Omega-3 polyunsaturated fatty acid supplementation, sports performance and recovery

by

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Abstract

Strategies focused on the enhancement of recovery from exercise and the subsequent improvement in sport performance are continually being sought by athletes. One strategy that has received considerable attention within the sports nutrition literature is omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation. The myriad of proposed health benefits has led to the investigation of dietary supplementation to enhance recovery from exercise due to the anti-inflammatory properties and whether this ultimately results in improved sports performance. The overall aim of this thesis was to establish whether n-3 PUFA supplementation could improve exercise performance and recovery from strenuous exercise.

To achieve this aim a series of studies were conducted on trained and untrained males, in both lab and field-based studies. There was no significant effect of n-3 PUFA supplementation on cycling time trial performance or associated physiological parameters (chapter 4). This finding was also observed in strength and power indices in untrained individuals following 3 weeks supplementation (chapter 5) and trained rugby union players following 6 weeks supplementation (chapter 6). However, muscle damage, measured by the reduction in peak isometric torque following exercise-induced muscle damage (EIMD), was lower following 3 weeks of n-3 PUFA supplementation (chapter 5), suggesting supplementation may aid the recovery process in untrained individuals. No changes were observed in biomarkers of muscle damage post-exercise following supplementation (chapter 4, 5 and 6).

When supplementation was combined with resistance training there was no additive effect of supplementation in terms of strength and power performance or recovery from EIMD. The increase in strength and power observed as a result of the resistance training was not negatively impacted with the addition of supplementation in untrained males (chapter 5). When supplementation was implemented within an applied sports setting, elite rugby union players, the increases in power observed mid-season were not different between supplemented and non-supplemented groups (chapter 6). No changes were observed in the recovery from resistance-based training or game play with the addition of supplementation (chapter 6).

In conclusion, this thesis has observed an attenuation of the strength deficit caused by EIMD with n-3 PUFA supplementation in untrained males, but no positive impact of supplementation on performance or recovery within the context of cycling or rugby union. There was no additive effect of supplementation when combined with resistance training on improvements in strength and power. However, there was no negative impact of supplementation on any performance indicators suggesting that n-3 PUFAs do not blunt training adaptations.

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List of Abbreviations

AA	Arachidonic Acid
ALA	Alpha Linoleic Acid
BMI	Body mass index
CBA	Cytometric Bead Array
CK	Creatine kinase
CMJ	Countermovement Jump
COX	Cyclooxygenase
CRP	C-Reactive Protein
Cu	Curcumin
DHA	Docosahexaenoic Acid
DOMS	Delayed onset muscular soreness
DPA	Docosapentaenoic Acid
EDTA	Ethylene diamine tetra-acetic acid
EIMD	Exercise induced muscle damage
ELISA	Enzyme-linked immunosorbent assays
EPA	Eicosapentaenoic Acid
FAME	Fatty acid methyl esters
GCMS	Gas Chromatography Mass Spectroscopy
HR	Heart rate
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
IL-1 ra	IL-1 receptor antagonist

IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-10	Interleukin 10
IPAQ	International Physical Activity Questionnaire
LA	Linoleic acid
LDH	Lactate dehydrogenase
Mb	Myoglobin
MD	Muscle damage
MPS	Muscle protein synthesis
mRNA	messanger Ribonucleic Acid
MVC	Maximal Voluntary Contraction
MVCC	Maximal Voluntary Concentric Contraction
MVEC	Maximal Voluntary Eccentric Contraction
MVIC	Maximal Voluntary Isometric Contraction
n-3 PUFA	Omega-3 polyunsaturated fatty acid
n-6 PUFA	Omega-6 polyunsaturated fatty acid
NSAID	Non-steroidal anti-inflammatory drugs
PBMC	Peripheral Blood Mononuclear Cell
PT	Performance test
PUFA	Polyunsaturated Fatty Acid
RER	Respiratory Exchange Ratio
ROM	Range of Motion
RPE	Rating of Perceived Exertion

RPM	Revolutions per minute
SJ	Squat Jump
SOR	Muscle soreness
sTn1	Skeletal Troponin 1
TNFα	Tumour Necrosis Factor alpha
VAS	Visual Analogue Scale
V_{ESTPD}	Minute ventilation standardised temperature and pressure of a dry gas
VCO_2	Carbon dioxide production
VO_2	Oxygen uptake
Wmax	Maximal work rate
1RM	One repetition maximum

Chapter 1: Introduction

1.1 General Introduction

Over the last 50 years, research has investigated strategies for athletes to prepare for competition, promote performance during competition and recover from competition (Close *et al.*, 2016). Two factors that hinder athletes recovery from both competition and training are exercise induced muscle damage (EIMD) and inflammation. EIMD and inflammation are regularly experienced by athletes as they generally train or perform on most days of the week. As EIMD and inflammation are associated with losses in muscle function and increases in muscle soreness, any subsequent performance may be impaired (Cheung, Hume and Maxwell, 2003; Howatson and van Someren, 2008; Pereira *et al.*, 2018).

There are two physiological responses that characterise EIMD, firstly the mechanical challenge caused by exercise results in myofibrillar disruption, secondly an inflammatory response occurs that results in cell infiltration into the damaged tissues (Smith *et al.*, 2008; Deyhle *et al.*, 2016; Harty *et al.*, 2019) to initiate subsequent tissue repair and remodelling (Peake *et al.*, 2017; Owens *et al.*, 2019). A temporal association exists between the magnitude of the loss of muscle strength immediately post exercise and the length of time it takes to fully restore muscle strength (Peake *et al.*, 2017). Strength is generally recovered within 2 days when muscle strength decreases by ≤ 20% immediately post-exercise (Malm *et al.*, 2004; Crameri *et al.*, 2007), whereas, recovery can still be impaired 7 days post-exercise when muscle strength decreases by ~50% (Paulsen *et al.*, 2010). The recovery process can be impaired and prolonged by the inflammatory response as an exaggerated production of pro-inflammatory cytokines can occur, causing more damage than the exercise itself.

A number of strategies have been developed to attenuate EIMD and the inflammatory response to exercise including stretching, massage, cryotherapy, ultrasound, light exercise, rest and anti-inflammatory medication (Cheung, Hume and Maxwell, 2003). Over the past three decades several studies have investigated nutritional or pharmacological interventions in order to reduce the negative impact of

EIMD (Sousa, Teixeira and Soares, 2014; Heaton *et al.*, 2017; Maughan *et al.*, 2018; Owens *et al.*, 2019; Bongiovanni *et al.*, 2020). There has been a particular focus on oral supplements that exhibit anti-inflammatory and/or antioxidant effects (Sousa, Teixeira and Soares, 2014; Merry and Ristow, 2016; Delecroix *et al.*, 2017) and supplements that facilitate muscle repair and regeneration (Harty *et al.*, 2019; Owens *et al.*, 2019) Non-steroidal anti-inflammatory drugs (NSAIDS) are commonly used by athletes and have been shown to decrease muscle soreness and pain (Warden, 2010). However evidence suggests NSAIDS can blunt muscle protein synthesis (Trappe *et al.*, 2002) and attenuate strength and muscle hypertrophic adaptations from resistance training (Schoenfeld, 2012; Lilja *et al.*, 2018). Markworth *et al.* (2013) suggested that the use of NSAIDs to enhance post resistance exercise recovery may be at the expense of delayed and/or diminished natural resolution of the exercise induced inflammatory response. Furthermore, regular use of NSAIDs is associated with gut ulcers and gastrointestinal bleeding (Marlicz *et al.*, 2014). Therefore, it is not advised to habitually consume NSAIDs, as it could reduce the long-term adaptations to training and cause gastrointestinal problems.

An alternative, non-pharmacological intervention is to consume fish oil, more specifically omega-3 polyunsaturated fatty acid (n-3 PUFA), which has anti-inflammatory properties (Calder, 2015). Anecdotally it is known that many athletes consume n-3 PUFA supplements regularly and case studies have supported this (Milsom *et al.*, 2014; Rosimus, 2018). The main goal for athletes taking these supplements is to improve performance either directly, or indirectly by reducing the time it takes them to recover from exercise. While a number of studies have assessed the effects of n-3 PUFA supplementation on muscle damage (Gray *et al.*, 2014), inflammation (Toft *et al.*, 2000; Tartibian, Maleki and Abbasi, 2011) and metabolism (Hingley *et al.*, 2017; Jannas-Vela *et al.*, 2020) during exercise, there are a limited number of studies directly reporting the effects of n-3 PUFA supplements on exercise performance (Mickleborough, 2013; Gravina *et al.*, 2017; Philpott *et al.*, 2018).

1.2 Thesis Summary

Chapter 2 includes an overview of what n-3 PUFAs are, how they are incorporated into human tissue and how they are measured. There is a review of the literature that has investigated the effects of n-3 PUFA supplementation on both endurance and resistance exercise-based performance and the associated muscle damage and inflammation.

Chapter 3 provides the general methods used throughout the experimental chapters within the thesis. Detail is provided on participant recruitment, pre-trial measures, collection and preparation of blood, analysis of blood and statistical analysis.

