of NZBC extract, providing new perspectives for possible acute dosing protocols. Future research is warranted to establish whether a chronic dose can provide additional benefits with regards to plasma uptake or whether a ceiling effect exists. Lastly, the timeframe of this current investigation lasted until 6 h, with VA, GA and PCA plasma levels still elevated at this time point. Previous studies focusing on the degradation products of cyanidin have reported a

presence of VA in serum at ~42 h post isotopically labelled cyanidin 3-*O*-glucoside bolus ingestion (Czank et al. 2013). Therefore, it is probable that the phenolic acids in this study could have remained present in plasma until ~42 h. Finally, no placebo/control group was used in this present investigation, however, given the purpose of the study to observe the appearance of VA, GA and PCA in plasma following a habitual, non-polyphenol restricted diet, we believed that the inclusion of the pre-supplementation time point (0 h) and fasting control throughout the study timeframe provided sufficient control and that increase in appearance of VA, GA and PCA in plasma was a result of the NZBC extract intake.

7.7 Conclusion

In conclusion, this study presents new information regarding the presence of anthocyanin phenolic acids, VA, GA and PCA from acute NZBC extract supplementation when superimposed onto a habitual non-polyphenol restricted diet in humans. The time course of phenolic acid accumulation peaks between 1.5 and 4 h post-NZBC extract ingestion depending on the phenolic acid of interest, however, large inter-individual variability is apparent. This information could inform future *in vivo* work that examines the health and performance related benefits with acute NZBC extract supplementation by indicating the times at which peak plasma concentrations are likely to be achieved and the overall plasma uptake of VA, GA and PCA. This could in turn be used to suggest appropriate timings of supplementation prior to exercise intervention onset when superimposed onto a non-polyphenol restricted habitual diet.

Two key questions that remain from this Chapter are whether a longer supplementation period of NZBC extract, such as 10-days, would result in similar or greater plasma concentrations of VA, GA and PCA. Secondly, whether an increased dosage of NZBC extract, such as 600 $mg \cdot d^{-1}$, alongside a non-polyphenol restricted diet would result in similar or greater plasma concentrations of VA, GA and PCA.

8. Plasma Uptake of Selected Phenolic Acids Following New Zealand Blackcurrant Extract Supplementation Pre- and Post-Drop Jump Exercise in Humans: A Retrospective Investigation

8.1 Abstract

Introduction. New Zealand blackcurrants (NZBC) are a rich source of anthocyanin, however, limited evidence is available on the appearance and time course of key anthocyanin phenolic acids from NZBC extract supplementation and their relationship to functional measures of EIMD. This Chapter investigated the total area under the curve and plasma uptake of three phenolic acids, vanillic acid (VA), protocatechuic acid (PCA) and gallic acid (GA), following 7-days intake of NZBC supplementation pre- and up to 72 h following a 100 drop-jump protocol (100-DJP) and the relationship between the phenolic acid plasma concentrations and muscle function measures before and following completing a 100-DJP. **Methods.** Twelve recreationally active men volunteered and were pre-conditioned to eccentric exercise by performing 100-DJP. Then, following a double-blind, repeated crossover design, participants ingested either 600 mg·day^{-1} capsules with a NZBC extract (CurraNZ™; containing a total of 210 mg anthocyanins) or a visually matched placebo (PLA) 7-days prior and 3-days after completing 100-DJP. Measures of muscle function (maximal voluntary isometric contraction; MVIC and CMJ) and venous blood samples were drawn pre- (baseline), 0-, 24-, 48- and 72 h-post 100-DJP and plasma concentrations of VA, GA and PCA were analysed by reversed-phase HPLC. **Results.** MVIC peak force was reduced immediately-post 100-DJP, compared to baseline and returned to baseline at 24 h with no difference between groups (P=0.940). CMJ outcome and neuromuscular variables were unaffected by the 100-DJP or NZBC extract (P>0.05). Total $AUC_{PRE\text{-}72h}$ was greater following NZBC extract supplementation compared to PLA for GA and PCA (P<0.05) but not for VA (P>0.05). Phenolic acid plasma uptake of VA remained unchanged across the intervention (P>0.05) for both NZBC and PLA. The GA plasma time-concentration curve was increased at 24, 48 and 72 h compared to baseline (pre) for NZBC (P<0.05) but no group or interaction effects were apparent (P>0.05). The PCA plasma time-concentration curve was increased at 24, 48 and 72 h compared to baseline for NZBC group (P<0.05) and a group*time interaction was present (P=0.001), but no main effects of group were apparent (P=0.052). No relationships were observed for any of the phenolic

acids with measures of muscle function (MVIC and CMJ) (P>0.05). **Conclusion.** This Chapter presents new information on the appearance and time-course of anthocyanin phenolic acids following NZBC extract supplementation and demonstrates these are not related to recovery of MVIC and CMJ following EIMD.

8.2 Introduction

In Chapters 3, 4, 5 and 6 it was shown that NZBC extract was unable to facilitate recovery from EIMD at the group level but may show some potential to facilitate recovery at the individual level. This was highlighted particularly in Chapters 4 and 6 where the SWC and response CIs highlighted the intra- and inter-individual variation in response to the half-marathon event and a 100-DJP, respectively, with NZBC extract and PLA supplementation. Furthermore, Chapter 7 demonstrated that acute NZBC extract supplementation increased plasma concentration of VA, GA and PCA and inter-individual variability was apparent, suggesting that this may be a contributing factor to the lack of observable recovery benefit at the group level in Chapters 3 and 5. However, as discussed previously other studies have shown that polyphenol-rich supplements may enhance recovery following EIMD, including Montmorency cherry (Beals et al. 2017; Bell et al. 2014; Bell et al. 2015; Bowtell et al. 2011; Connolly et al. 2006; Howatson et al. 2010; Kuehl et al. 2010; Levers et al. 2016; McCormick et al. 2016), pomegranate (Machin et al. 2012; Trombold et al. 2011; Trombold et al. 2012), blueberry (McLeay et al. 2012) and blackcurrant (Hutchinson et al. 2016; Coelho et al. 2017; Rowland, 2018).

The majority of studies that investigate the effects of polyphenol supplementation on recovery from strenuous exercise have not quantified plasma phenolic metabolites after supplementation. The only exception is for several studies employing cocoa or chocolate supplementation in which, plasma epicatechin and catechin concentrations were measured (Decroix et al. 2017; Davison et al. 2012). A number of published studies have measured acute changes in plasma phenolic metabolites or the parent compounds after single doses of Montmorency cherry (Keane et al. 2016), blueberry (Rodriguez-Mateos et al. 2013), blackcurrant (Chapter 7 and Rechner et al. 2002; Roehrig et al. 2019) and pomegranate (Seeram et al. 2008). It has been suggested that future work in this field should ideally quantify exercise performance outcomes alongside measurement of plasma phenolic metabolites to

enable identification of the bioactives metabolites and inform optimisation of the polyphenol blends consumed (Bowtell and Kelly, 2019).

The data presented in Chapter 7 showed that an acute dose of NZBC extract increases plasma concentrations of select phenolic acids. However, it is not abundantly clear whether longer supplementation periods (e.g., 7-days) would result in greater systemic appearance of phenolic acids than what has been observed with acute intakes. In a pilot study, Hurst et al. (2020) demonstrated how consuming a dose of daily NZBC extract relative to body mass (3.2 $mg \cdot kg^{-1} \cdot d^{-1}$) for five-weeks was able to improve the efficacy to resolve acute inflammation and increase anti-inflammatory mediators to a greater extent than following a single, acute relative dose (3.2 $mg \cdot kg^{-1} \cdot d^{-1}$) taken 1 h before and 1 h after the same exercise. The authors made this observation following a 30-minute row at 70% $\dot{V}O_{2max}$ in 34 healthy males and females (Hurst et al. 2020). However, what was not demonstrated was whether the improved efficacy of NZBC extract supplementation was due to the five-week supplementation period enhancing plasma concentrations of anthocyanin or its downstream metabolites to a greater extent than the acute supplementation. Previously, Hurst et al (2019), showed that the same relative dose of NZBC extract (3.2 $mg \cdot kg^{-1} \cdot d^{-1}$), which equates to ~240 mg anthocyanins, increased plasma anthocyanin concentration to 9.8±3.2 nM compared to lower doses of 0.8 and 1.6 $mg \cdot kg^{-1}$ (3.7±1.3 nM and 6.9±1.8 nM, respectively). Thus, it appears that the greater the dose of NZBC extract ingested, the greater the plasma concentration of anthocyanin and/or metabolites. It could be hypothesised that utilising the greater dose of NZBC extract and utilising a longer supplementation, such as five-weeks, would increase the plasma concentration of anthocyanin and possibly exert greater health and physiological benefits.

In Chapter 7, the plasma concentrations of VA, GA and PCA following acute ingestion of a single dose (300 mg) of NZBC extract were measured in individuals following a non-polyphenol restricted diet. It was apparent that the time course of these selected phenolic acids varies depending on the phenolic acid of interest and large inter-individual variability is apparent. Further, Chapters 5 and 6 demonstrated that both intra- and inter-individual variation

are apparent in muscle function response to a 100-DJP. However, the supplementation protocol in Chapters 5 and 6 was 7-days intake of 600 mg·d^{-1} NZBC extract before and for the 3-days following a 100-DJP. Therefore, the present Chapter will examine (1) the total $AUC_{PRE-72h}$ for VA, PCA and GA following NZBC extract supplementation compared to a placebo, (2) the time course of plasma concentrations VA, PCA and GA over 10-days of 600 mg·d^{-1} NZBC extract supplementation alongside a non-polyphenol restricted diet compared to a placebo, (3) the relationship between VA, GA and PCA plasma concentration and muscle function measures of MVIC and CMJ, pre- and following a 100-DJP.

The following hypothesis were tested:

- The NZBC extract supplementation strategy would increase the total $AUC_{PRE-72h}$ for VA, PCA and GA to a greater extent than placebo.
- Supplementing with 600 mg·d^{-1} NZBC extract for 10-days would increase plasma concentrations of VA, PCA and GA at the measured time points (pre, 0 h, 24 h, 48 h and 72 h post-100DJP) greater than placebo.
- That there would be an observable relationship between VA, PCA and GA plasma concentration and muscle function measures of MVIC and CMJ, pre- and following a 100-DJP.

8.3 Methods

8.3.1 Participants, experimental design, blood sampling, familiarisation, muscle damaging exercise, MVIC, CMJ, supplementation protocol and dietary and exercise control

The data gathered in this Chapter are the same as Chapter 5 and 6, which are being presented again with a specific focus on examining the relationships between functional changes and plasma concentrations of select phenolic acids. Please refer to Section 5.3.1 and 5.3.2 for participant characteristics and the experimental design (Figure 8.1), respectively. Please refer

to the Section 5.3.3 for details of the familiarisation trial, Section 5.3.4 for details of the muscle damaging exercise, Section 5.3.6 for details of the MVIC measurement, Section 5.3.10 for details of the CMJ measurement, Section 5.3.15 for details of the supplementation protocol and Section 5.3.16 for details of the dietary and exercise control.

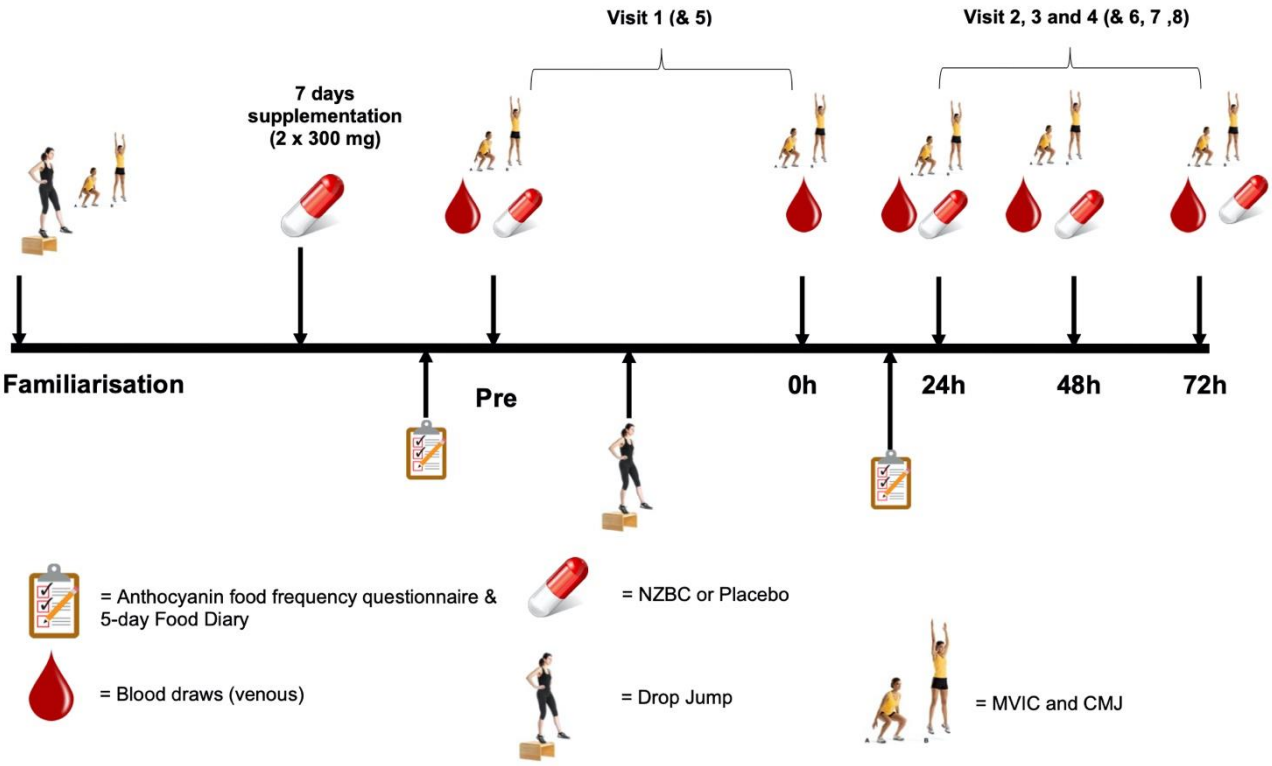


Figure 8.1 Double-blind, repeated crossover design. First four-day block visits 1-4 and second four-day block visits 5-8, separated by a two-week washout

8.3.2 Plasma Extraction

Please refer to Section 7.3.5 for details on the plasma extraction method used. However, the recovery ranges for this Chapter were 89.56-97.36%, 90.14-100.15% and 92.37-102.16% for low, mid- and high fortified levels, respectively. The final results were collected for recovery at the low fortification level.

8.3.3 High Performance Liquid Chromatography (HPLC) analysis

Please refer to Section 7.3.6 for details on the HPLC method used. However, in the present Chapter, the HPLC method was used for the detection and quantification of selected phenolic compounds in the plasma samples for pre-DJP, 0, 24, 48 and 72 h post 100-DJP for both NZBC and PLA conditions (Figure 8.1).

8.3.4 Data analysis

Statistical analyses were completed using Statistical Package for Social Sciences 23.0 (SPSS, Chicago, Illinois).

To address the first aim of this Chapter, the total $AUC_{PRE\text{-}72h}$ of VA, GA and PCA following NZBC extract supplementation vs. PLA were assessed using paired samples *t*-tests.

To address the second aim of the Chapter, all dependent variables (VA, GA and PCA) were analysed using a treatment (600 mg NZBC dose or PLA) by time (PRE, 0, 24, 48 and 72 h) two-way, repeated measures analysis of variance (ANOVA). Mauchly's test of sphericity was used to check homogeneity of variance for all variables; where necessary, any violations of the assumption were corrected using the Greenhouse-Geisser adjustment. Main effects for time were followed up using Bonferroni post hoc analysis. To ensure no order effects were present, paired samples *t*-tests were used comparing the pre-100-DJP trials for both supplementation groups (e.g., received NZBC 1st vs. received PLA 1st) for each dependent variable (VA, GA and PCA). Further analysis was conducted to identify maximum plasma concentrations (C_{max}) and times to achieve maximum plasma concentrations (t_{max}), which were directly obtained from the plasma concentration-time profiles (Toutain and Bousquet-Melou, 2004). As a measure of overall plasma uptake of individual phenolic acids, the area under the plasma concentration-time curve ($AUC_{PRE\text{-}72h}$) for each participant was estimated by using the linear trapezoidal rule, with the total sum and mean of each individual being reported. To assess the sum of change of PCA and GA, two phenolic acids that demonstrated the greatest increase in plasma concentration in Chapter 7 following acute supplementation with 300 mg

NZBC extract intake after an overnight fast, delta from pre was evaluated for both NZBC and PLA groups.

To address the third aim of this Chapter, Pearson (r) correlation coefficients were calculated for the relationship between habitual anthocyanin intake and each phenolic acid (VA, GA and PCA), between each phenolic acid (VA, GA and PCA) and between percentage change from baseline for MVIC and CMJ outcome variables (jump height, time to take off and RSImod) and raw values for CMJ neuromuscular variables (concentric peak force, net impulse, average power, duration and eccentric peak force, net impulse, displacement and duration) (as determined in Chapter 5) with each phenolic acid (VA, GA and PCA).

The alpha level for statistical significance was set at 0.05 *a priori* and Partial-eta^2 (ηp^2) effect sizes (ANOVA) and Cohen's *d* (paired *t*-tests) are reported to indicate the magnitude of observed effects (Lakens, 2013). Partial-eta^2 (ηp^2) effect sizes of 0.01 – 0.06, 0.06 – 0.14 or ≥0.14 are considered small, medium and large changes, respectively (Lakens, 2013) and 0.2, 0.5 and 0.8, small, medium and large changes, respectively (Cohen, 1988). Data in text and tables are reported as mean (95% confidence intervals) and data in figures as mean with individual data points (Weissgerber et al. 2015) unless otherwise specified.

8.4 Results

VA results are based on 8 and PCA and GA on 11 participants', respectively, due to their values falling below the limits of detection (LOD) during HPLC analysis.

8.4.1 Comparisons between total $AUC_{PRE\text{-}72h}$ for VA, GA and PCA for NZBC and PLA

Figure 8.2 shows the increase in total $AUC_{PRE\text{-}72h}$ for PCA and GA over the 10-days NZBC supplementation compared to PLA, whilst VA remained unchanged in both groups. The total $AUC_{PRE\text{-}72h}$ was greater following NZBC extract supplementation compared to PLA for GA

($t_{(10)}$=3.66, P=0.004, d=1.1) and for PCA ($t_{(10)}$=3.91, P=0.003, d=1.2) but not for VA (P=0.350, d=-0.4).

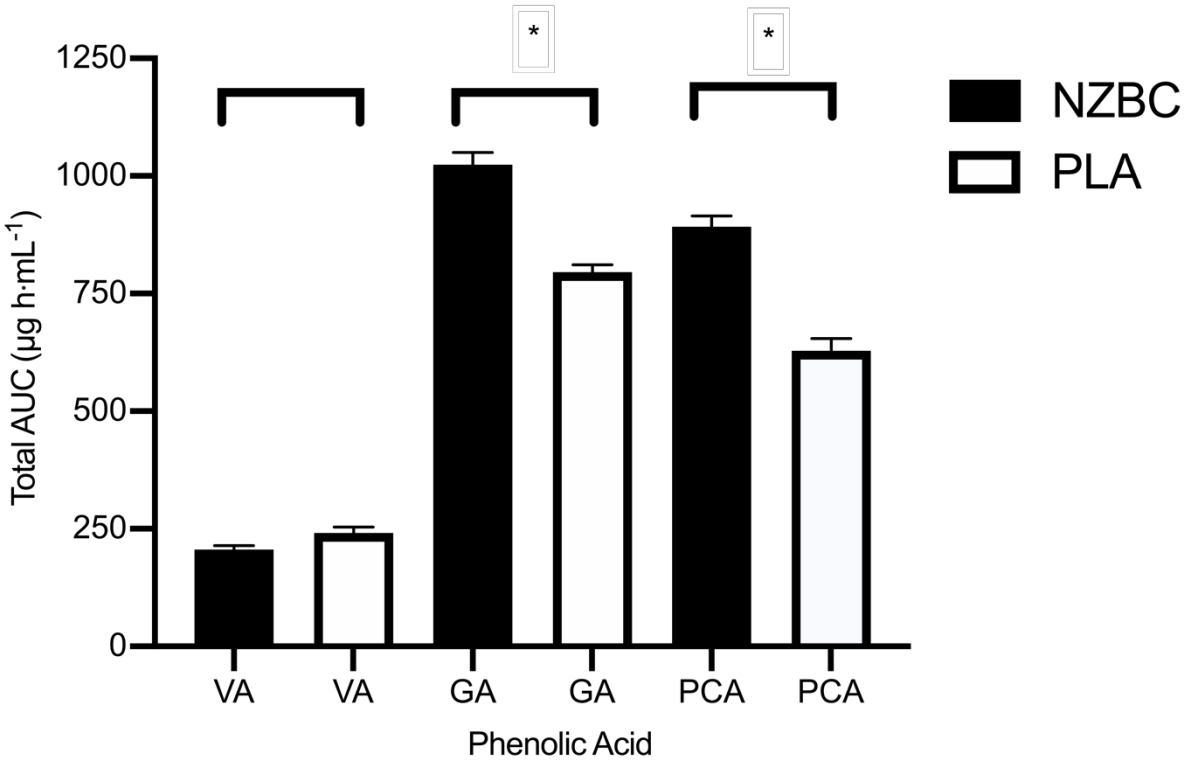


Figure 8.2 Total $AUC_{PRE-72h}$ for VA, GA and PCA following 10-days NZBC and PLA supplementation. Asterisk signifies statistical difference between NZBC and PLA groups (P<0.05). Data are presented as mean±SD; VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid; AUC, area under the curve; NZBC, New Zealand blackcurrant; PLA, placebo.

8.4.2 Plasma time-concentration curves

Vanillic acid (VA)

Figure 8.3 (panel A) shows there was no change in plasma VA concentration across PRE to 72 h post the 100-DJP following 10 days NZBC extract and PLA supplementation. There was no main effect of time on VA plasma concentration (P=0.887, ηp^2=0.017) group (P=0.185, ηp^2=0.107) or interaction effects (P=0.841, ηp^2=0.022). No order effects were observed for any of the VA, irrespective of whether NZBC or PLA was received first (P=0.531 and P=0.457, respectively). Vanillic acid t_{max}, c_{max}, total $AUC_{PRE\text{-}72h}$ and mean of each individual $AUC_{PRE\text{-}72h}$ can be located in Table 8.1.

Gallic acid (GA)

Figure 8.3 (panel B) illustrates the increase in plasma GA concentration from PRE to 72 h post the 100-DJP following 10 days of NZBC extract supplementation and no change in PLA. There was a main effect of time on GA plasma concentration ($F_{(2.2,\ 44.1)}$=5.05, P=0.009, ηp^2=0.202). Post-hoc analyses revealed GA plasma concentration levels were elevated above baseline at 24, 48 and 72 h (P=0.008, P=0.009, P=0.037, respectively) in NZBC group only. However, no group (P=0.063, ηp^2=0.162) or interaction effects (P=0.395, ηp^2=0.046) were present. No order effects were observed for any of the GA, irrespective of whether NZBC or PLA was received first (P=0.241 and P=0.909, respectively). Gallic acid t_{max}, c_{max}, total $AUC_{PRE\text{-}72h}$ and mean of each individual $AUC_{PRE\text{-}72h}$ can be located in Table 8.1.

Protocatechuic acid (PCA)

Figure 8.3 (panel C) illustrates the increase in plasma PCA concentration across PRE to 72 h following 10 days NZBC extract supplementation only. There was a main effect of time on PCA plasma concentration ($F_{(2.8,\ 56.5)}$=7.34, P=0.001, ηp^2=0.268). Post-hoc analyses revealed that PCA concentrations were elevated above baseline at 24, 48 and 72 h (P=0.015, P=0.023, P=0.004, respectively) in NZBC group only. Further, a group*time interaction was observed

($F_{(2.8, 56.5)}$=8.13, P=0.001, ηp^2=0.289) but no main effects of group were apparent (P=0.052, ηp^2=0.176). No order effects were observed for any of the PCA, irrespective of whether NZBC or PLA was received first (P=0.233 and P=0.520, respectively). Protocatechuic acid t_{max}, c_{max}, total $AUC_{PRE-72h}$ and mean of each individual $AUC_{PRE-72h}$ can be located in Table 8.1.