Chapter 4 presents the results of an experimental study to determine the effects of 4 weeks n-3 PUFA supplementation on cycling time trial performance and the recovery in the following 24 hours post-exercise. The study aimed to improve on some of the limitations of previous research by recruiting trained cyclists and employing a cross-over experimental design involving the same participants completing both trials, pre- and post-intervention, with a washout period. Importantly, the bioavailability of the n-3 PUFA was measured to ensure the n-3 PUFA supplements were being incorporated into whole blood and peripheral blood mononuclear cells (PBMCs).

Chapter 5 presents the results from a two-part study investigating the effects of n-3 PUFA supplementation on resting performance measures of strength and power and whether n-3 PUFA attenuated the eccentric exercise induced muscle damage in untrained males. The second part of the study investigated the same parameters but this time following 8 weeks of eccentric training in addition to supplementation.

Chapter 6 aimed to investigate the effects of n-3 PUFA supplementation in a sport specific setting; rugby union and evaluate performance and recovery from rugby game play and rugby specific gym sessions. The participants in this chapter were elite athletes.

Chapter 7 summarises the results of the thesis, bringing together the main strands of exercise performance, exercise induced muscle damage and inflammation. The

findings are discussed in the context of previous research and future directions, with practical recommendations being provided for athletes.

Chapter 2: Literature Review

2.1 Introduction

The overarching aim of this thesis, formulated as a result of the literature presented in this review, is to investigate whether exercise performance and recovery from exercise in healthy males can be improved by n-3 PUFA supplementation. Firstly, muscle damage and inflammation resulting from exercise will be discussed in the context of sports performance. Secondly, n-3 PUFAs will be introduced and an explanation of how n-3 PUFA supplementation results in the incorporation of n-3 PUFA into tissues. A brief background of the use of n-3 PUFAs by athletes will be presented and how n-3 PUFA supplementation may alleviate inflammation and muscle damage resulting in improved performance. Finally, evidence from the literature relating to n-3 PUFA supplementation and both endurance and resistance-based exercise performance in young healthy individuals will be presented, aiming to provide a rationale for n-3 PUFA supplementation for athletes.

2.2 Exercise induced muscle damage and inflammation

2.2.1 Remodelling of skeletal muscle in athletes

Athletes train on most days of the week, often twice per day, and training can cause exercise induced muscle damage (EIMD) with the greatest damage occurring following unaccustomed and eccentric exercise (Gibala *et al.*, 2000; Deyhle *et al.*, 2016; Harty *et al.*, 2019). However, it is crucial that athletes perform unaccustomed exercise as this contributes to the overload principle of training (McNicol *et al.*, 2009) and eccentric exercise increases muscle hypertrophy most effectively (Schoenfeld *et al.*, 2017). EIMD is a temporary decrease in muscle force production, a rise in passive tension, increased muscle soreness and swelling and an increase in circulating intramuscular proteins following exercise (Howatson and van Someren, 2008). The most damage occurs with the greatest number of eccentric contractions (Nosaka *et al.*, 2003), at the longest muscle length (Nosaka and Sakamoto, 2001; Fochi *et al.*, 2016) and when contractions are performed maximally (Nosaka and Newton, 2002).

Historically, inflammation has been viewed as detrimental for recovery from exercise, however, it is now thought that providing inflammatory processes are regulated, they are integral to muscle repair and regeneration (Peake *et al.*, 2017). It is well established that exercise of sufficient intensity and duration can induce an acute inflammatory response that facilitates muscle recovery and enhances adaptive immunity (Gleeson *et al.*, 2011; Peake *et al.*, 2017). Therefore, it is an important requirement of an athletes training to induce inflammation so that regeneration and growth of muscle can occur, however, this must be regulated to ensure athletes subsequent performance is not impaired.

2.2.2 Exercise-Induced Muscle Damage (EIMD)

It has been proposed that EIMD occurs in a biphasic manner with the first phase, typically referred to as the primary event, involving mechanical and metabolic damage induced during the exercise and the second phase; secondary muscle damage, encompassing the ensuing biochemical changes in the hours and days following the primary event (Howatson and van Someren, 2008; Smith *et al.*, 2008). Illustrating these two events is a bimodal strength reduction after eccentric exercise, with the first (larger) force loss occurring immediately post-exercise, then a period of force recovery, followed by a second period of force loss occurring several hours after exercise (MacIntyre *et al.*, 1996). Immediately after eccentric exercise the number of muscle fibres showing disruption is increased (Gibala *et al.*, 1995), with the disruption of Z-disks and sarcomeres peaking between 1 to 3 days post exercise (Newham *et al.*, 1983; Crameri *et al.*, 2007).

The primary event has been subdivided into two possible pathways: mechanical and metabolic (Ebbeling and Clarkson, 1989; Armstrong, Warren and Warren, 1991). However, more recent evidence has suggested metabolic factors seem to be unlikely candidates as a basis for EIMD with the literature reporting these metabolic factors using exercise modalities that involve eccentric contractions meaning it is more likely that the mechanical stress is the main cause of EIMD (Howatson and van Someren, 2008). One theory behind the mechanical pathway was identified by (Morgan, 1990) as the 'popping sarcomere hypothesis', which proposes that EIMD is caused by direct mechanical stretch of sarcomeres. Lengthening of sarcomeres is non-uniform

under eccentric conditions, resulting in some myofilaments being stretched and thick and thin filament being unable to overlap (Hyldahl and Hubal, 2014). Eccentric actions are more damaging than isometric or concentric contractions and it is believed that this is due to the increased tension as the muscle lengthens, this results in a higher load spread across the same number of muscle fibres (Clarkson and Hubal, 2002). Within the overloaded sarcolemma and t-tubules structures, stretch activated channels are opened, membrane disruption and excitation-contraction coupling dysfunction occurs (Hyldahl and Hubal, 2014).

The secondary muscle damage phase appears to be initiated by a disruption of the intracellular Ca^{2+} homeostasis resulting from sarcolemma damage and opening of stretch activated channels (Armstrong, Warren and Warren, 1991; Hyldahl and Hubal, 2014). This triggers a cascade of events that further damage skeletal muscle cells by initiating alteration to the cytoskeleton, sarcoplasmic reticulum, mitochondria and myofilaments (Gissel and Clausen, 2001). Rises in serum Creatine Kinase (CK), lactate dehydrogenase (LDH) and myoglobin (Mb) occur when the sarcolemma and Z-disks are damaged and the intramuscular proteins are leaked out of the damaged cells (Baird *et al.*, 2012; Koch, Pereira and Machado, 2014).

An alternative theory proposed by Warren *et al.* (2002) suggested that early strength loss following exercise is due to failure of the excitation-contraction coupling process, rather than damage to the contractile elements proposed in the popping sarcomere hypothesis and that there is no secondary loss of strength. However, it is important to note that most of the data to support this hypothesis was derived from animal models of contraction-induced muscle injury and it is important to be cautious when generalising these findings to humans.

In human skeletal muscle, there appears to be a continuum of muscle fibre damage following eccentric actions, with minor perturbations resulting in cell signalling-mediated adaptive responses, through to more intensified eccentric stimuli increasing the likelihood of a more severe inflammatory response (Hyldahl and Hubal, 2014). At the extreme end of the continuum, supposed adaptive responses may become maladaptive and severe muscle strain may occur, characterised by necrosis, incomplete healing and fibrotic scar tissue formation (Butterfield, 2010). The magnitude of the response to the eccentric action is likely to be further impacted