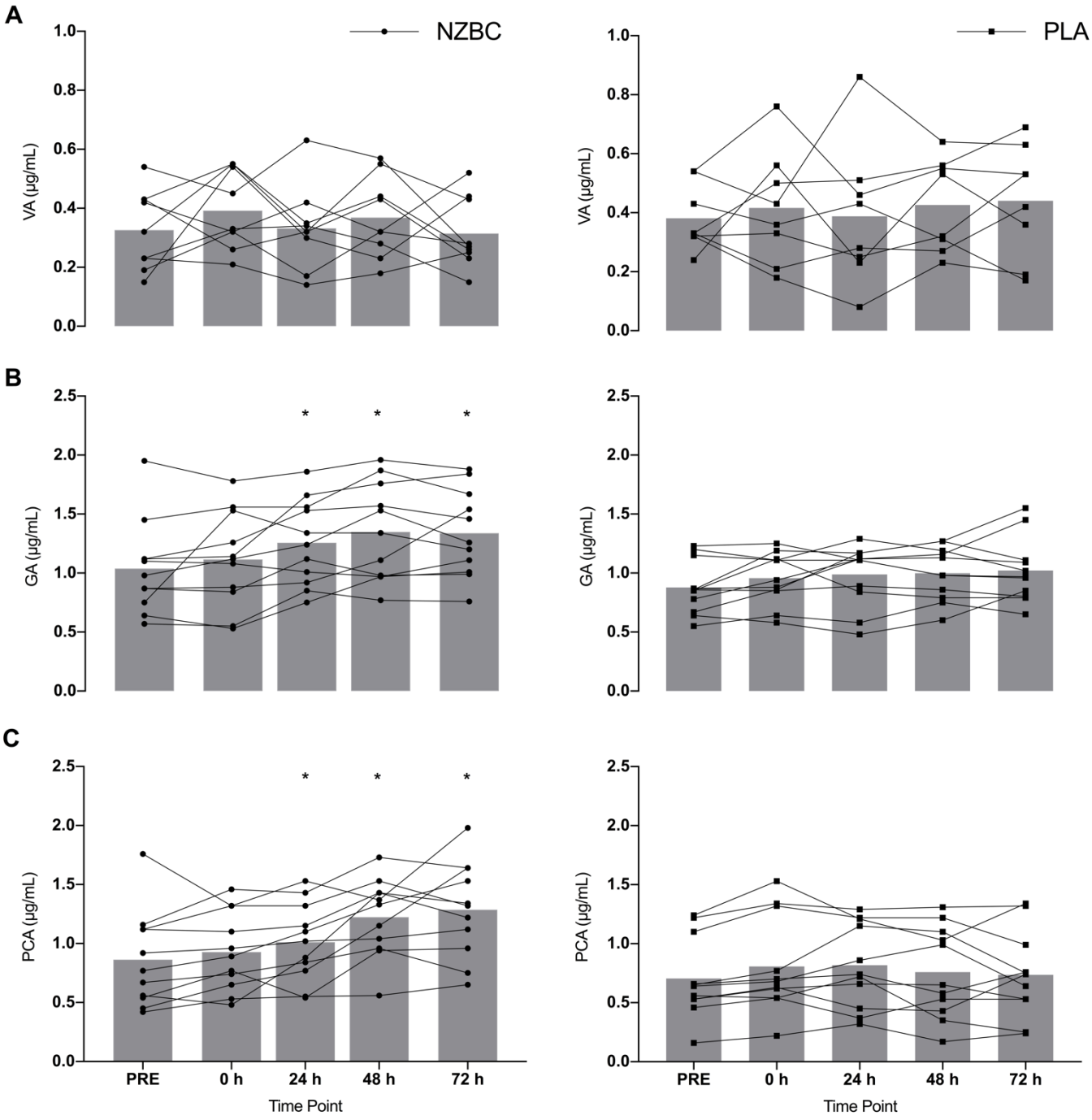


Figure 8.3 Vanillic acid (Panel A, n=8), gallic acid (Panel B, n=11) and protocatechuic acid (Panel C, n=11) responses from PRE to supplementation with 600 mg NZBC extract capsule or PLA to 72 h post 100-DJP. Absolute baseline (PRE) values were 0.33 (95%CI: 0.10-0.55) µg·mL^{-1} and 0.43 (95%CI: 0.01–0.84) µg·mL^{-1} for VA NZBC and PLA groups, 1.05 (95%CI: 0.42–1.67) µg·mL^{-1} and 0.90 (95%CI: 0.49–1.31) µg·mL^{-1} for GA NZBC and PLA groups and 0.92 (95%CI: 0.20–1.63) µg·mL^{-1} and 0.72 (95%CI: 0.31–1.13) µg·mL^{-1} for PCA NZBC and PLA groups. Asterisk indicates a significant time effect compared to baseline (P<0.05); data are presented as mean (bars) and individual data points.

Table 8.1 Vanillic acid, gallic acid and protocatechuic acid responses to 10-days of 600 mg·d^{-1} NZBC or PLA supplementation

Phenolic acid	**T_{max} (Time point post 100-DJP)**	**C_{max} (µg g·mL^{-1})**	**Total $AUC_{PRE-27h}$ (µg h·mL^{-1})**	**Individual mean $AUC_{PRE-27h}$ (µg h·mL^{-1})**
Vanillic acid				
NZBC	0 h post	0.39 (95%CI: 0.38-0.40)	233.0 (95%CI: 232.4–233.7)	25.9 (95%CI:25.3–26.5)
PLA	0 h post	0.43 (95%CI: 0.38-0.47)	278.3 (95%CI: 277.1–279.5)	30.9 (95%CI: 29.7–32.2)
Gallic acid				
NZBC	48 h post	1.36 (95%CI: 1.23 –1.48)	1126.8 (95%CI: 1118.1–1135.6)	93.9 (95%CI: 85.1–102.7)
PLA	72 h post	1.04 (95%CI: 0.99–1.09)	883.4 (95%CI: 881.4–885.3)	73.6 (95%CI: 71.7–75.6)
Protocatechuic acid				
NZBC	72 h post	1.36 (95%CI: 1.25–1.46)	1055.2 (95%CI: 1047.6–1062.7)	87.9 (95%CI: 80.4–95.5)
PLA	24 h post	0.82 (95%CI: 0.77–0.87)	686.81 (95%CI: 682.4–691.3)	57.2 (95%CI: 52.8–61.7)

t_{max}, time to maximum concentration; c_{max}, concentration maximum; $AUC_{PRE-72h}$, area under the curve PRE – 72 h post 100-DJP; NZBC, New Zealand blackcurrant; PLA, placebo.

8.4.3 Combined PCA and GA plasma concentration

As PCA and GA demonstrated the greatest increase in plasma concentration in Chapter 7 following acute supplementation with 300 mg NZBC extract intake after an overnight fast (see Figure 7.2), delta from PRE was evaluated for both NZBC and PLA groups. Figure 8.4 shows the increase in summed plasma PCA and GA concentrations across PRE to 72 h following 10 days NZBC supplementation only. There was a main effect of time on summed PCA and GA plasma concentration ($F_{(2.3, 46.3)}$=14.16, *P*=0.001, ηp^2=0.415). Post-hoc analyses revealed that summed PCA and GA plasma concentration were elevated above baseline (pre) at 0, 24, 48 and 72 h (*P*=0.031, *P*=0.001, *P*=0.001, *P*=0.001 respectively) in NZBC group only. Further, a group*time interaction and main effects of group were observed ($F_{(2.3, 46.3)}$=7.69, *P*=0.001, ηp^2=0.278; $F_{(1, 20)}$=7.92, *P*=0.011, ηp^2=0.284, respectively).

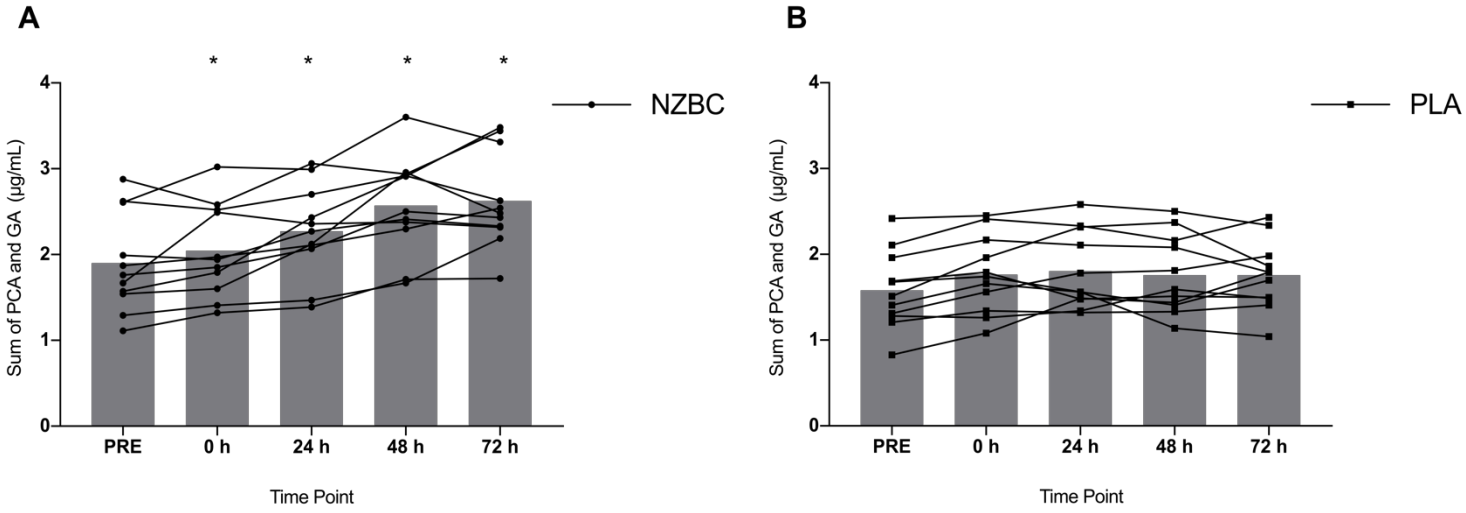


Figure 8.4 Sum (Σ) of protocatechuic acid (PCA) and gallic acid (GA) responses from baseline to supplementation with 600 mg NZBC (A) extract capsule or PLA (B). Absolute baseline (PRE) values were 1.90 (95%CI:1.56-2.24) µg·mL and 1.58 (95%CI:1.32–1.85) µg·mL for NZBC and PLA, respectively. Asterisk indicates a significant time effect compared to baseline (PRE) (*P*<0.05); data are presented as mean (bars) and individual data points.

Figure 8.5 highlights the summed PCA and GA plasma concentration delta from PRE to 72 h following 10-days NZBC extract and PLA supplementation.

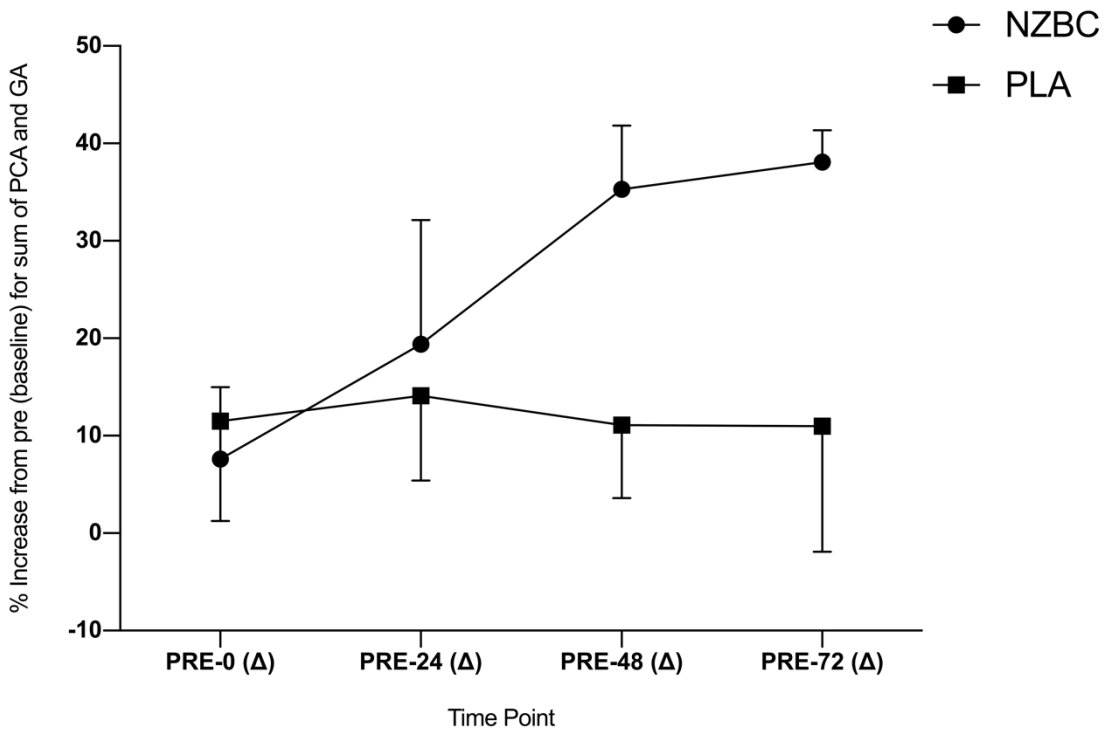


Figure 8.5 Sum (Σ) of PCA and GA plasma concentration expressed as percentage change from baseline (PRE) for 0 h post 100-DJP and 24, 48 and 72 h; PCA, protocatechuic acid; GA, gallic acid; NZBC, New Zealand blackcurrant; PLA, placebo.

8.4.4 Relationships between phenolic compounds

No relationships were observed between phenolic acids, VA, GA and PCA in the NZBC or PLA groups (Table 8.2). However, relationships were observed between NZBC group GA and PLA group VA and NZBC group GA and PLA group GA but as these were from the opposing supplementation group, these relationships are deemed spurious (Table 8.2).

Table 8.2 Pearson's correlation coefficient matrix of vanillic acid, gallic acid and protocatechuic acid plasma concentration for NZBC and PLA from Pre- to 72 h post 100-DJP.

	NZBC VA	**NZBC GA**	**NZBC PCA**	**PLA VA**	**PLA GA**	**PLA PCA**
NZBC VA		*r*=0.213 *P*= 0.582	*r*=-0.660 *P*=0.867	*r*=0.290 *P*=0.487	*r*=0.268 *P*=0.485	*r*=0.238 *P*=0.538
NZBC GA	*r*=0.213 *P*=0.582		*r*=0.139 *P*=0.666	*r*=-0.739* *P*=0.023	*r*=0.673* *P*=0.017	*r*=0.010 *P*=0.975
NZBC PCA	*r*=-0.066 *P*=0.867	*r*=0.139 *P*=0.666		*r*=0.204 *P*=0.599	*r*=-0.413 *P*=0.309	*r*=0.492 *P*=0.104
PLA VA	*r*=0.290 *P*=0.487	*r*=-0.739* *P*=0.023	*r*=0.204 *P*=0.599		*r*=-0.486 *P*=0.184	*r*=0.218 *P*=0.574
PLA GA	*r*=0.268 *P*=0.485	*r*=0.673* *P*=0.017	*r*=-0.413 *P*=0.309	*r*=-0.486 *P*=0.184		*r*=0.010 *P*=0.974
PLA PCA	*r*=0.238 *P*=0.538	*r*=0.010 *P*=0.975	*r*=0.492 *P*=0.104	*r*=0.218 *P*=0.574	*r*=0.010 *P*=0.974	

Asterisk highlights statistical significant relationships between phenolic acid plasma concentration ($P<0.05$). NZBC, New Zealand blackcurrant; PLA, placebo; VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid.

8.4.5 Relationships between habitual dietary intake and phenolic compounds

Please refer to Section 5.4.1, Table 5.1 for details of reported dietary intake. There were no relationships between estimated habitual anthocyanin intake and the total $AUC_{PRE-72h}$ for the phenolic acids (VA, GA and PCA) for either the NZBC or PLA groups (Table 8.3).

Table 8.3 Pearson's correlation coefficients for habitual anthocyanin intake and phenolic acid (VA, GA, PCA) total $AUC_{PRE-72h}$

	NZBC VA	NZBC GA	NZBC PCA	PLA VA	PLA GA	PLA PCA
Habitual anthocyanin intake (mg·day^{-1})	*r*=-0.299 *P*=0.565	*r*=-0.282 *P*=0.499	*r*=0.597 *P*=0.118	*r*=0.251 *P*=0.588	*r*=-0.413 *P*=0.309	*r*=0.352 *P*=0.392

Note: NZBC, New Zealand blackcurrant; PLA, placebo; VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid

8.4.6 Relationships between phenolic acid concentration in plasma and measures of maximal voluntary isometric contraction and countermovement jump

Table 8.4 shows there were no relationships between VA, GA and PCA and measures of MVIC or CMJ in the NZBC group. Table 8.5 shows there were no relationships between VA, GA and PCA and measures of MVIC or CMJ in the PLA group. There were no relationships between any of the phenolic acids (VA, GA and PCA) and measures of MVIC or CMJ ($P>0.05$).

Table 8.4 Pearson's correlation coefficient table of muscle function measures for all time points (PRE-72 h) and VA, GA and PCA plasma concentration for NZBC.

	VA	GA	PCA
MVIC (N)	r = -0.534, P = 0.353	r = 0.367, P = 0.554	r = 0.454, P = 0.442
Jump height (cm)	r = 0.427, P = 0.473	r = 0.227, P = 0.651	r = 0.231, P = 0.708
RSImod (index)	r = 0.468, P = 0.426	r = 0.576, P = 0.310	r = 0.541, P = 0.346
Time to take off Jump height (s)	r = -0.319, P = 0.602	r = -0.402, P = 0.503	r = -0.501, P = 0.390
Concentric peak force (N·kg)	r = 0.550, P = 0.337	r = 0.599, P = 0.286	r = 0.489, P = 0.403
Concentric net impulse (Ns·kg)	r = 0.495, P = 0.397	r = 0.173, P = 0.781	r = 0.081, P = 0.897
Concentric average power (W·kg)	r = -0.111, P = 0.859	r = 0.031, P = 0.960	r = 0.007, P = 0.991
Concentric phase duration (s)	r = 0.473, P = 0.421	r = -0.723, P = 0.168	r = -0.876, P = 0.051
Eccentric peak force (N·kg)	r = 0.300, P = 0.624	r = -0.125, P = 0.841	r = 0.167, P = 0.788
Eccentric net impulse (Ns·kg)	r = -0.343, P = 0.572	r = 0.644, P = 0.241	r = 0.860, P = 0.062
Eccentric phase displacement (braking phase) (m)	r = -0.509, P = 0.381	r = -0.198, P = 0.750	r = -0.276, P = 0.653
Eccentric phase duration (s)	r = -0.463, P = 0.432	r = 0.430, P = 0.469	r = 0.349, P = 0.564

Maximal voluntary isometric contraction (MVIC); Reactive strength index modified (RSImod); VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid

Table 8.5 Pearson's correlation coefficient table of muscle function measures for all time points (PRE-72 h) and VA, GA and PCA plasma concentration for PLA.

	VA	GA	PCA
MVIC (N)	r = 0.172, P = 0.782	r = 0.240, P = 0.697	r = -0.607, P = 0.278
Jump height (cm)	r = 0.054, P = 0.931	r = -0.370, P = 0.540	r = -0.784, P = 0.117
RSImod (index)	r = -0.276, P = 0.653	r = -0.551, P = 0.335	r = -0.510, P = 0.380
Time to take off Jump height (s)	r = 0.655, P = 0.230	r = 0.411, P = 0.492	r = -0.610, P = 0.275
Concentric peak force (N/kg)	r = 0.855, P = 0.114	r = -0.321, P = 0.599	r = -0.680, P = 0.207
Concentric net impulse (Ns/kg)	r = 0.236, P = 0.702	r = -0.182, P = 0.769	r = -0.850, P = 0.068
Concentric average power (W/kg)	r = 0.296, P = 0.629	r = -0.210, P = 0.734	r = -0.664, P = 0.222
Concentric phase duration (s)	r = 0.358, P = 0.554	r = 0.050, P = 0.936	r = -0.774, P = 0.125
Eccentric peak force (N/kg)	r = -0.259, P = 0.674	r = -0.708, P = 0.181	r = -0.678, P = 0.209
Eccentric net impulse (Ns/kg)	r = -0.163, P = 0.794	r = -0.612, P = 0.272	r = -0.439, P = 0.460
Eccentric phase displacement (braking phase) (m)	r = 0.870, P = 0.055	r = 0.597, P = 0.288	r = -0.396, P = 0.509
Eccentric phase duration (s)	r = 0.313, P = 0.608	r = 0.530, P = 0.359	r = -0.380, P = 0.529

Maximal voluntary isometric contraction (MVIC); Reactive strength index modified (RSImod); VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid

8.5 Discussion

This is the first study to show that 10-days supplementation with 600 $mg \cdot d^{-1}$ NZBC extract increases plasma concentrations of PCA and GA compared to PLA in individuals following a non-polyphenol restricted diet. This observation is in support of the first and second hypotheses. The plasma concentrations of PCA and GA continued to increase 24, 48 and 72 h following the 100-DJP in the NZBC group compared to baseline (PRE). However, in contrast for the third hypothesis, there were no relationships between the change in muscle function measures (MVIC and CMJ) and the plasma concentration of VA, PCA and GA following the 100-DJP.

Although direct comparisons with the literature are problematic due to differences in study design, namely having no exercise component and polyphenol-restricted diets, the C_{max} values of VA, PCA and GA observed within this Chapter are within similar ranges reported previously within the literature. Keane et al. (2016) observed that in in 12 healthy male participants who followed a low-polyphenol diet for 48 h prior to consuming either 30 or 60 mL of Montmorency tart cherry concentrate, that plasma VA plasma C_{max} was 0.30±0.01 μg·mL and 0.29±0.03 μg·mL for both groups, respectively. In the same study, PCA plasma C_{max} was 2.76±0.10 μg·mL and 2.75±0.13 μg·mL for the 30 and 60 mL Montmorency tart cherry concentrate groups, respectively. Furthermore, Roehrig et al. (2019) observed in one healthy male participant who followed an anthocyanin-free diet in the 72 h prior to and for the duration of the experimental trial, GA plasma C_{max} was 12.9±2.5 nmol·L (~1.2±0.2 μg·mL) in the 8 h following ingestion of 1.5 g of blackcurrant powder extract with 200 mL water. The higher PCA plasma C_{max} values reported by Keane et al. (2016) are likely due to the greater cyanidin content within Montmorency tart cherry concentrate compared to NZBC extract, which contains greater delphinidin content (Blando et al. 2004). What is interesting, is that Roehrig et al. (2019) observed similar GA C_{max} values following blackcurrant extract ingestion as what was observed within the present Chapter, despite the participant following an anthocyanin restricted diet. However, future research is warranted to observe whether the similar GA C_{max}

values are likely to be a consistent observation following blackcurrant ingestion given the low sample size in Roehrig et al. (2019).

It is important to note that the majority of dietary anthocyanins are not absorbed at the upper GI level, thus upon reaching the intestinal microbiota, they are bio-transformed into their metabolites, which are absorbed (Faria et al. 2014). In the intestinal microbiota, anthocyanins are extensively metabolised. This conversion is often essential for absorption and modulates the biological activity of these anthocyanin metabolites, not only due to the direct bioactivity from that of the parent compounds, but also because of their prebiotic activity in modulating the microbiota composition (Vendrame et al. 2011). Microbiota catabolism results in the production of new phenolic compounds, which may be absorbed and exhibit different bioactivity from that of the parents and plays an important role in systemic and local health effects (Williamson and Clifford, 2010). Various anthocyanins are mainly catabolised into PCA, *p*-coumaric acid, and vanillic acid in the intestine (Faria et al. 2014). Anthocyanins are metabolised in the colonic microflora, initially by deglycosylation and in a second phase by degradation into simple phenolic acids.

Although still in its infancy, there is a growing body of evidence which suggests that plasma levels of gut-derived phenolic acids may be linked to both acute and chronic exercise influences (Medina et al. 2012; Nieman et al. 2013; Nieman et al. 2018; Pereira-Caro et al. 2017). Nieman et al. (2018) demonstrated how in a targeted metabolomics approach, two-weeks flavonoid supplementation (329 $mg \cdot d^{-1}$) resulted in enhanced translocation of 15 gut-derived phenolics into circulation following a brisk 45-minute walk (60% VO_{2max}) compared to a sitting/rest condition in 77 healthy males and females who walked regularly. Further, in a sub-group analysis in the same study utilising 19 trained runners (55.8±2.7 $mL^{-1} \cdot kg^{-1} \cdot min$), a double dose (658 $mg \cdot d^{-1}$) of flavonoid supplementation for two-weeks resulted in increases in the translocation of 15 of the 76 gut-derived phenolics following a 2.5 h run at (70% VO_{2max}). The authors reported that the participants who completed the 2.5 h run demonstrated an amplified translocation of gut-derived phenolic acids compared to the brisk walking group and

attributed this to several possible underlying mechanisms, which included, increased gut permeability, a selective change in gut transporter density and function, changes in gut microbiota population diversity, and altered gastrointestinal motility and transport rate (Pereia-Caro et al. 2017; Karl et al. 2017; Lai, Jay and Sweet, 2018). Although purely speculative, it is possible that the increase in plasma concentrations of PCA and GA in the NZBC group and not in the PLA group following 100-DJP in this present study is due to anyone of these aforementioned mechanisms of action. Undoubtedly, for further understanding of the relative importance of each of these mechanisms, whether supplementation with NZBC extract is able to modulate these mechanisms and if a 100-DJP alters gut motility in a similar fashion to brisk walking and running, future research is required. However, what is important to acknowledge is that the observations in Nieman et al. (2018) were apparent when participants were following a polyphenol-restricted diet, whereas, in this present study, participants followed their habitual diets (i.e., non-polyphenol-restricted). Thus, it is interesting to see the increases in plasma PCA and GA concentration still occur when NZBC extract supplementation was superimposed on top of a habitual diet. Furthermore, future research should consider not analysing single phenolic acid appearance and activity as it has been suggested that combinations of bioactive substances, such as phenolic acids, exert effects at target sites that are greater than the sum of individual components (Lila et al. 2005). It has been previously shown how vascular smooth cell migration *in vitro* was 36±12% greater compared to control when PCA and VA were combined (Keane et al. 2016). As plasma concentrations of PCA and GA increased to a greater extent following NZBC extract supplementation compared to PLA (Figure 8.2 and Figure 8.3), future research should assess whether combined PCA and GA would result in similar *in vitro* increases and whether this translates *in vivo* to a physiological benefit.