by the novelty of the stimulus, the size of the muscle group exercised, the mode of muscle activation (i.e. voluntary or stimulated), genetic variability (Hyldahl and Hubal, 2014), velocity (Chapman *et al.*, 2006), length (Child, Saxton and Donnelly, 1998; Fochi *et al.*, 2016) and force of contraction (Nosaka, Newton and Sacco, 2002) (Figure 1).
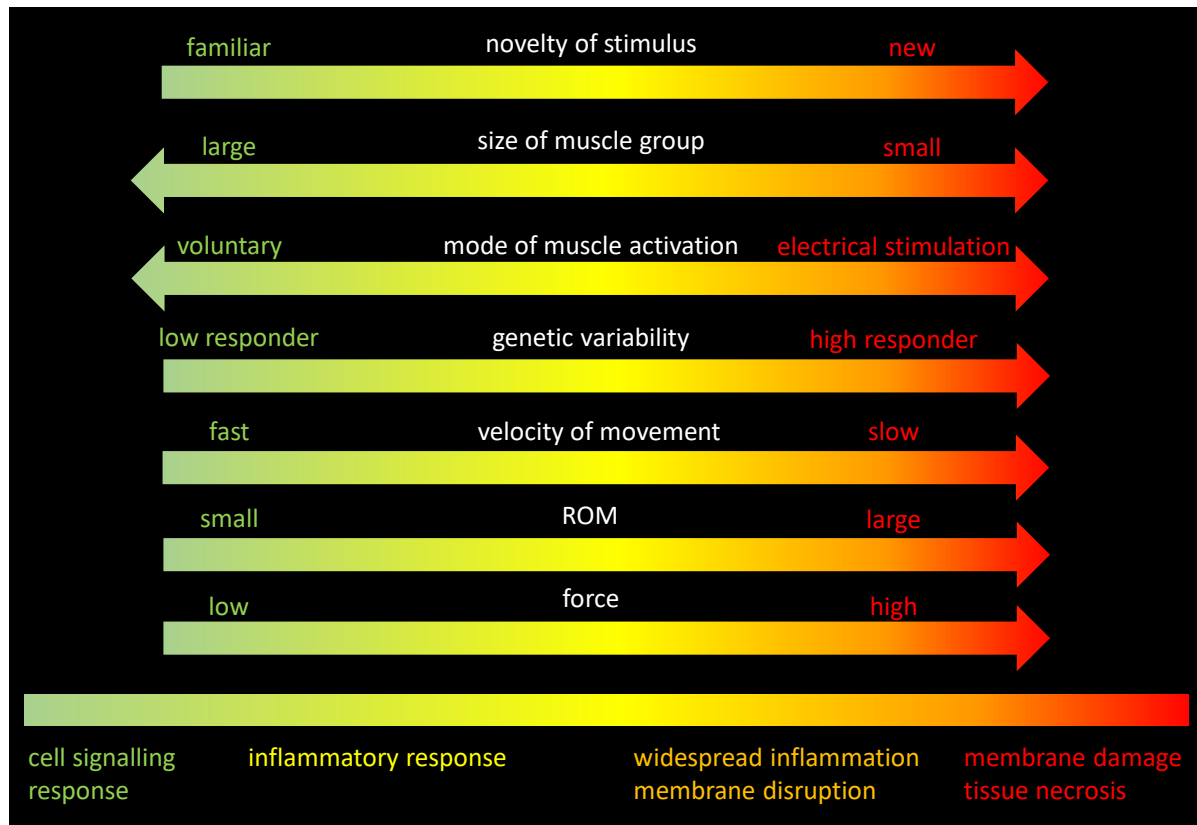
Figure 1 – Proposed continuum of skeletal muscle fibre damage following eccentric contractions. Figure adapted from Hyldahl and Hubal, (2014). Lines with bidirectional arrows indicate that the response under each condition can occur anywhere along the continuum, e.g., electrical stimulation results in a greater inflammatory response and more severe myofibrillar disruption than voluntary contractions but may also result in a lesser injury under circumstances of low intensity/volume/novelty etc.

EIMD causes the release of proteins from the muscles into the systemic circulatory system due to increased sarcolemma permeability. These proteins can be measured to serve as a proxy for evaluating EIMD. The systemic concentration of CK is the most frequently measured intramuscular protein to provide a marker of muscle damage (Ebbeling and Clarkson, 1989), with Mb and LDH also regularly reported in the literature. The reference intervals for CK at rest, when there is no EIMD, in male

athletes are 82 to 1083 U/L and 47 to 513 U/L in female athletes (Mougios, 2007). However, CK levels can rise substantially in response to muscle damaging exercise (1000 to 5000 U/L) and this response is highly variable in terms of time-course and magnitude (Ebbeling and Clarkson, 1989; Clarkson and Hubal, 2002). The perception of soreness after eccentric exercise is inconsistent with serum CK levels (Newham *et al.*, 1988). Furthermore the level of CK activity does not appear to correlate well with decrements in muscle function (Warren, Lowe and Armstrong, 1999). Although it could be determined that measuring CK does not therefore give a useful measure of EIMD, Lee and Clarkson, (2003) observed that subjects with lower increases in plasma CK following eccentric exercise returned to baseline strength levels more rapidly. The lack of consensus could be due to the different timings of CK measurement or varying muscle damaging protocols.

Mb is an oxygen-binding heme protein of striated muscles that is rapidly released after muscle damage (Sorichter, Puschendorf and Mair, 1999). Mb increases in the systemic circulation following eccentric exercise are common. Eccentric exercise studies have identified peaks of Mb of 135 ng/ml at 6 hours after downhill running (Byrnes *et al.*, 1985), 7857 μg/l 48 hours after eccentric contractions of the hamstrings and quadriceps (Croisier *et al.*, 1999), ~650 ng/ml 72 hours after eccentric calf raises (Kanda *et al.*, 2013) and 525 ng/ml 96 hours after eccentric contractions of the biceps (Lee and Clarkson, 2003; Chen *et al.*, 2011). Assays used to determine Mb concentrations cannot distinguish between Mb release from the heart or skeletal muscle (Sorichter, Puschendorf and Mair, 1999), which is why levels of Mb should not be used as a marker of muscle damage in isolation.

LDH is measured in the serum with reported increases occurring 6 to 12 hours post muscle damaging exercise, returning to baseline within 8 to 14 days (Sorichter, Puschendorf and Mair, 1999). Peaks in LDH of 228 U/L were observed 24 h post marathon (Withee *et al.*, 2017) and peaks of 455 U/L were shown 24 h post resistance exercise session (Rodrigues *et al.*, 2010). Significant LDH activities are found in almost every tissue, especially skeletal muscle, liver, heart, kidneys, brain, lungs and erythrocytes (Sorichter, Puschendorf and Mair, 1999), making it difficult to distinguish if the damage has actually taken place in the muscle. Again, this is why it

is important to measure a range of intramuscular proteins to ascertain whether EIMD has taken place.

The main symptoms that an athlete may experience in terms of EIMD are elevated muscle soreness and a loss of muscle force (Newham, Jones and Clarkson, 1987). The soreness can reduce the capacity of the muscle to efficiently absorb shock at impact, which places the joints and tissue structures under unaccustomed loading (Smith, 1992). Soreness increases within the first 24 hours post exercise and peaks between 24 and 72 hours (Close *et al.*, 2006), disappearing by 5 to 7 days post-exercise (Cheung, Hume and Maxwell, 2003). A reduction in force output by a damaged part of the muscle may lead to compensatory recruitment from an undamaged part, or from another muscle, leading to unaccustomed stress on compensating muscle groups, increasing the risk of injury (Smith, 1992). If a training session is set at a specific percentage of one repetition maximum (1RM) then individuals may have to work harder to achieve the same percentage of 1RM due to a reduction in strength and power (Smith, 1992) and this could increase the risk of injury. There is a temporal association with the magnitude of the loss of muscle strength immediately post exercise and the length of time it takes to fully restore muscle strength (Peake *et al.*, 2017). Strength is generally recovered within 2 days when muscle strength decreases by ≤ 20% immediately post-exercise (Malm *et al.*, 2004; Crameri *et al.*, 2007) but recovery can still be impaired 7 days post-exercise when muscle strength decreases by ~50% (Paulsen *et al.*, 2010).

An athletes principle goal during recovery is to restore physical performance and this is likely why performance itself is the preferred marker of EIMD (Byrne, Twist and Eston, 2004). Reductions in maximal voluntary contraction (MVC) give a reliable estimate of the EIMD (Warren, Lowe and Armstrong, 1999), however, the magnitude of change can vary widely depending on the nature of the exercise task and the training status of the individual. Nevertheless, using MVCs (normally performed on an isokinetic dynamometer) as a marker of EIMD is limited as the actions are typically performed by isolated muscle groups and at low velocities; therefore they are unlikely to provide a true reflection of the loss of function of actual sporting activity (i.e. sprinting, jumping) (Byrne, Twist and Eston, 2004). To overcome this, sport specific movements should also be assessed such as jump tests and 1RM of

whole-body resistance-based exercises such as squats. Few studies have used sports specific tests as markers of EIMD, making it difficult to assess the extent of the EIMD on sports performance. Whilst a cluster analysis demonstrated that the indirect markers of EIMD; MVC, soreness, CK activity, range of motion (ROM) and limb swelling, were consistent with the clusters that they had determined (i.e. small changes for the low responders, larger changes for medium responders and large changes for the highest responders) (Damas *et al.*, 2016). For this thesis both sports specific tasks and the generic markers, identified above, will be used to assess the efficacy of n-3 PUFA on the effects of EIMD.

2.2.3 Exercise-Induced Inflammation

With high training volumes, repeated post-exercise inflammation may contribute towards post-exercise muscle soreness and impaired physical performance. Delayed recovery of muscle function measured 1 – 4 days following 300 unilateral, maximal eccentric contractions of the quadriceps was associated with local accumulation of leukocytes. However, there was a negative correlation between leukocyte accumulation and muscle soreness (Paulsen *et al.*, 2010). Accumulated leukocytes are mobilised into the circulation following EIMD (Peake, Nosaka and Suzuki, 2005). Neutrophils and macrophages produce pro-inflammatory cytokines and these are expressed in skeletal muscle up to five days after exercise (Peake, Nosaka and Suzuki, 2005).

A set of 'common' or 'general' markers of inflammation that can be measured in circulating blood have been identified from a wide range of inflammatory diseases, and are recommended for use when evaluating inflammation in human nutritional studies (Calder *et al.*, 2013). Techniques including counts of leukocytes taken from whole blood, serum counts of inflammatory proteins (such as cytokines) or directly from muscle biopsy tissue are used to measure exercise-induced inflammation.

The post-exercise inflammatory response is often characterised by an increase in the concentration of cytokines within the bloodstream (Northoff and Berg, 1991). Cytokines are the most commonly measured biomarker of exercise-induced

inflammation and can be easily analysed using commercially available enzyme-linked immunosorbent assays (ELISA). Interleukin-6 (IL-6) is the major cytokine released by contracting skeletal myocytes during exercise (Ostrowski *et al.*, 1999; Hiscock *et al.*, 2004; Fischer, 2006) acting as a metabolic sensor as well as a recruiter of other inflammatory cells (Hiscock *et al.*, 2004). Circulating levels of IL-6 are increased in larger amounts than any other cytokine in relation to exercise (Pedersen, 2000) and it is likely that this is why IL-6 levels are most often measured in exercise based studies. In relation to concentric exercise, the increase in IL-6 is tightly related to the duration of exercise (Ostrowski *et al.*, 1998; Goussetis *et al.*, 2009) and levels decline to reach pre-exercise levels within a few hours (Pedersen, Steensberg and Schjerling, 2001). In contrast, eccentric exercise induces modest increases in plasma IL-6 and peaks sometime after cessation of exercise, remaining elevated for several days (Phillips *et al.*, 2003). Prolonged exercise (> 2.5 hours) can increase plasma levels of IL-6 by 100-fold, with more modest increases occurring with shorter duration exercise (Fischer, 2006). Increases range from 0.4 pg·ml^{-1} following 45 min cycling at 50% VO$_2$ max (Shojaei, Farajov and Jafari, 2011) to 70 pg·ml^{-1} post marathon (Ostrowski *et al.*, 1999). Tumour necrosis factor alpha (TNF-α) is also increased post-exercise (Deminice *et al.*, 2013; Mickleborough *et al.*, 2015; Ulven *et al.*, 2015) and it is known that pro-inflammatory cytokines such as TNF-α inhibit muscle protein synthesis (MPS) by decreasing mRNA translation initiation (Lang *et al.*, 2002), suggesting inflammation may attenuate the anabolic response to resistance exercise.

2.3 n-3 PUFA supplementation and incorporation

Many important fatty acids can be synthesised within the body; however, some must be included in the diet and they are described as essential fatty acids. Humans are unable to synthesise the n-6 PUFA linoleic acid (LA) and the n-3 PUFA alpha-linolenic acid (ALA), instead, we rely on these essential fatty acids to have entered the food chain from plants. Linoleic acid is the principal PUFA in most Western diets (Innes and Calder, 2018) and is found in vegetable oils including sunflower, corn and soybean oil. Conversely, typical intakes of ALA among Western adults is low, approximately 0.5 to 2g/d. ALA can be found in plant sources such as linseed oil,

rapeseed oil, soya oil, mustard oil and walnuts. LA is typically consumed in 5- to 20-fold greater amounts than ALA (Calder and Yaqoob, 2009).

Dietary consumption of LA and ALA is important because they are precursors to longer highly unsaturated and biologically relevant PUFAs. Biochemical pathways exist to convert LA to Arachidonic acid (AA) and ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in a series of elongation and desaturation steps (Figure 2). The substrates LA and ALA compete within the same pathway for the enzymes Δ6-desaturase and Δ5-desaturase, with ALA suppressing n-6 PUFA metabolism (Calder and Yaqoob, 2009; Innes and Calder, 2018). When incorporated into the membrane phospholipids, n-3 and n-6 PUFAs act as substrates for the synthesis of lipid derived mediators of inflammation, termed eicosanoids (Calder, 2015). Eicosanoids are inflammatory mediators and precursors for prostaglandins, thromboxanes and leukotrienes. They are considered hormone-like substances because they are produced when stimulated, rapidly utilised and metabolised and not stored in cells. Eicosanoids derived from n-3 PUFAs have less biological potency for inducing cellular responses than those derived from n-6 PUFAs, resulting in decreased inflammatory responses (Alexander, 1998). The endogenous conversion of ALA to EPA and DHA is limited in humans: between 0.2% and 8% of ALA is converted to EPA and 0% to 4% of ALA to DHA (Mozaffarian and Wu, 2011). There is also a limited storage of n-3 PUFAs in the adipose tissue suggesting a continued dietary supply is required (Arterburn, Hall and Oken, 2006). The main dietary sources of EPA and DHA come from oily fish (e.g. mackerel, salmon, pilchards and sardines) and fish oil supplements.
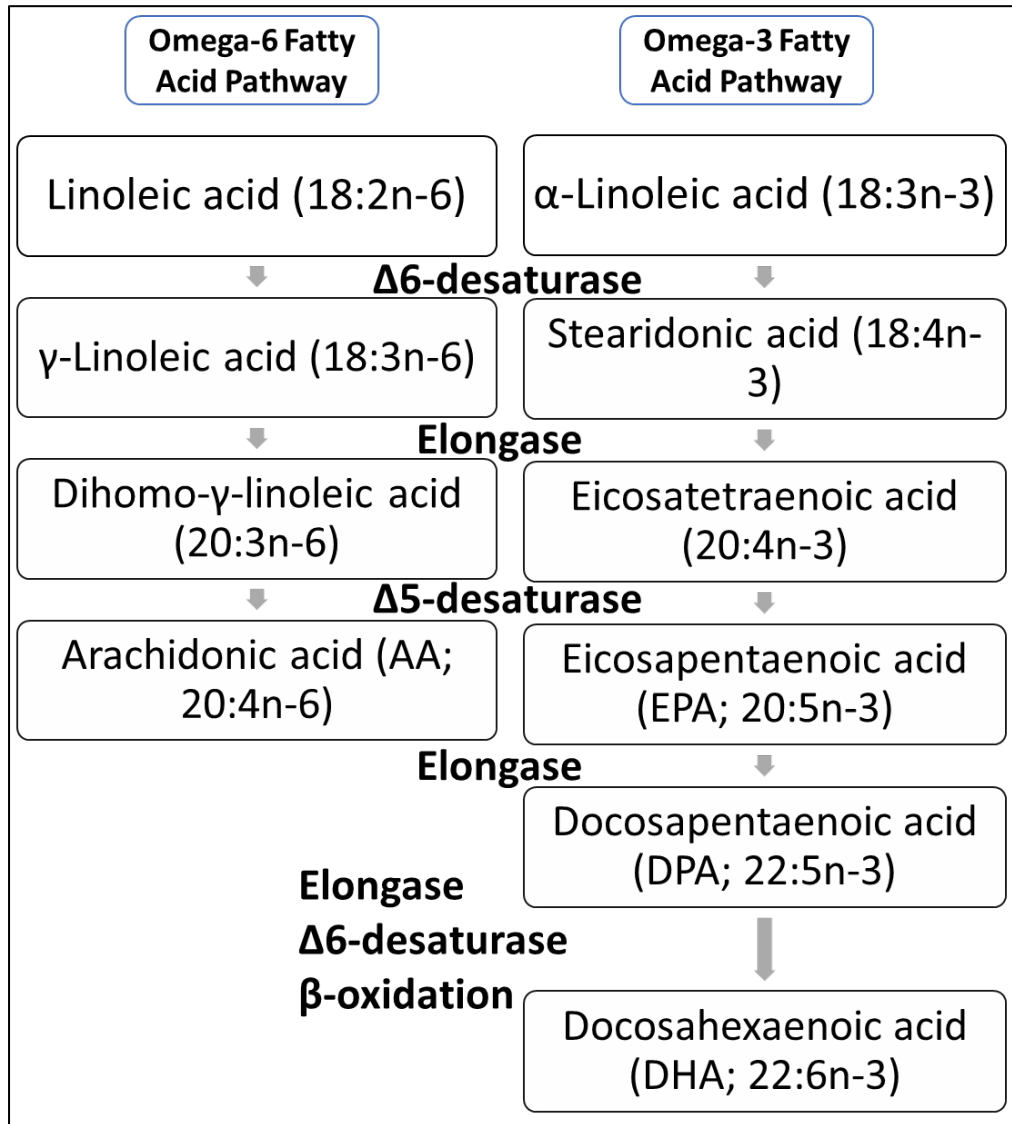
Figure 2 – Pathways of biosynthesis n-6 and n-3 PUFAs. Figure adapted from Calder, (2015).

The biophysical properties of the cell membrane are influenced by the composition of its phospholipids. Saturated fatty acids provide a rigid state, whereas *cis* double bonds cause the membrane to be more fluid, which affects many features of cell membranes and functions (Nikolaidis and Mougios, 2004). It is generally accepted that for n-3 PUFAs to influence processes within the human body they must be incorporated into the phospholipids of the cell membranes. Cell function can be affected in a variety of ways by increasing cell and tissue n-3 PUFA content. The physical properties of the cell membranes such as fluidity and raft structure can influence the activity of membrane proteins including receptors, transporters, ion

channels, and signalling enzymes (Calder and Yaqoob, 2009). Cell signalling pathways are affected, through modifying the expression, activity, or avidity of membrane receptors or modifying intracellular signal transduction mechanisms, resulting in altered transcription factor activation and modified gene expression (Calder, 2012b).