Whilst it is not possible to directly compare the VA, PCA and GA responses in this Chapter to those observations in Chapter 7 due to the differences in study design, it is interesting to note that despite a moderate positive relationship (r =0.59, P=0.014) being observed between VA

and PCA in Chapter 7 when participants supplemented acutely with 300 mg of NZBC extract, no relationships were observed between VA and PCA in the current Chapter. Given that this Chapter utilised a longer supplementation period (10-days) and an increased dose (600 $mg \cdot day^{-1}$), it is surprising that such an observation was not apparent. As discussed in Chapter 7, it has been demonstrated that PCA can be metabolised into metabolites such as VA (Gao et al. 2006; de Ferrars et al. 2014). Further, in Chapter 7, following an overnight fast, plasma concentrations of VA were 0.27 (95%CI: 0.13-0.68) μg·mL prior to the acute 300 mg NZBC extract ingestion, whereas in the current Chapter, following 7-days of 600 mg NZBC extract or PLA ingestion and a 2 h fast prior to the laboratory visit, plasma concentrations of VA were 0.33 (95%CI: 0.10-0.55) μg·mL and 0.43 (95%CI: 0.01–0.84) μg·mL for NZBC and PLA groups, respectively. Although caution must be exercised when drawing comparisons between the Chapters due to the differences in study design and participants, it would appear that plasma VA concentration is more indicative of participant's habitual diet, compared to resulting from enterohepatic metabolism of PCA to VA following NZBC extract supplementation. This suggestion is further corroborated by the PLA group in this current Chapter demonstrating plasma VA concentrations that are in a similar, if not higher, range to the NZBC extract group (Figure 8.3, panel A). Both Chapter 7 and the present Chapter permitted participants to follow non-polyphenol restricted habitual diets to maintain ecological validity. Comparatively, it has been previously shown in 12 healthy male participants who followed a low-polyphenol diet for 48 h prior to consuming either 30 or 60 mL of Montmorency tart cherry concentrate, that prior to ingesting the supplement, baseline plasma VA concentration was 0.16±0.03 and 0.09±0.02 μg·mL (mean±SEM), respectively (Keane et al. 2016). Although speculative, the findings of Keane et al (2016) compared to the observations in Chapter 7 and the present Chapter suggest that plasma VA concentrations are indeed reflective of participant's habitual polyphenol consumption.

Similarly to Chapter 7, no relationships between individual estimated habitual anthocyanin intake and the $AUC_{PRE\text{-}72h}$ for VA, PCA or GA were apparent for either NZBC or PLA groups

despite estimated habitual anthocyanin intake being greater in this Chapter than in Chapter 7 (232±93 vs. 168±140 mg·day^{-1}, respectively). This consistent finding suggests that whilst it may be useful to document estimated habitual anthocyanin intake, in a similar way to capturing estimated macronutrient intake by means of a food-diary, to assess low compared to high consumers or group participants, the anthocyanin food frequency questionnaire (FFQ) utilised here and in the previous experimental Chapters is not indicative of subsequent VA, PCA or GA plasma concentration. It is likely that the anthocyanin FFQ is prone to similar limitations inherent with other dietary recall methods such as under and overestimation of foods consumed and issues with memory recall of dietary intake (Schoeller, 1995). Thus, until validation of the anthocyanin FFQ is completed in future research, the estimates obtained from the FFQ must be interpreted with caution.

Despite the increase in plasma PCA and GA concentration following the 100-DJP in the NZBC group, no relationships were apparent between the functional measures of recovery, MVIC and CMJ outcome and neuromuscular variables (Table 8.1). It is plausible that the lack of any relationships between the phenolic acids and functional measures of recovery following the 100-DJP, is due to the 100-DJP only inducing a modest decrement in MVIC peak force and CMJ outcome and neuromuscular variables (Figure 5.8 and Table 5.3, respectively). To date, no other study has assessed concentrations of plasma phenolic acids alongside functional measures of EIMD following a strenuous exercise bout such as the 100-DJP utilised within this Chapter (Bowtell and Kelly, 2019), thus, comparisons to the wider literature are limited.

However, it has previously been shown how an individual's training status can influence resting levels of gut-derived phenolic metabolites compared to lesser trained individuals (Nieman et al. 2018; Medina et al. 2012). In a secondary analysis to the primary targeted metabolomics analysis, Nieman et al. (2018) highlighted that pre-study plasma concentration of the gut-derived phenolic metabolites (all 76 that were detected) was 40% higher in the trained running group than in the brisk walking group. The authors concluded that the data indicated that acute exercise bouts (both brisk walking and intensive running) combined with

flavonoid supplementation, and the elevated fitness status associated with habitual running, are linked to elevations in plasma levels of gut-derived phenolics. Furthermore, Medina et al (2012) showed how compared to a control group of non-trained volunteers, the trained triathlete group, demonstrated a five-fold increase in flavanone metabolites excretion in urine following polyphenol-rich, aronia-citrus juice ingestion on-top of a flavanol-rich diet over a 24 h period. The authors attributed this increase in flavanone metabolite excretion to the triathlete's having an increased gut permeability and perhaps allowing maximum exposure and absorption of polyphenols, leading to increased bioavailability. Although the participants within this Chapter were non-resistance trained, how much other exercise they engaged with prior to study recruitment remains unknown but it appears plausible that differences in participant training history (e.g., habitual running status) could have contributed to the inter-individual variability apparent with VA, PCA and GA plasma concentration in this current Chapter (Medina et al. 2012). Further, as it has been demonstrated that polyphenols, namely anthocyanins, have the ability to modulate colonic bacteria growth (Parkar, Trower and Stevenson, 2013), it is possible that combined increased physical activity and polyphenol intake could modify the activity of the colon microbiota and therefore facilitate increased polyphenol bioavailability (Medina et al. 2012).

It is pertinent to consider that if using the PLA group as a baseline to assess the effect of the 10-days NZBC extract supplementation, the baseline (pre) plasma concentrations of PCA and GA were 0.70 (95%CI: 0.50-0.91) μg·mL and 0.88 (95%CI: 0.74-1.01) μg·mL, respectively. Whereas, in the NZBC group, plasma concentrations of PCA and GA just prior to completing the 100-DJP in the NZBC condition (i.e. after 7 days of NZBC supplementation) were 0.86 (95%CI:0.62-1.10) μg·mL and 1.04 (95%CI: 0.81-1.27) μg·mL suggesting an increase of ~0.16 μg·mL for both PCA and GA plasma concentration following 7-days (168 h) of NZBC supplementation. Comparatively, following the 100-DJP, the plasma concentrations of PCA and GA plasma concentration increased from 0.86 (95%CI:0.62-1.10) μg·mL and 1.04 (95%CI: 0.81-1.27) μg·mL at baseline (pre) to 1.29 (95%CI: 1.05-1.52) μg·mL and 1.34

(95%CI: 1.12-1.56) μg·mL, respectively, following an additional 3-days (72 h) NZBC supplementation (Figure 8.4). Although purely speculative, it would appear that the slope of the line increase in PCA and GA plasma concentation is greater following the 100-DJP, indicating that the 100-DJP may have increased gut motility and/or permeability following NZBC extract supplementation (Nieman et al. 2018). Therefore, in combination with previous literature, this observation suggests that combining supplementation with an exercise stimulus may result in the greatest increases in systemic levels of PCA and GA for indviduals.

8.6 Limitations

As the information presented in this Chapter was based on the retrospective analysis of data from previous chapters, it is important to acknowledge the limitations of the research presented herein that might affect the applicability of the findings. Similarly, to Chapter 7, the analysis was not exhaustive and so not every possible anthocyanidin metabolite was analysed and instead sought to extend the observations from Chapter 7 by following 10-day supplementation with 600 mg of NZBC extract. As the observations from this Chapter show, the plasma concentration of PCA and GA continued to rise up to 72 h following the 100-DJP. It remains unclear at what time point the plasma concentrations of PCA and GA would begin to decline once the NZBC extract supplementation had ceased. However, Czank et al (2013) highlighted how phenolic acids are still detectable in serum ~42 h post isotopically labelled cyanidin 3-*O*-glucoside bolus ingestion, so it is probable that plasma concentrations of VA, PCA and GA could have remained detectable at ~42 h post final NZBC extract ingestion. Furthermore, both the NZBC and PLA groups participated in the 100-DJP and thus, there was no comparator control or rest group making it difficult to ascertain the direct impact of the 100-DJP on the PCA and GA plasma concentration increase (Figure 8.4). However, there were no apparent order effects suggesting that the 2-week washout period was adequate and that the increase in PCA and GA plasma concentration was due to the 10-day NZBC extract supplementation. Lastly, as it has been shown that as polyphenol, namely flavonoid, dietary intake increases, it can diversify the gut microbiota, which is key to flavonoid metabolism

(Kardum and Glibetic, 2018; Murota et al. 2018), it is probable that where participants within this Chapter were free to consume their habitual diets alongside the NZBC extract supplementation, it cannot be ascertained how much the NZBC extract supplementation was directly responsible for the plasma increases in PCA and GA following the 100-DJP and how much the non-polyphenol restricted habitual diets were contributing to this observation. However, as aforementioned, there were no apparent order effects suggesting that the observed increases in PCA and GA plasma concentration following the 100-DJP in fact a result of the 10-day NZBC extract supplementation.

8.7 Conclusion

The primary finding of this Chapter was that 10-days supplementation with 600 $mg \cdot d^{-1}$ NZBC extract increases plasma concentrations and the total $AUC_{PRE\text{-}72h}$ of PCA and GA compared to a consuming a PLA in individuals following a non-polyphenol restricted diet. The plasma concentrations of PCA and GA continued to increase 24, 48 and 72 h following the 100-DJP in the NZBC group compared to PRE. However, there were no relationships between muscle function measures (MVIC and CMJ) and the plasma concentration of VA, PCA and GA following the 100-DJP. The increase in plasma concentrations and total $AUC_{PRE\text{-}72h}$ of PCA and GA increased following the 100-DJP after NZBC extract supplementation only, coupled with findings from other studies this suggests that the 100-DJP may have facilitated the appearance of the gut-derived phenolic acids. However, whether this was due to increased translocation across the gut barrier or whether it was due to increased gut microbiota biodiversity remains unclear. Lastly, the observations from Chapter 7 and the current Chapter suggest that plasma concentrations of VA are more indicative of habitual polyphenol intake than as a direct anthocyanin metabolite by-product from NZBC extract supplementation *per se*. Future research should consider the gut microbiota specifically as many phenolic acids are metabolised here and increase the observable systemic levels. In addition, utilising measures of gut permeability such as in Nieman et al (2018) would help develop the

understanding of the role of different exercise modalities on polyphenol metabolism and bioavailability.

9. General Discussion

9.1 Aims and summary of thesis

The overarching aim of the research presented in this thesis was to investigate the effects of NZBC extract supplementation on recovery following EIMD. As it was anticipated that there would be inter-individual variability in the group response and recovery from the EIMD protocols, targeted analyses were used in an attempt to explain the reason for some of the apparent intra- and inter-individual variability.

Previous research published in the last two decades was used to inform the hypothesis that supplementing with NZBC extract before and following exercise, might attenuate some of the effects of EIMD such as neuromuscular function and muscle soreness. This previous research has presented evidence that polyphenol-rich food and supplements can attenuate EIMD and enhance exercise recovery (Bell et al. 2015; Bowtell et al. 2011; Clifford et al. 2015; Coelho et al. 2017; Connolly et al. 2006; Howatson et al. 2009; McLeay et al. 2012; Rowland, 2018; Trombold et al. 2010; Trombold et al. 2011). The interest in these foods and supplements originates from the fact that they contain bioactive substances, namely polyphenols, which have purported anti-inflammatory and antioxidant properties. New Zealand blackcurrant is a rich source of polyphenols, primarily anthocyanins, but it's effects on recovery following EIMD had not previously been studied in detail. Previous research has also shown that EIMD and polyphenol supplementation have inherent inter-individual variability, with the term responder and non-responder being cited more frequently (Chen et al. 2012; Gulbin and Gaffney, 2002; Damas et al. 2016; Hubal et al. 2007; Nosaka and Clarkson, 1996; Paulsen et al. 2012; Sayers et al. 2003). The precise mechanism for the inter-individual variability is not abundantly clear, but an individual's genotype (Baumert et al. 2016; Clarkson et al. 2005), gut microbiota composition (Kardum and Glibetic, 2018; Murota et al. 2018; Tomas-Barberan, Selma and Espin, 2018) and polyphenol bioavailability (Keane et al. 2016; Rechner et al. 2002; Rodriguez-Mateos et al. 2013; Seeram et al. 2008) may all have contributing roles. This inter-individual variability in the response to NZBC extract on recovery from EIMD had not previously been investigated.