The 'Omega-3 Index' was originally developed to provide an independent graded risk factor for death from coronary heart disease (Harris and Von Schacky, 2004), but it has been used more recently to evaluate the levels of n-3 PUFA in tissue cell membranes (Lembke *et al.*, 2014). The omega-3 index expresses the combined amount of EPA and DHA (in weight %) present in the red blood cell membrane lipid fraction (von Schacky, 2010), with an index of ≤ 4 considered at higher cardiovascular risk, 4 – 8 characterised as medium risk and > 8 are at low risk (Harris and Von Schacky, 2004). Whilst some of the n-3 PUFA supplementation literature has used this method to quantify incorporation of n-3 PUFA into the tissues, a large proportion of the literature has simply expressed the n-3 PUFAs as a percentage of total fatty acids identified, as presented in this thesis (section 3.4.3).

There is a dose response relationship between n-3 PUFA supplement intake and the incorporation of EPA and DHA into plasma, cells and tissues, although the incorporation into different tissues occurs at different rates and to different extents (Calder and Yaqoob, 2009). The mechanisms that this thesis focuses on is provided in more detail in section 2.6, but as the focus will be on inflammation, the incorporation of n-3 PUFA into immune cells will now be presented, with the caveat that despite the numerous studies conducted on n-3 PUFA and the immune system, only a minority report the fatty acid composition of the cells being investigated (Yaqoob *et al.*, 2000). Similar to other tissues, such as adipose tissue and muscle, the EPA and DHA content of the immune cell membranes can be manipulated by consumption of these fatty acids (Galli and Calder, 2009). Studies using a range of intakes from 1 to 4 g/day have found incorporation of EPA and DHA into neutrophils (Healy *et al.*, 2000) and EPA into peripheral blood mononuclear cells (PBMCs) (Rees *et al.*, 2006) occurred in a linear dose response manner. There was a fourfold increase in the proportion of EPA in PBMCs which plateaued after 4 weeks in healthy adults supplementing with 2.1 g/d EPA and 1.1 g/d DHA (Yaqoob *et al.*,

2000). Whilst Toft *et al.* (2000) found an increased incorporation of n-3 PUFA and decreased AA in the PBMCs of male runners supplemented with 3.6 g/d of n-3 PUFA for 6 weeks. Thus, the general consensus is that EPA and DHA are incorporated into immune cells when their intake is increased (Faber *et al.*, 2011; Browning *et al.*, 2014).

A major concern for athletes supplementing with n-3 PUFAs and studies where the supplements are 'off the shelf' are that the truth of label claims for encapsulated oils is not regulated, therefore the amount of EPA and DHA being consumed cannot be determined by the consumer or investigator. Future studies should analyse the EPA and DHA content of the capsules in the same way that the incorporation of the n-3 PUFAs are determined within the body. Moreover, even if the n-3 PUFA intake from the capsules is known, humans are metabolically unique, with idiosyncrasies in digestion, absorption, tissue distribution and cellular metabolism. Accordingly, n-3 PUFA levels can be different within individuals consuming the same dosage of n-3 PUFAs. Harris & Von Schacky (2004) illustrate the varied response with an Omega-3 Index between 3 to 7% in the placebo group, 4 to 10% in the 500 mg/day group and from 5 to 13% in the 1 g/day, following supplementation with the respective doses of EPA and DHA for 5 months.

2.4 Use of n-3 PUFA supplements by athletes

Anecdotally it is known that many athletes consume n-3 PUFA supplements on a regular basis and case studies have supported this (Milsom *et al.*, 2014; Rosimus, 2018). Ritz *et al.*, (2020) identified that 15% of National Collegiate Athletic Association Division 1 athletes (sports included baseball, basketball, cross country, American football, football, golf, gymnastics, softball, swimming, track and field and volleyball) use n-3 PUFA supplements. In fact, n-3 PUFAs are one of the most popular dietary supplements used by elite athletes (Shaw, Slater and Burke, 2016). A review focusing on nutritional strategies in team sports suggested that n-3 PUFA supplements may be efficacious in improving immune status over the course of a season (Heaton *et al.*, 2017). Furthermore, a recent systematic review on the benefits of n-3 PUFA supplements in athletes revealed positive effects on cognition, cardiovascular dynamics in cyclists, and muscle recovery (Lewis *et al.*, 2020).

However, the recent consensus statement by the International Olympic Committee on dietary supplements and the high performance athlete states there is limited support for n-3 PUFAs in blunting inflammation and functional changes after eccentric exercise induced muscle damage (Maughan *et al.*, 2018). There is a clear lack of agreement evident within the literature, nonetheless athletes continue to take n-3 PUFA supplements due to the possibility that they may enhance their performance.

2.5 Why athletes supplement with n-3 PUFAs

The health benefits of n-3 PUFAs were originally discovered in epidemiological studies of the Greenland Eskimos who consumed a high seafood diet and had low rates of coronary heart disease, asthma, type 1 diabetes mellitus and multiple sclerosis (Kromann and Green, 1980), despite consuming large amounts of fat. Stark et al. (2002) quantified this high seafood diet and found Canadian women had 54% lower levels of n-3 PUFA than Greenland Inuit women. More recently further evidence regarding the beneficial effect of n-3 PUFAs on human health have been investigated. They have been recognised to exert positive effects on atherosclerosis (Calder, 2012b) cardiovascular disease (Lemaitre *et al.*, 2003), Rheumatoid arthritis (Goldberg and Katz, 2007), asthma (Mickleborough, Ionescu and Rundell, 2004; Galli and Calder, 2009), brain function (Bourre, 2004), and the prevention of acute and chronic inflammation (Calder, 2015). These conditions/diseases/phenotypes are mostly inflammatory-related and appear to be benefitting from n-3 PUFA supplementation.

Athletes experience muscle fatigue, soreness, muscle structural damage, free radical damage, neutrophilia and muscle swelling as a result of intense exercise training (Armstrong, Warren and Warren, 1991), see section 2.2 for more detail. Despite the transient nature of these symptoms, they can be debilitative particularly for athletes involved in competitions involving multiple events in a short time frame, or athletes that have multiple training sessions per day. EIMD and exercise induced inflammation can hinder the ability to perform subsequent bouts of exercise and therefore reduce adherence to an exercise training programme. Given the beneficial effect of n-3 PUFA on inflammation shown in clinical populations, it is plausible that

athletes may also benefit from n-3 PUFA supplementation to reduce exercise-induced inflammation. This would enable quality training sessions to be completed that are not hindered by soreness or pain caused by the exercise-induced inflammation and potentially result in improved performance.

Athletes that supplement with n-3 PUFAs can alter the composition of their cell membranes, increasing the amount of EPA and DHA in the cell membrane phospholipids and decreasing the amount of AA as they compete within the same pathway for the enzymes Δ6-desaturase and Δ5-desaturase (Figure 2). EPA and DHA are substrates for production of anti-inflammatory and inflammation resolving mediators called resolvins, protectins and maresins. Healthy volunteers supplemented with 2.4g n-3 PUFA for 3 weeks had increased levels of resolvins in the blood (Mas *et al.*, 2012). Increasing the contents of EPA and DHA in the cell membranes alters the production of resolvins, protectins and maresins (Calder, 2012a). Resolvins, protectins and maresins are agonists of resolution that do not evoke unwanted side effects such as immunosuppression (Serhan, 2017). Increasing these lipid mediators within athletes may help to resolve the high levels of inflammation associated with intense exercise and allow athletes to recover quicker.

2.6 Mechanisms for improved performance with n-3 PUFA supplementation

Cross-sectional data suggests that individuals with higher levels of circulating n-3 PUFA have better physical function than individuals with lower levels of n-3 PUFA (Fougère *et al.*, 2018). Potential mechanisms for improving physical performance by increasing circulating n-3 PUFA include facilitating transport of red blood cells across the capillary bed (Bruckner *et al.*, 1987), increasing muscle protein synthesis (MPS) (Smith *et al.*, 2011a), reducing muscular dysfunction (Ochi, Tsuchiya and Yanagimoto, 2017) and reducing exercise induced inflammation (Marques *et al.*, 2015). Due to the broad spectrum of these mechanisms, this thesis will focus on the latter two of these potential mechanisms; reducing muscle dysfunction and exercise

induced inflammation, although it is understood that there are often interactions between all these mechanisms.

2.6.1 Reducing exercise-induced inflammation and muscle dysfunction

The supplementation of the diet with fatty acids, particularly n-3 PUFAs, has been demonstrated to promote an anti-inflammatory phenotype and reduce the concentration of inflammatory cytokines (Vedin *et al.*, 2008, 2012; Calder, 2015). Inflammation post exercise may be modulated by n-3 PUFA supplementation (Simopoulos, 2007; Da Boit, Hunter and Gray, 2016). Endres *et al.*, (1989) found that the synthesis of Interleukin 1 beta (IL-1β), Interleukin 1 alpha (IL-1α) and TNF in PBMCs was suppressed by 6 weeks of 18 g/d of fish oil concentrate and this suppression was still present 10 weeks post supplementation. Furthermore, n-3 PUFA supplementation reduced post exercise levels of C-Reactive Protein (CRP) (Phillips *et al.*, 2003; Bloomer *et al.*, 2009; Lembke *et al.*, 2014), TNF-α, (Bloomer *et al.*, 2009; Mickleborough *et al.*, 2015), IL-6 (Phillips *et al.*, 2003; DiLorenzo, Drager and Ankin, 2014; Marques *et al.*, 2015) and Interleukin 1 receptor antagonist (IL-1ra) (Marques *et al.*, 2015), with many of these reduced levels of cytokines being linked to reduced muscle dysfunction (DiLorenzo, Drager and Ankin, 2014; Lembke *et al.*, 2014; Mickleborough *et al.*, 2015). Thus, it is possible that supplementing with n-3 PUFA prior to the exercise that causes inflammation and damage will assist in the regulation of the inflammatory response enabling effective muscle repair and regeneration.

Whilst the precise biochemical mechanisms of action relating to the anti-inflammatory effects of n-3 PUFAs are yet to be defined, there are two likely mechanisms: firstly a greater proportion of n-3 PUFAs compared to n-6 PUFAs will result in a decreased proportion of eicosanoids produced from the n-6 PUFA AA, which are known to be pro-inflammatory (Calder, 2012a), and secondly n-3 PUFA incorporation into skeletal muscle alters lipid raft formation (Hou, McMurray and Chapkin, 2016) and manipulates mechanical and nutritional cues to the translational machinery (McGlory, Vliet, *et al.*, 2019). Hou, McMurray and Chapkin (2016) proposed that the ability of n-3 PUFA to alter plasma membrane and cytoskeleton-dependent signalling may impact CD4^{+} T cell differentiation. Therefore, there would

be a shift in differentiation from pro-inflammatory effector subsets to anti-inflammatory subsets.

2.7 Impact of n-3 PUFA on endurance exercise

There is a wealth of literature investigating the impact of n-3 PUFA supplementation on physiological markers associated with endurance exercise performance, however, the data on actual exercise performance is limited. The main physiological markers that have been investigated are heart rate (HR), rating of perceived exertion (RPE), oxygen uptake (VO_2) and exercise-induced inflammation. In terms of exercise performance, time to exhaustion is the main measure that has been used with the modality typically being cycling. The majority of studies have found no effect of n-3 PUFAs on time to exhaustion (Huffman *et al.*, 2004; Raastad, Hastmark and Strømme, 2007; Peoples *et al.*, 2008; Buckley *et al.*, 2009; Boss *et al.*, 2010). This finding is reinforced in studies that have determined performance through time trials and found no effect of n-3 PUFAs on time trial performance (Oostenbrug *et al.*, 1997; Nieman *et al.*, 2009; Poprzecki *et al.*, 2009; Da Boit *et al.*, 2015; Lewis *et al.*, 2015). Interestingly, despite the lack of effect of n-3 PUFAs directly on performance, many of these studies did find improvements in terms of physiological markers. It could be that the performance tests used in these studies were not sensitive enough to detect statistically significant differences following supplementation. Therefore, it is important to consider the effects of n-3 PUFA supplementation on physiological markers in more detail.

2.7.1 Heart Rate (HR) and Rating of Perceived Exertion (RPE)

In a cross-sectional study of 1,008 men aged 42 to 60 years, higher serum n-3 PUFA concentrations were associated with lower resting HR (Tajik *et al.*, 2018). Furthermore, Mozaffarian et al., (2005) conducted a meta-analysis of randomized, double-blind, placebo controlled trials on the effect of fish oil on resting HR in humans and found that fish oil reduces HR by 2 beats·min^{-1}, particularly in those with higher baseline HR or longer duration supplementation. When looking at HR during exercise, however, research into the effect of n-3 PUFAs on submaximal heart rate

is equivocal, with some studies showing that fish oil can reduce submaximal HR (Ninio *et al.*, 2008; Peoples *et al.*, 2008; Buckley *et al.*, 2009; Boss *et al.*, 2010; Macartney *et al.*, 2014), and some studies showing no effect (Huffman *et al.*, 2004; Walser and Stebbins, 2008; Bloomer *et al.*, 2009; Poprzecki *et al.*, 2009; Kawabata *et al.*, 2014). There is no obvious discrepancy between these contrasting findings in terms of duration of supplementation with all studies having at least 4 weeks supplementation period, increasing to 12 weeks, which would suggest a long enough supplementation period for n-3 PUFAs to be incorporated into the tissues. There is also a mix of populations (i.e., old and young, trained and untrained) in the studies that have found an effect of n-3 PUFA supplementation and those that have found no effect. Therefore the discrepancy is likely to be due to the differences in exercise protocols employed in the various studies.

A few studies have investigated the effects of n-3 PUFAs on maximal HR and there appears to be no effect (Raastad, Hastmark and Strømme, 2007; Buckley *et al.*, 2009; Macartney *et al.*, 2014; Tajik *et al.*, 2018), while Delodder et al., (2015) reported a negative effect in terms of performance as maximal HR decreased following supplementation. The doses of n-3 PUFA in this study were incredibly high and administered over a short timescale, with a 3-hour infusion of 32 to 55 g and a matching oral supplementation over 3 days dependent on the participants body mass. The levels of incorporation into the erythrocytes and the platelets were low. This is potentially due to the short duration of supplementation, as there was insufficient time for EPA and DHA to be incorporated into the platelet and erythrocytes membranes. Therefore, it was unlikely to have produced any significant physiological effects, especially since platelet function tests were unaltered.

Despite finding no effect of n-3 PUFAs on submaximal HR, Walser & Stebbins, (2008) did find an increased stroke volume (n-3 PUFA 32.3 ± 8.7 vs placebo 14.1 ± 6.3 ml) and cardiac output (n-3 PUFA 10.3 ± 1.2 vs placebo 8.5 ± 1.0 L·min^{-1}). Further research into the mechanisms of this have not been performed in humans, however Stebbins, Hammel, Marshal, Spangenberg, & Musch, (2010) found that n-3 PUFAs augmented the contraction-induced increases in skeletal muscle blood flow and conductance in rats during moderate treadmill running. These increases in conductance and blood flow occurred without an associated change in mean arterial

pressure or heart rate indicating that n-3 PUFAs may alter vasoreactivity in the vasculature of skeletal muscle. They suggested that the source of the elevated skeletal-muscle blood flow is an increased cardiac output caused by a decrease in systemic vascular resistance that is concomitant with n-3 PUFA induced increases in muscle conductance.

Collectively, the evidence suggests a potential decrease in submaximal HR following n-3 PUFA supplementation but no effect on maximal HR. In terms of exercise performance, a lower submaximal heart rate would suggest the athlete will find the exercise easier, so have a lower RPE, allowing the athlete to exercise for longer durations or work at higher intensities. There is limited literature in terms of endurance exercise, n-3 PUFA supplementation and RPE but in the two studies identified, RPE and pain were lowered. During an incremental exercise test and a submaximal exercise test, a group supplemented with n-3 PUFA for 8 weeks had significantly lower RPE than a control group (Kawabata *et al.*, 2014). In addition, male and female runners supplemented with n-3 PUFA for 11 weeks had significantly lower pain scores than a placebo (1.1 ± 0.4 units lower than placebo) group after a 30km run at 70% VO_2 max (Baum, Telford and Cunningham, 2013). Athletes finding the exercise easier in terms of lower RPE and HR due to n-3 PUFA supplementation may be able to work at a higher intensity (i.e., run faster or cycle at a higher power output), thus enabling them to finish a race quicker.

2.7.2 Oxygen Uptake (VO$_2$)

There appears to be a consensus in the literature that VO_2 max is not improved by n-3 PUFA supplementation (Huffman *et al.*, 2004; Raastad, Hastmark and Strømme, 2007; Bloomer *et al.*, 2009; Poprzecki *et al.*, 2009; Boss *et al.*, 2010; Delodder *et al.*, 2015; Jannas-Vela *et al.*, 2017). Nevertheless, following 3 weeks of 1.3 g/day n-3 PUFA supplementation, male cyclists did have a significant increase in VO_2 max compared with a placebo group (74.8 ± 5.6 vs 71.0 ± 4.1 ml·kg^{-1}·min^{-1}) (Żebrowska *et al.*, 2015). Critically, the nature of the participants in the study by the Żebrowska *et al.* (2015) were highly trained male cyclists (VO_2 max = 70 ml·kg^{-1}·min^{-1}), whereas participants in the other studies were either sedentary or only recreationally active, but not specifically trained cyclists. In another study conducted on trained cyclists,

submaximal VO_2 during a time to exhaustion (TTE) test at 55% maximal power output was lower in the n-3 PUFA supplemented group compared with the placebo group (Peoples *et al.*, 2008). This finding was replicated in recreationally active males (Kawabata *et al.*, 2014). Contrary to this, Huffman et al. (2004) found no difference in submaximal oxygen uptake during 75 minutes running at 60% VO_2 max in recreationally trained healthy males and females. The mode of exercise could be the determining factor here, as the studies where n-3 PUFA supplementation has been effective are cycling studies, whereas studies that have shown no effect involve running. Interestingly, (Hingley *et al.*, 2017) reported a lower oxygen cost during a cycling time trial in trained male cyclists and runners that had supplemented with tuna fish oil (-154 ± 59 ml·min^{-1}·100W) compared to a placebo group (-23 ± 26 ml·min^{-1}·100W), despite there being no difference in submaximal VO_2 during 10 minutes steady state cycling. This is yet to be replicated but would be advantageous for an athlete to know if they were racing. The evidence suggests that n-3 PUFA supplementation is unlikely to improve VO_2 max, however, supplementation may reduce submaximal VO_2 and potentially cycling efficiency, which could result in a improved endurance performance.