Figure 9.1 is a repeat of the flow diagram first presented in the introduction to this thesis but instead with a specific focus on the key results from each study. The flow diagram illustrates how Studies 1 to 4 provided new insights into whether NZBC extract can accelerate recovery from EIMD at both the group and individual level. Study 5 quantified the appearance of anthocyanin-derived phenolic acids following an acute dose of NZBC extract at rest for the first time. These data were used in Study 6 to examine the time course of plasma concentrations of VA, PCA and GA following 10-days of 600 $mg \cdot d^{-1}$ NZBC extract alongside a non-polyphenol restricted diet compared to consuming a placebo over the same time course. In addition, Study 6 examined the total $AUC_{PRE\text{-}72h}$ for the key phenolic acids, VA, PCA and GA following NZBC vs. PLA and the relationship between VA, GA and PCA plasma concentration and muscle function measures of MVIC and CMJ pre and following a 100-DJP.

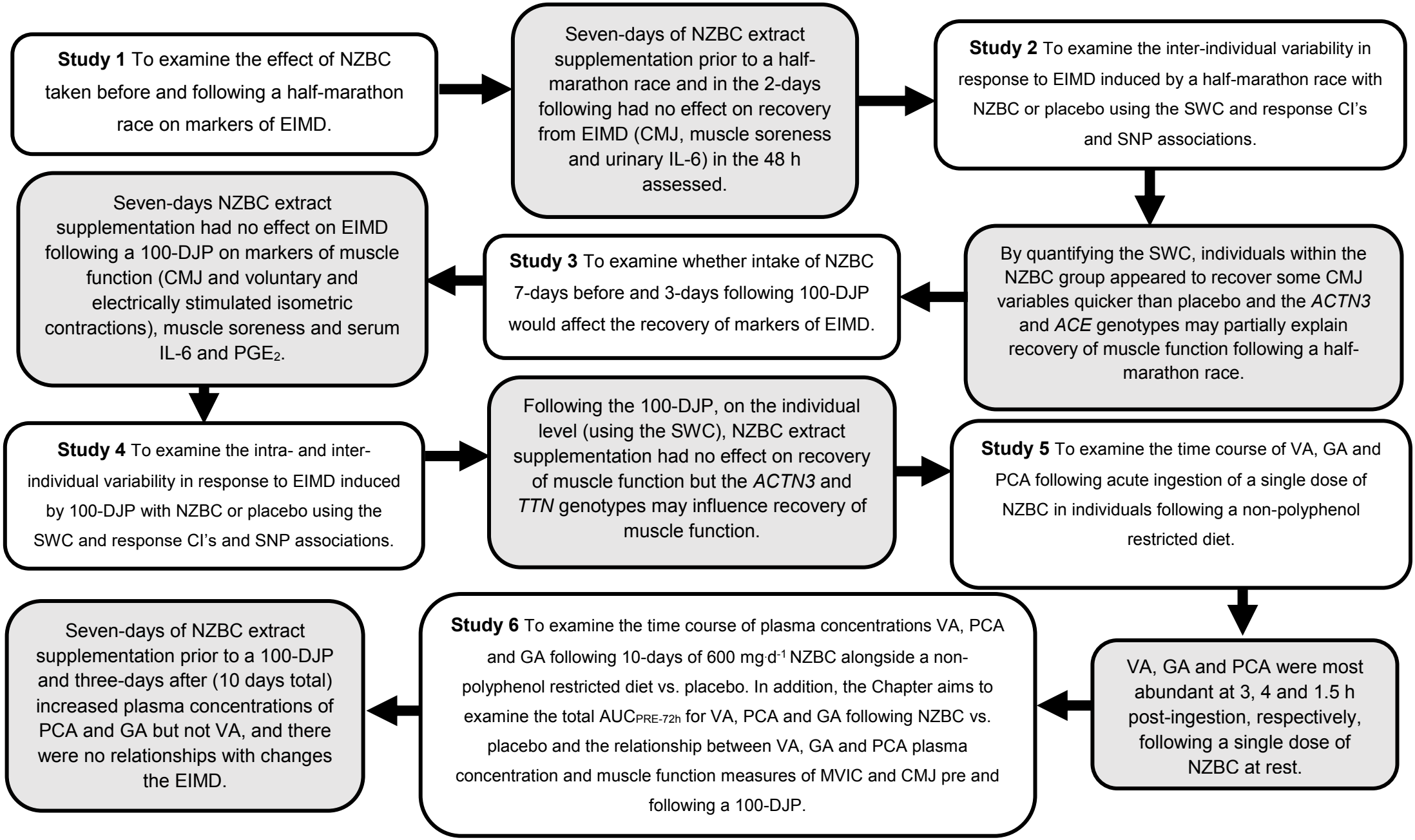


Figure 9.1 Overview of aims (white boxes) and key results (grey boxes) in Studies 1 to 6 within the thesis. NZBC, New Zealand blackcurrant; EIMD, exercise-induced muscle damage; SWC, smallest worthwhile change; CI, confidence interval; SNP, single nucleotide polymorphism; PLA, placebo; VA, vanillic acid; GA, gallic acid; PCA, protocatechuic acid; 100-DJP, 100-drop jump protocol; MVIC, maximal voluntary isometric contraction; CMJ, countermovement jump; AUCPRE-72h, total area under the curve pre to 72 h; *ACTN3,* alpha-actinin-3; *ACE,* angiotensin-I converting enzyme; *TTN,* titin; IL-6, interleukin-6; PGE_2, prostaglandin-E_2.

The series of investigations in this thesis led to a number of new contributions to the body of knowledge. Study 1 was the first study to examine NZBC extract efficacy as a recovery aid following EIMD induced by a half-marathon. Whilst it did not demonstrate any effect for NZBC extract to facilitate recovery following EIMD, it was acknowledged that, despite having strengths for its ecological valid design, it was likely that we were unable to detect any meaningful changes in recovery due to lack of extraneous variable control and having limited measures to assess recovery from EIMD. In Study 2, there was some evidence that supplementing with NZBC extract accelerated the recovery of dynamic muscle function for some individuals. However, this observation was not consistent across all studies, with Studies 3 and 4 suggesting no benefit of NZBC extract supplementation on muscle function recovery following EIMD at the group or individual level in a laboratory-controlled design experiment, respectively.

As the Studies within this thesis have implemented a habitual diet throughout, in an attempt to increase the ecological validity, it seemed pertinent to examine whether the lack of recovery benefit from EIMD with NZBC extract supplementation was perhaps due to the apparent poor bioavailability of anthocyanin, which is abundant in NZBC. Study 5 was the first study to examine the phenolic acid appearance in plasma following acute NZBC extract supplementation at rest. It was also the first to demonstrate that this acute NZBC extract supplementation was able to increase plasma concentrations of key phenolic acids, despite large inter-individual variability being present. Therefore, it was important to address whether a longer supplementation period, such as that used in Studies 1, 2, 3 and 4, was also able to increase plasma concentrations of the key phenolic acids. Study 6 was the first study to examine the phenolic acid appearance in plasma following 10-days NZBC extract supplementation before and following strenuous exercise. It was also the first to demonstrate that this 10-day NZBC extract supplementation was able to increase plasma concentrations of key phenolic acids, despite no relationships between phenolic acid appearance and markers of muscle

function recovery. Furthermore, there was no benefit of NZBC extract on muscle soreness or markers of inflammation 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 NZBC extract may not be a useful supplementation strategy in accelerating exercise recovery at the group or individual level following a 100-DJP but may confer some benefits at the individual level following a half-marathon race on dynamic jumping exercise, such as a CMJ. Furthermore, Study 6 does highlight the importance of quantifying exercise performance outcomes alongside measurements of plasma phenolic metabolites.

9.2 Main findings

The results presented within this thesis have been previously discussed throughout each of the experimental Chapters, and thus, this section will discuss the main findings of this thesis in the context of existing literature, the strengths and limitations of the work as a whole and potential future areas for investigation.

9.3 Effects of NZBC on muscle function recovery

Study 1 showed that at the group level, seven-days of NZBC supplementation had no effect on recovery from EIMD as assessed by CMJ outcome and neuromuscular variables in the 48 h following a half-marathon running race. However, Study 2 highlighted how some individuals in the NZBC group recovered CMJ eccentric peak force, net impulse, phase duration and concentric power quicker following the half-marathon than those in the PLA group. Nonetheless, it is important to highlight that this observation was made from an independent-groups design and in order to have full confidence in NZBC as a recovery aid, replication would be required with the same participants. Furthermore, this observation was not consistent as Studies 1, 3 and 4 demonstrated that there were no benefits on the recovery of muscle function (voluntary and involuntary contractions or CMJ) recovery for NZBC extract supplementation

following EIMD. The lack of benefit of NZBC on muscle function recovery is in accordance with previous research where it was observed that 12-days NZBC extract supplementation was unable to attenuate declines in MVIC peak force following EIMD caused by 4 sets of 15 maximal concentric and eccentric elbow flexor contractions on an isokinetic dynamometer (Rowland, 2018). However, previous research utilising different polyphenol-rich supplements 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) have found benefits on muscle function recovery following EIMD. The reason for the equivocal findings between the research presented in this thesis between Studies 2 and 1, 3, and 4 could be attributable to several factors, including the differences in the exercise models used to induce EIMD, training status of participants, genotype and sex differences. Nonetheless, perhaps the most logical explanation is related to the variation in the magnitude of EIMD experienced between running a half-marathon and the 100-DJP. With regards to Studies 3 and 4, in which NZBC extract supplementation did not enhance voluntary and involuntary isometric contractions or CMJ performance following 100-DJP, the magnitude of muscle damage was much smaller, and the rate of recovery much quicker than in the Study 2 where some individuals appeared to recover quicker with NZBC extract supplementation. Furthermore, a secondary loss in muscle function was observed in Study 2 for some individuals in the PLA group; as shown in Chapter 4 (Figures 4.4, panels A and B, 4.5, panels A and B and 4.6, panels A and B), where CMJ JH, TTT, concentric power, eccentric peak force, net impulse and phase duration actually deteriorated in the immediately post to 48 h period, following the half-marathon without NZBC supplementation. Whereas, in Studies 3 and 4, when NZBC supplementation was ineffective, MVIC returned to almost baseline (pre-100-DJP) values by 24 h (Figure 5.8 and 6.5), whereas CMJ outcome and neuromuscular variables remained largely unaffected by the 100-DJP (Table 5.3 and Figures 6.1 - 6.9).

The variation in EIMD and rate of recovery could also help to explain, at least in part, why NZBC extract supplementation appeared to enhance the rate of recovery in some individuals of CMJ performance and outcome variables in Study 2, but not in Studies 3 and 4. However, due to the lack of benefit of NZBC extract supplementation observed in Studies 3 and 4, following a repeated crossover design, it should be highlighted that greater participant numbers are required to confirm this observation before concluding that NZBC extract supplementation has limited use for recovery from EIMD.

As the overall loss of muscle function for CMJ performance was far less in Studies 3 and 4 than in Study 2, this could have rendered NZBC extract supplementation less effective for accelerating the recovery of this measure. In light of these observations, it could be speculated that NZBC extract was ineffective in Studies 1, 3 and 4 because either, a) the muscle function loss was less and, thus, group and individual differences were more difficult to detect without greater participant numbers, and/or; b) there was no further loss in muscle function and, therefore, the secondary EIMD response was minimal and not of sufficient magnitude for NZBC extract supplementation to be beneficial. These findings would suggest that the magnitude of muscle force loss in the days after the muscle damaging bout could be a key determinant of the effectiveness of NZBC extract as a recovery aid. Thus, subsequent research is warranted to consider if the exercise stimulus is sufficiently strenuous to actually warrant an intervention. Nonetheless, the inconsistent findings imply that a definitive conclusion cannot be achieved as to the efficacy of NZBC extract supplementation for enhancing muscle function recovery on these studies alone.