2.7.3 Exercise-Induced Inflammation

The impact of n-3 PUFA supplementation on circulating inflammatory markers has been investigated in a range of endurance sports, however the findings are currently inconclusive. Positive results have been observed during and for 48 hours after 60 minutes of weighted backpack walking where TNF-α and CRP were lower when participants supplemented with n-3 PUFA for 6 weeks, compared with when they supplemented with a placebo (Bloomer *et al.*, 2009). This result was not replicated in wheelchair basketball, where TNF-α and CRP, as well as IL-1β and Interleukin-4 (IL-4) were unchanged after 60 minutes of wheelchair basketball training (Marques *et al.*, 2015), however, none of these markers were actually increased by the acute exercise so there was little scope for n-3 PUFAs to have an effect. Although a positive finding in this study was that the exercise induced increases in IL-6 and IL-1ra were attenuated following 4 weeks of n-3 PUFA supplementation (Marques *et al.*, 2015). In contrast, there were no reductions in CRP, IL-1ra, IL-6 or IL-8 after 3 days of high intensity cycling following 6 weeks n-3 PUFA supplementation (Nieman

et al., 2009). In addition, there was no attenuation in exercise-induced increases in TNF-α, IL-6, IL-1ra, TGF-β$_1$ in males supplemented with 3.6 g/day n-3 PUFA compared with a control group after a marathon (Toft *et al.*, 2000). The dose and duration of the supplementation between the studies discussed above differed and therefore, alongside the differences in the type of exercise, this could have impacted the differing outcomes.

2.8 Impact of n-3 PUFA on resistance exercise

Several studies have been conducted investigating the impact of n-3 PUFA on resistance exercise. There are three main models of investigation for this area:

1. Providing participants with supplements or placebo and assessing the effects on resistance exercise-based performance tests,

2. Inducing muscle damage through eccentric contractions and assessing the recovery of participants that have supplemented with n-3 PUFA or placebo,

3. Supplementing individuals with n-3 PUFA and monitoring resistance-based training and the adaptations resulting from this training. The literature has largely focused on older adults due to the higher levels of sarcopenia observed in these individuals and therefore the greater potential for n-3 PUFA supplementation to have an effect. However, this section will focus on younger individuals as this is the population that will be investigated in the experimental chapters of this thesis.

Similar to endurance exercise, tests of performance on younger adults are also limited, with the majority being conducted in athletes. Peak torque from isometric voluntary quadricep contractions are unchanged by n-3 PUFA supplementation in males (Gray *et al.*, 2014; Lewis *et al.*, 2015, 2017; Hingley *et al.*, 2017). Likewise, there does not appear to be an effect of n-3 PUFA on peak torque in young females (Lenn *et al.*, 2002; Lembke *et al.*, 2014; McKinley-Barnard *et al.*, 2018). Despite the lack of improvement in strength McGlory *et al.*, (2019) found n-3 PUFA supplementation attenuated the skeletal muscle atrophy following 2 weeks muscle disuse in young females. The females decline in muscle volume was greater in the

control group compared to the n-3 PUFA group (14 vs 8%). Young resistance trained males that supplemented with n-3 PUFA for 6 weeks increased 1RM leg extension (6.1 ± 3.4%) in the non-dominant leg following 2 weeks of a 60% energy-restricted diet compared with no change in the control group (Philpott *et al.*, 2019). Thus, increases in strength due to n-3 PUFA supplementation appears most evident under catabolic conditions (Smith *et al.*, 2011a; Lalia *et al.*, 2017). There is a clear gap in the literature with regards to n-3 PUFA supplementation whilst strength training, but it would be interesting to investigate whether n-3 PUFA can augment strength training considering its beneficial effect in catabolic conditions.

There is limited evidence investigating n-3 PUFA supplementation and sports specific performance tests and as an athlete's main goal is to restore physical performance it is sensible to assess athletes on these tasks, giving a better indication of sports specific recovery. There was a greater countermovement jump peak force (4.6 ± 5.9%) in professional male rugby union players following 5 weeks of 2.2 g/day n-3 PUFA supplementation (Black *et al.*, 2018). However, this was not replicated in well-trained males following 8 weeks of a fatty acid mix containing EPA and DHA (Pumpa *et al.*, 2011), or in male and female football players following 4 weeks n-3 PUFA supplementation (Gravina *et al.*, 2017). Despite the lack of effect on countermovement jump, football players supplemented with n-3 PUFA did cover a greater distance on the Yo-Yo Intermittent Recovery Test 1 (Gravina *et al.*, 2017). Conversely, there was no effect of n-3 PUFA supplementation on the Yo-Yo test and the Loughborough Soccer Passing Test in just male football players (Philpott *et al.*, 2018). There was a reduced power drop of 4.8 ± 3.4 % vs placebo in the Wingate test following 3 weeks of n-3 PUFA supplementation in healthy male athletes that competed in a range of Olympic sports (Lewis *et al.*, 2015) but this was not replicated in trained male cyclists and runners supplemented with tuna fish oil (Hingley *et al.*, 2017).

The evidence to date is equivocal with further research required, particularly in team-based sports, to determine whether n-3 PUFA supplementation can aid anaerobic performance. Interestingly, despite some of the performance-based studies demonstrating null findings, they often report a beneficial effect of n-3 PUFA on physiological parameters such as markers of muscle damage, inflammation, and

soreness. It is possible that the performances tests employed were not sensitive enough to detect differences following supplementation. Therefore, it is important to consider the effects of n-3 PUFA supplementation on physiological markers in more detail.

2.8.1 Muscle Recovery

A recent systematic review and meta-analysis investigating the effectiveness of n-3 PUFA supplements on muscle soreness following eccentric exercise reported significantly decreased delayed onset muscle soreness (DOMS) (Lv, Zhang and Zhu, 2020). Muscle soreness resulting from eccentric exercise was attenuated in untrained males (Tartibian, Maleki and Abbasi, 2009; Rajabi *et al.*, 2013; Mickleborough *et al.*, 2015; Tsuchiya *et al.*, 2016; Ochi, Tsuchiya and Yanagimoto, 2017) and females (Jouris, Mcdaniel and Weiss, 2011; Lembke *et al.*, 2014; Corder *et al.*, 2016) following n-3 PUFA supplementation. This finding was replicated in competitive male football players following 6 weeks supplementation, with soreness 58% lower in a n-3 PUFA group compared with a carbohydrate group during the 72 hour recovery period from maximal eccentric hamstrings contractions (Philpott *et al.*, 2018). In contrast, there was no effect of n-3 PUFA supplementation on muscle soreness in untrained males (Lenn *et al.*, 2002; Phillips *et al.*, 2003; Gray *et al.*, 2014), females (Tinsley *et al.*, 2017) and novice resistance-trained females (Hayward *et al.*, 2016). It is important to note that when the results from (Tinsley *et al.*, 2017) were analysed by calculating effect sizes rather than an ANOVA, static and functional soreness 48 hours post exercise were actually 33 to 42% lower in the n-3 PUFA versus placebo group, suggesting classic tests of significance may not show the true picture in terms of smaller group changes. Surprisingly, McKinley-Barnard *et al.*, (2018) found increased muscle soreness up to 24 hours post eccentric exercise in physically active females that had supplemented with 4.2 g/day of n-3 PUFA compared with a placebo. However, measurements were not performed beyond 24 hours post-exercise and therefore potentially missed the peak of muscle soreness, which has been suggested to occur between 24 – 48 hours post-exercise (Ebbeling and Clarkson, 1989).

The evidence to date regarding muscle soreness following eccentric exercise is unclear which is likely due to the variety of eccentric protocols used, with some protocols less 'damaging' than others, and also there are differing supplementation protocols in terms of duration and dosage, with not all studies measuring n-3 incorporation. The research by Philpott *et al.*, (2018) is the only study conducted on competitive athletes where the exercise induced muscle damage is prescribed using an eccentric muscle damaging protocol. It is notoriously difficult to ask competitive athletes to perform exercise that is going to cause them damage and does not really enhance their training or sports performance, however, this method is the only way to quantify the muscle damaging exercise and allow a direct comparison between a supplement and placebo.