To assess whether NZBC extract supplementation was able to intervene in the secondary cascade of EIMD, markers of inflammation were measured systemically. In line with other polyphenol supplements showing anti-inflammatory benefits, it was initially hypothesised that NZBC extract supplementation would attenuate EIMD by mitigating the inflammatory cascade following a muscle damaging exercise bout. However, contrary to this hypothesis, biomarkers of inflammation (IL-6 and PGE_2) were not

different after NZBC extract or a PLA supplementation in any of the studies within this thesis. It is important to note however, that these markers were all taken systemically and, thus, it is possible that NZBC extract mitigated inflammation at the local level, but that this was not detectable in plasma. Furthermore, although IL-6 is frequently used as a biomarker of inflammation following muscle-damaging exercise, due to its pleiotropic nature, an increase in its appearance when measured systemically can also be an indicator of reduced muscle glycogen content (Pedersen et al. 2004), an anti-inflammation response (Fischer, 2006; Paulsen et al. 2012) or an infection (Tanaka, Narazaki and Kishimoto, 2014). Given that the inflammatory cascade occurs in response to an increase in oxidative stress within damaged cells, without a broader range of biomarkers indicative of both the pro- and anti-inflammatory response and oxidative stress, drawing definitive conclusions is problematic (Owens et al. 2018). Future research should look to include a comprehensive range of biomarkers from different cellular targets of pro-inflammation such as IL-6, TNF-α, CRP, anti-inflammation such as IL-10, IL-1ra, oxidative stress such as protein carbonyls, F_2-isoprostanes and endogenous redox enzyme activity such as SOD and GPx when assessing the efficacy of NZBC supplementation following EIMD (Close, Ashton, McArdle and MacLaren, 2005; Cobley et al. 2017; Gleeson et al. 2011; Powers et al. 2010). Similarly, PGE_2 is known to act as a noxious stimulus contributing to muscle tenderness (Hyldahl and Hubal, 2014; Mizumura and Taguchi, 2016), play a role in skeletal muscle protein turnover (Palmer, 1990; Rodemann and Goldberg, 1982; Vandenburgh et al. 1995), and act as a MuSCs proliferation moderator (Ho et al. 2017). Thus, although systemic concentrations of PGE_2 can act as indicators of inflammation precipitating from the COX-2 pathway (Reddy et al. 2005), due to its multifaceted roles within the aforementioned biological systems, future research should look to measure levels of COX-2 enzyme activity in conjunction with PGE_2 to obtain a more complete picture following NZBC supplementation and EIMD (Reddy et al. 2005; Seeram et al. 2001).

However, it has been previously demonstrated how polyphenol supplementation has improved muscle function recovery (Clifford et al. 2015; Clifford et al. 2016; Trombold et al. 2010) and CK response (Coelho et al. 2017) following muscle damaging exercise, in the absence of any changes in systemic levels of inflammation. Therefore, the lack of systemic changes in inflammatory biomarkers with NZBC extract within this thesis raises the possibility that mechanisms other than an anti-inflammatory effect could have been responsible for the improvement in CMJ performance and outcome variables for some individuals in Study 2.

Those data presented for Study 6 demonstrated that seven-days intake of 600 $mg \cdot day^{-1}$ NZBC extract was able to increase the AUC of GA and PCA compared to PLA, in addition to the unexpected finding of an increase in the rate of appearance of GA and PCA, in the 72 h following the 100-DJP. As discussed in Study 6, it could be speculated that a synergistic relationship exists between the NZBC extract supplementation and 100-DJP, which was able to increase gut motility and/or permeability, providing individuals with greater whole-body exposure to the gut-derived phenolic acids. The increased exposure to the gut-derived phenolic acids may have provided not only anti-inflammatory but also antioxidant benefits (Larrosa et al. 2010) and possible enhancement of muscle function (Ryu et al. 2016) as has been previously shown in rodent models. However, as aforementioned, this is purely speculative and future research should look to explore whether this concept holds true in human exercise models and with a broader range of inflammatory and oxidative stress biomarkers.

9.4 Effects of NZBC on muscle soreness

In contrast to previous research, NZBC extract supplementation was unable to attenuate the ratings of muscle soreness following EIMD in Studies 1 and 3 within this thesis. Rowland (2018) and Coelho et al. (2017) both demonstrated that NZBC extract supplementation was able to attenuate feelings of muscle soreness following muscle

damaging protocols. The reasons for the contrasting observations between those within this thesis and Rowland (2018) and Coelho et al. (2017) is not abundantly clear but is likely due to a number of factors, including differences in the muscle damaging protocol used, training status of participants, sex differences, genotype and methods used to measure muscle soreness/pain (0–10 vs. 0–100 mm VAS scale). With regards to the latter, both Rowland (2018) and Coelho et al. (2017) required participants to flex and extend their dominant arm, which had been subjected to an elbow flexor muscle damaging protocol on an isokinetic dynamometer, and then mark a point on the VAS scale between 0 'no pain whatsoever' and 100 mm 'worst pain imaginable'. In contrast, Studies 1 and 3 within this thesis required participants to perform a squat to 90^{o} with hands on hips and whilst in the squat position, rate their feelings of lower limb soreness on a VAS scale between 0 'no soreness' to 10 'extreme soreness'. Whilst it is not immediately clear how this could have affected the participant's ability to rate their feelings of muscle soreness following the half-marathon and 100-DJP; due to a lack of experimental comparisons between different VAS scale approaches, it is possible that the VAS scale was not a sensitive enough measure to capture any potential benefit on muscle soreness and pain in the lower limbs with NZBC extract supplementation. It has been previously shown how use of a pressure pain threshold test (PPT) along with rating feelings of soreness on a VAS scale between 0 – 10 (no soreness to extreme soreness) was able to accurately capture quadricep muscle tenderness following a single-leg repeated eccentric quadricep contract protocol in 20 healthy male participants following naproxen or PLA supplementation (Lecomte, Lacroix and Montgomery, 1998). Furthermore, Clifford et al. (2015) demonstrated how following high or low-beetroot supplementation in the 72 h following a 100-DJP, a PPT was able to demonstrate that participants felt significantly less pain with the beetroot supplementation compared to PLA. Thus, it could suggested that when assessing participant's feelings of muscle soreness in larger muscle groups such as the quadriceps, following a muscle damaging protocol, a PPT in isolation or in combination with ratings of muscle soreness (VAS),

appears to be a more sensitive measure, which future research should look to explore further when considering a polyphenol supplementation strategy. Nonetheless, another possible explanation for the lack of a perceivable benefit of NZBC extract supplementation on feelings of muscle soreness is due to both the half-marathon and 100-DJP only inducing moderate increases VAS ratings (Figures 3.3 a and 5.10). However, the ratings of muscle soreness in both Studies 1 and 3 are in similar ranges to Jakeman et al. (2017), who observed VAS ratings no higher than 7 in the 96 h following a 100-DJP with omega-3 fish oil supplementation, on a VAS scale that was similar to the one used within this thesis. Thus, the inconsistent findings between previous NZBC research and the observations within this thesis, imply that a definitive conclusion cannot be drawn as to the efficacy of NZBC extract supplementation for attenuating feelings of muscle soreness and further research is warranted.

As discussed in Section 5.5, although the precise mechanisms that cause muscle soreness remain unclear, previous research has demonstrated that intramuscular generation of noxious stimuli such as nerve growth factor (NGF), bradykinin, and PGE_2, are likely to play important roles (Hyldahl and Hubal, 2014; Murase et al. 2010; Mizumura and Taguchi, 2016). Arachidonic acid (AA) is synthesized by the COX-1 and COX-2 enzymes to form chemical mediators of pain and inflammation such as PGE_2. Furthermore, Seeram et al. (2001) observed that anthocyanins found in blackberries, which share similar anthocyanin profiles to NZBC, demonstrated a 45.7% inhibition of COX-2 enzyme. Although not a primary aim of this thesis, in Study 5, measures of PGE_2 were collected to assess whether there would be any relationship between NZBC extract supplementation and measures of muscle soreness and levels of PGE_2. However, contrary to the hypothesis that, as NZBC extract is abundant in anthocyanin, levels of PGE_2 would correlate to ratings of muscle soreness, and be suppressed in the NZBC group, no relationships were apparent. The reason for the lack of a relationship demonstrated in this thesis is not abundantly clear, but could be related to several factors

including, a) the precision within the PGE_2 assay used within this thesis (10% CV) and b) insufficient participant numbers to detect subtle changes in PGE_2 levels. Although no relationships were demonstrated within this thesis between ratings of muscle soreness and PGE_2 following NZBC extract supplementation, future research could still consider including measures of PGE_2 and/or NGF and bradykinin along with measures of muscle soreness/pain in a more comprehensive approach to assessing the efficacy of a polyphenol supplement on self-perceptual muscle soreness, due to the likely multifaceted nature of the mechanism/s driving delayed onset muscle soreness (DOMs).

9.5 Plasma uptake of NZBC

Studies 5 and 6 quantified the phenolic acid uptake following supplementation with NZBC extract. The presence of VA (C_{max} 0.49 and 0.39 μg g·mL^{-1}), GA (C_{max} 1.88 and 1.36 μg g·mL^{-1}) and PCA (C_{max} 1.66 and 1.36 μg g·mL^{-1}) were identified in the plasma following NZBC extract supplementation. To date, the majority of previous investigations have focussed on the absorption of primary anthocyanins. As the bioavailability of anthocyanins is poor compared to other flavonoids (McGhie and Walton, 2007), exploring the presence and absorption time of their primary downstream metabolites may be more appropriate when trying to determine the compounds provoking favourable physiological benefits. Gallic acid and PCA are the main degradation products of delphinidin and cyanidin, the most abundant anthocyanins present in NZBC extract (Slimestad and Solheim, 2002; Matsumoto et al. 2001). Study 5 demonstrated that concentrations of VA, GA and PCA were most abundant at 3, 4 and 1.5 h post-ingestion NZBC extract, respectively. Whereas, in Study 6, NZBC extract supplementation increased the plasma concentration and total AUC of GA and PCA over the 72 h following the 100-DJP, however, this observation was not apparent for VA. As discussed in Section 8.5, plasma concentrations of VA appear to be more indicative of an individual's habitual diet rather than as a result of enterohepatic metabolism from PCA following NZBC extract supplementation. Perhaps the most interesting observation from Study 6,

was of how the 100-DJP appeared to facilitate a greater rate of increase in plasma concentration of GA and PCA compared to purely NZBC extract supplementation alone. As discussed in Section 8.5, the current evidence to date appears to suggest an almost facilitative role of exercise on gut permeability, which in turn, contributes to the increase in appearance of phenolic acids following polyphenol supplementation (Nieman et al. 2018). Clearly, evidence in this area is lacking due to the observation only becoming apparent within the last few years and so, future research should look to assess the possible synergistic relationship between phenolic acid appearance and an exercise stimulus.

A large inter-individual variability in phenolic acid appearance was apparent within the group response for Study 5. This observation may be partially explained by the findings of Manach et al. (2017) who suggested that the gut microbiota is a key determinant of the inherent inter-individual variability when studying the cardiovascular health effects of dietary polyphenol intake. It has been hypothesised that variations in the physiological benefits observed after polyphenol intake, could be associated with differences in the production of bioactive compounds (e.g., phenolic acids) by the gut microbiota, which in turn depends on the variations in microbial ecology that colonise the colon of each individual person (Tomas-Barberan et al. 2018). Thus, this suggestion implies that dietary intake of any polyphenol, could potentially lead to different metabolites depending on each individual's specific gut microbiota composition, and therefore, to different physiological effects. Clearly, implementing a polyphenol supplementation strategy does not come without a myriad of possible further questions and avenues of possible exploration. However, one possible way to address the complex problem is through the use of the 'omics' technology. Foods that are rich in polyphenols contain hundreds of polyphenolic compounds that give rise to a multitude of different metabolites when ingested and can subsequently be detected in a wide range of biological tissues, in a wide range of concentrations. This complexity has proven troublesome for researchers

to establish the true extent of the exact number of different polyphenolic metabolites that exist and how precisely these compounds can exert physiological effects. However, in the past two decades, developments in technology, namely 'metabolomics' have become more accessible to researchers, which allows comprehensive assessment of the whole metabolome and measurements of metabolite concentrations in cells and tissues (Stewart et al. 2007). Arguably, if access to metabolomics is possible, future research should look to utilise the method to help deepen our understanding of the mechanisms behind the inter-individual variability inherent with dietary polyphenol intake and possible physiological benefits.

9.6 Strengths and Limitations

It is possible that the allowance of habitual diet consumption may have altered the appearance of phenolic acids from NZBC extract supplementation because of synergistic or antagonistic effects (Niki et al. 1991). However, it was decided that all Studies within this thesis would utilise an ecologically valid design to assess NZBC extract supplementation effectiveness, given that most polyphenol supplementation studies enforce strict polyphenol-deplete diets, which may affect the observations. Thus, it is still promising to see that both acute and 10-days NZBC extract supplementation were able to increase the plasma concentrations of key phenolic acids alongside participant's habitual diets. Several factors, such as environment, are known to affect the polyphenol content of foods (as described in Section 2.1). The NZBC extract capsules used in all studies herein came from the same batch. However, it was not financially or logistically possible to determine the polyphenol content of the NZBC extract capsules used, instead the information supplied from the manufacturer's certificate of HPLC analysis was utilised. Where feasible, future studies should look to quantify the polyphenol content of supplements alongside intervention-based designs to ensure full confidence in the supplement contents.

Another key limitation that must be addressed is that the analysis in Studies 5 and 6 was not exhaustive and so, not every polyphenol was analysed. Instead, the focus was on the appearance in plasma of the degradation products of two of the main anthocyanidins reported in NZBC and VA, the methylated degradation compound of PCA, that have purported health and physiological benefits (Rechner et al. 2002; Slimestad and Solheim, 2002; Matsumoto et al. 2001). In addition, it is recognised that plasma AUC is not indicative of whole-body AUC and it is likely phenolic acids were present in urine or faeces (de Ferrars et al. 2014). Furthermore, due to their instability and poor bioavailability, parent anthocyanins were not investigated. However, it is possible that these compounds could have contributed to any potential physiological effects from NZBC extract and cannot be ruled out.

Although it was shown how key phenolic acids from anthocyanin appear in the plasma in the hours and days following NZBC extract supplementation, the precise mechanisms of how NZBC would exert its beneficial effects, remains unknown. Furthermore, it is acknowledged that another limitation of this thesis was the inability to obtain muscle tissue samples to assess the magnitude of EIMD within the experimental Chapters to further explore the precise mechanisms. Given that it has been demonstrated, in rodents at least, that anthocyanins can be stored within organ tissues, in order to fully understand the underpinning mechanisms behind both polyphenols and EIMD, future research should consider obtaining muscle biopsy samples from the affected tissue. The NZBC extract supplement was unable to attenuate any of the measured systemic markers of inflammation after exercise in any of the Studies in this thesis. Thus, this provides rationale for future studies to instead focus on changes in these biomarkers at the local (muscle) level or look to investigate the potential influence of a PLA effect. Lastly, it is acknowledged that there was a small number of participants in the genotype analysis in Studies 2 and 4. However, given the difficulty in performing these types of intervention

studies, the data presented is still of great scientific value and should be utilised as a catalyst to reliably validate and replicate these findings.

Although there were limitations in the work conducted in the thesis, there were also many strengths that made the work unique and valuable to future research. The use of portable force plates in Studies 1, 2, 3 and 4 allowed greater insight into how participants may adopt different CMJ strategies following both half-marathon running and 100-DJP, potentially highlighting aspects of relevance to real-world sporting performance that may be masked when only considering variables such as jump height. The incorporation of both group and individual responses has allowed greater understanding into the variability that exists following EIMD and provides a potential framework that could be utilised in the future when assessing an individual's susceptibility to EIMD and whether a nutritional supplementation intervention may have any recovery benefits. The identification of key anthocyanin-derived phenolic acids following acute and 10-days NZBC extract supplementation provides new information following reports that the downstream metabolites are likely responsible for observed performance and physiological responses (Cook and Willems, 2018). Lastly, the removal of the RBE following implementation of the 100-DJP, allowed for assessment of NZBC extract supplementation efficacy on EIMD in a more ecologically valid study design where athletes are more likely to partake in training regimes that they are accustomed too (Currell and Jeukendrup, 2008; Hopkins, 2000).

9.7 Future work

The aims of this thesis have been addressed in the six studies and have provided a unique contribution to the existing literature. The research presented has demonstrated a lack of recovery benefit from NZBC extract supplementation at both the group and individual level and provided an insight into the appearance of phenolic acids from

anthocyanin following intake of NZBC extract, which had previously remained unknown. Consequently, a number of potential possibilities for future research have been identified.

Studies 5 and 6 of this thesis have established the time course at which some phenolic acids are most bioavailable (peak concentration) following acute and 10-days of NZBC extract intake, respectively. The peak concentration demonstrated in Study 5 for VA, GA and PCA was 0.49, 1.88 and 1.66 μg $g \cdot mL^{-1}$, respectively, following acute ingestion of 300 mg NZBC extract. In contrast, the peak concentration demonstrated in Study 6 for VA, GA and PCA was 0.39, 1.36 and 1.36 μg $g \cdot mL^{-1}$, respectively, following 10-days intake of 600 mg NZBC extract. Direct comparisons between studies 5 and 6 must be interpreted with caution due to the duration of the sampling time points (acutely over 6 h vs. over several days) and dose (300 $mg \cdot d^{-1}$ vs. 600 $mg \cdot d^{-1}$), respectively. However, a few key questions that remain which, future research needs to address are a) whether there is a 'ceiling effect' for how high plasma concentration of phenolic acids can go b) can these phenolic acids be preferentially stored in human tissues and c) how does supplementation of NZBC extract, alongside a habitual diet, alter the gut microbiome and does this in turn, have a role to play in the appearance of the phenolic acids at the systemic level?

With regards to the first two points, previous research has demonstrated how anthocyanins from blackcurrant (Matsumoto et al. 2006), whole tart cherry (Kirakosyan et al. 2015) and blackberry (Felgines et al. 2009) can be located within the organ tissues of rodents and rabbits following ingestion. Matsumoto et al. (2006) highlighted how, blackcurrant anthocyanins were detected within the plasma and whole eye after oral and intraperitoneal administration of blackcurrant powder in rats and rabbits. Furthermore, anthocyanins could be located in the bladder, kidney, liver, heart and brain of healthy rats following ingestion of the whole tart cherry (Kirakoyan et al. 2015). Similarly, Felgines et al. (2009) observed how rats fed a blackberry enriched diet for 12 days resulted in accumulation of anthocyanins in the bladder, prostate, testes and heart.

Interestingly, the authors also illustrated how cyanidin-3-glucoside and methylated derivatives were present in the adipose tissue. Taken together, these findings suggest that when ingested, anthocyanin and the phenolic acid derivatives, are not only present systemically, but also accumulate within organ tissue, which may correlate with some of the health-enhancing benefits of anthocyanin. Further, the accumulation in tissues, may partially explain why plasma levels of phenolic acids did not exceed ~ 3 μg $g \cdot mL^{-1}$ in Studies 5 and 6 of this thesis. However, what remains unknown is whether NZBC anthocyanins are able to accumulate in any of the aforementioned organs or skeletal muscle in humans. The identification of target tissues, such as skeletal muscle, of anthocyanins may then help to understand the true metabolic fate and mechanisms of action of the anthocyanins *in vivo.*

As aforementioned in Section 9.5, it has been previously demonstrated how the gut microbiota can be positively modulated via the diet, which increases the populations of the beneficial bacteria such as lactobacilli and bifidobacteria, which in turn, could lead to various health benefits through different mechanisms (Collins and Gibson, 1999). These mechanisms may include reducing gut pH through stimulation of the growth of the lactic acid-producing microbiota (Langhendries et al. 1995), improvement of immune function and stimulation of appropriate immunomodulatory cells (Isolauri et al. 1995) and direct antagonistic effects on pathogens (Macfarlane and Gibson, 1994). Furthermore, Molan et al. (2014) demonstrated in rats how two different blackcurrant extract powders (First Leaf and Cassis Anthomix 30 from Japan and New Zealand, respectively) were able to act as prebiotics, by their capacity to increase the numbers of beneficial bacteria such as lactobacilli and bifidobacteria. Taken together, these observations suggest that NZBC extract supplementation, may be able to facilitate the increase in gut microbiota populations, which may partially explain the observations in Studies 5 and 6. However, future research is needed to explore this further to confirm through gut microbiota

sampling and also explore the relationship between exercise and phenolic acid appearance systemically, in urine and in faeces (Nieman et al 2018).

Furthermore, it is important to acknowledge that there was a lack of female participants within the experimental work in this thesis. Apart from Studies 1 and 2 and 5, all the Studies in this thesis had only male participants. Given the suggestion that males and females might recover at different rates, possibly due to the menstrual cycle's effect on the inflammatory cascade, following EIMD (Hackney et al. 2019; McNulty et al. 2020; Clarkson and Hubal, 2001; Tiidus, 2000), future research should also look to compare the effects of NZBC extract on recovery in male and female participants.

Another important direction for future research is to establish the most effective supplementation strategy of NZBC extract for mitigating EIMD and enhancing recovery. It was beyond the scope of this thesis to address this question in detail. Studies 1 and 3 both used 600 mg of NZBC extract for 9- and 10-days, respectively. Thus, it remains to be established whether the lack of an observable group benefit of NZBC extract on recovery in both studies 1 and 3 was due to an insufficient dose of NZBC extract being supplemented and perhaps a greater one (e.g. 900 $mg \cdot d^{-1}$; Cook et al. 2017) was required to elicit recovery benefits from EIMD. Other important features relating to NZBC extract dosage strategy that require further investigation are the frequency and timing of NZBC extract supplementation before and after exercise. As this thesis was one of the first to investigate NZBC extract supplementation in terms of its effects on EIMD and recovery, there was a lack of literature on which to base a dosage strategy on, as such the frequency and timing of supplementation in each study was based on a) observations from previous work on NZBC extract and exercise performance from our laboratory; b) the time course of secondary EIMD and likely pattern of recovery and c) other polyphenol-rich supplements demonstrating favourable effects on EIMD. Notwithstanding this rationale, it is unclear whether this approach was the most effective with NZBC extract and, therefore, future studies should look to compare this strategy to

more acute or chronic strategies, including a longitudinal strategy as used by Hurst et al. (2020) where NZBC extract was supplemented for five weeks at a dose of 3.2 $mg \cdot kg^{-1}$ (~240 mg) anthocyanin prior the experimental visits.

Lastly, another area which has remained relatively unexplored, but is worth consideration, is whether polyphenol supplementation interventions can enhance the recovery of the connective tissues (i.e., tendons). Collagen fibres are one of the main components of tendons and as such, the sparsity in the literature is surprising given the high incidence of tendon injuries from physical training and/or sports (Wilkinson et al. 2011; Scott et al. 2013). Two studies have previously demonstrated that quercetin and epigallocatechin gallate are able to prevent collagen breakdown by inhibiting collagenase (Sin and Jim, 2005; Yamakawa et al. 2004). Given the possible protective role that the muscle tendon unit (MTU) plays in EIMD (Narici et al. 1996), it would be pertinent for future research to assess whether polyphenol supplementation is able to enhance collagen synthesis and/or mitigate damage to the tendons as another approach to supporting an individual's recovery from EIMD.

9.8 Practical recommendations

Based on the observations from the studies presented within this thesis, some practical recommendations for the use of NZBC as a recovery supplementation strategy following muscle-damaging exercise are offered:

1. As no detrimental effects were reported within the studies (i.e., participants did not report any negative side effects of the NZBC extract supplementation), supplementing acutely (immediately prior to) or chronically (over a 10-day period) may be useful for athletes when fruit and vegetable intake may be inhibited. As the plasma uptake data from Chapters 7 and 8 highlight, either supplementing acutely with 300 mg or over 10-days with 600 $mg \cdot d^{-1}$ NZBC extract increases plasma concentrations of key anthocyanin phenolic acids (PCA and GA) to a

similar extent compared to a habitual diet and/or placebo. This in turn could lead to improving markers of recovery that were not measured within this present thesis for the athlete who is travelling and/or competing in foreign countries.

2. Although no beneficial effects were observed of NZBC extract supplementation on the markers of recovery from EIMD within this thesis, if an athlete were to utilise NZBC as part of a nutrition supplementation strategy, it would be advisable to consider implementing a pragmatic approach where supplementation would be used during congested training and/or competition periods where the recovery window is minimal rather than continuously where fruit and vegetable intake likely provide sufficient polyphenol intake. Further, for the practitioner, it would be prudent to consider implementing a stratification approach with individual athletes or teams where first they would be screened to assess their habitual fruit and vegetable status via plasma concentrations of α- and β-carotene (Lewis et al. 2018; Margaritelis et al. 2020). If the plasma concentrations of α- and β-carotene were 'low', then initiating a supplementation strategy with NZBC may then be beneficial to provide the athlete with a source of polyphenols, which may help mitigate a slowed recovery from muscle-damaging exercise.

9.9 Conclusion

The research presented within this thesis has provided the first evidence that a NZBC extract supplement is unlikely to provide any recovery benefits following EIMD following a half-marathon or a 100-DJP. Further, both acute (single dose) and chronic (10-day) supplementation of NZBC extract results in increased plasma concentrations of key phenolic acids alongside a habitual non-polyphenol restricted diet. Future research should utilise a protocol that evokes a large EIMD response and in more trained individuals before ruling NZBC extract supplementation out as a potential recovery aid. This is particularly important given that NZBC extract was able to increase phenolic acid plasma concentration and total AUC, even when participants were following their habitual

diets, which may be linked to phenolic acid tissue accumulation/storage in other tissues (Kirakoyan et al. 2015; Matsumoto et al. 2006). Lastly, the two-phase approach to assessing inter-individual and intra-individual response used in this thesis provides a potential approach, which future research could utilise to assess an individual's susceptibility and recovery response from EIMD, with a polyphenol supplement intervention. This in turn, could help inform practitioners to provide personalised recommendations on the use of polyphenol supplements to enhance performance.

10. References

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