Changes in MVC following muscle damaging exercise tends to be used as a proxy for global neuromuscular fatigue and indeed much of the literature has focused on this to determine the effectiveness of n-3 PUFA in aiding recovery from exercise-induced muscle damage. Supplementation attenuated the decrements in peak isometric torque of the biceps 1 day (percentage of MVC: EPA 90.5 ± 14.0% vs Control 76.8 ± 16.0%) (Ochi, Tsuchiya and Yanagimoto, 2017) and 2 to 5 days (Tsuchiya *et al.*, 2016) post muscle damaging exercise in healthy males. Mickleborough *et al.*, (2015) also found higher peak isometric torque in the quadriceps following downhill running in the n-3 PUFA supplemented group compared with the placebo. Following 4 sets of 20 repetitions of leg press, n-3 PUFA also reduced the exercise-induced decrements in leg press 1RM score in healthy young males (Rajabi *et al.*, 2013). However, some of the literature in this area suggests n-3 PUFA has little effect on attenuating strength deficits in the days following muscle damaging exercise (Lenn *et al.*, 2002; Pumpa *et al.*, 2011; DiLorenzo, Drager and Ankin, 2014; Gray *et al.*, 2014; Lembke *et al.*, 2014; Lewis *et al.*, 2015, 2017; Hingley *et al.*, 2017; Jakeman *et al.*, 2017; McKinley-Barnard *et al.*, 2018; Philpott *et al.*, 2018). The discrepancy in findings is likely due to the variations in muscle damaging protocols causing differing amounts of EIMD and different supplementation protocols leading to differences in incorporation of n-3 PUFA into the muscle.

Blood levels of myofibril proteins such as CK, LDH, Skeletal Troponin 1 (sTn1), slow myosin heavy-chain fragments and Mb have also been used as markers of muscle damage. In the 96 hours following 20 minutes downhill running, untrained males had smaller increases in sTn1 (n-3 PUFA 165.1 ± 139.1% vs placebo 260.2 ± 170.6%), CK   (n-3 PUFA 579.8 ± 287.4% vs placebo 1006.5 ± 631.2%) and Mb (n-3 PUFA 1109.5 ± 496.2% vs placebo 1917.6 ± 876.3%) in the n-3 PUFA supplemented group compared with to the placebo group (Mickleborough *et al.*, 2015). Myoglobin was lower in the supplement group compared with the placebo group in physically active females following eccentric quadriceps contractions (McKinley-Barnard *et al.*, 2018) and in healthy young males following eccentric biceps contractions (Tsuchiya *et al.*, 2016). CK levels were lower in n-3 PUFA groups compared with placebo groups following eccentric biceps curls (DiLorenzo, Drager and Ankin, 2014), eccentric hamstring contractions (Philpott *et al.*, 2018), 40 minutes bench stepping (Tartibian, Maleki and Abbasi, 2011) and eccentric leg press (Rajabi *et al.*, 2013). There were also lower levels of LDH in healthy young males following eccentric leg press (Rajabi *et al.*, 2013). In contrast, there were no differences in levels of CK between n-3 PUFA and placebo groups post eccentric exercise (Lenn *et al.*, 2002; Phillips *et al.*, 2003; Pumpa *et al.*, 2011; Gray *et al.*, 2014; Lembke *et al.*, 2014; Jakeman *et al.*, 2017). Interestingly Tsuchiya *et al.*, (2016) did not see a simultaneous lower level of CK, to match the Mb results, in the n-3 PUFA group. The lack of significance in CK likely occurred due to high variability of CK within the two groups as it was an independent groups design with no pre-supplementation testing. Furthermore n-3 PUFA failed to attenuate increases in Mb following downhill running (Pumpa *et al.*, 2011) or LDH following eccentric arm curls (Phillips *et al.*, 2003). It is possible that the downhill running protocol was insufficient to cause a large degree of damage in the trained men and the small muscle mass used in the eccentric unilateral arm curls did not cause enough damage for the n-3 PUFA supplementation to have an effect.

The increases in systemic myofibril proteins following exercise appear to be reduced in some n-3 PUFA supplementation studies and unchanged in others. As detailed above there is a wide range of different modes of muscle damaging exercise employed in the different studies. It may be that some studies did not cause enough EIMD for n-3 PUFA to modulate the release of proteins, or the dosage and/or duration of n-3 PUFA supplementation may have been insufficient to cause

significant changes in n-3 PUFA incorporation in the cells. Moreover, the release of muscle specific proteins (e.g. CK) is not always proportional between the protein released and the muscle damage per se, since release reflects numerous other processes in addition to actual damage (Clarkson *et al.*, 1986; Endres *et al.*, 1989; Warren, Lowe and Armstrong, 1999; Stupka *et al.*, 2001; Malm *et al.*, 2004). Therefore, it is important that these proteins are not used in isolation as a marker of muscle damage.

2.8.2 Exercise-induced inflammation

Levels of plasma IL-6 in untrained males were reduced for up to 3 days following eccentric exercise in an n-3 PUFA group compared with placebo (Tartibian, Maleki and Abbasi, 2011; DiLorenzo, Drager and Ankin, 2014; Tsuchiya *et al.*, 2016). Lembke *et al.*, (2014) found attenuated CRP following eccentric bicep contractions in healthy male and female college students following 4 weeks n-3 PUFA supplementation. Similarly, increases in TNF-α post exercise were lower in n-3 PUFA groups compared with placebo groups in untrained young males (Tartibian, Maleki and Abbasi, 2011; Mickleborough *et al.*, 2015).

However, a systematic review published in 2012 concluded that exercise-related n-3 PUFA supplementation studies (with doses of 0.9 – 2g/day) included in the review did not provide strong evidence of anti-inflammatory effects in trained or untrained participants (Rangel-Huerta *et al.*, 2012). Interestingly, it has been suggested that innate immune responses in younger people are not affected by an EPA intake of ≤ 4.05g per day (Rees *et al.*, 2006), which may be the reason for the lack of evidence of anti-inflammatory effects. Since Rangel-Huerta *et al.*'s review further studies have found similar results with no effect of n-3 PUFA on exercise induced increases in CRP in untrained females supplemented with 3 g/d (Corder *et al.*, 2016) and competitive male football players supplemented with 2.2 g/d (Philpott *et al.*, 2018). Furthermore, exercise induced increases in CRP, TNF-α and IL-6 were not attenuated by n-3 PUFA supplementation in trained young males (Pumpa *et al.*, 2011). Paradoxically, McKinley-Barnard *et al.*, (2018) found a greater serum TNF-α in the n-3 PUFA supplemented group (4.2 g/d) compared with placebo following unilateral eccentric knee extensor exercise in physically active females. Differences

in the supplementation, as well as the mode and intensity of EIMD, must be considered when interpreting the differences observed in the inflammatory response after supplementation. Further research is required to provide a greater range of cytokine responses to exercise, ensuring that the exercise stimulus is large enough to generate muscle damage and inflammation and checking that the n-3 PUFAs have been incorporated into the immune cells. Due to the potential detrimental nature of exercise-induced inflammation on athletes, it is also important to investigate the effects of n-3 PUFA on inflammation following actual performance, for example a rugby match, rather than just simulated performance tests. Further research is also required to investigate the effect of resistance training and n-3 PUFA supplementation as to date this has only been conducted on older adults.

2.8 Curcumin supplementation

Whilst n-3 PUFA supplementation is the focus of this thesis, in chapter 6 curcumin is introduced as an additional supplement to n-3 PUFA, therefore a short summary of curcumin and previous research in the context of exercise recovery will be provided.

Curcumin is a polyphenol and the main constituent in the spice turmeric. Extensive research over the past quarter century has demonstrated that curcumin has promising effects on patients with inflammatory diseases including cancer, cardiovascular disease, arthritis, diabetes and lupus (Gupta, Patchva and Aggarwal, 2013). The potential anti-inflammatory properties of curcumin has resulted in research focused on the impact of curcumin on performance and post-exercise recovery (Fernández-Lázaro *et al.*, 2020; Campbell, Carlini and Fleenor, 2021). Supplementation of between 150 to 1500mg/day of oral curcumin, both before and up until 72 h after exercise has been shown to be effective on exercise performance, modulated by the reduction of EIMD and inflammation (Fernández-Lázaro *et al.*, 2020).

The combined effects of curcumin and piperine on MD following maximal downhill leg jumps were examined and found an attenuated loss of mean power during a 6-second single leg sprint (+175 Watts, 15% vs. placebo) 24 hours post MD (Delecroix *et al.*, 2017). Although there were no differences at timepoints 0, 48 or 72 h post MD or in jump height or muscle soreness, suggesting the effects may be time sensitive.

Whilst there were only 10 participants in this study, they were elite rugby players, and a robust study design (blinded, randomised, cross-over design) was employed. In another placebo-controlled, randomised, cross-over design study, 150mg of curcumin ingested before and 12 h after 50 maximal eccentric contractions of the elbow flexors attenuated the reduction in torque production (-33 ± 8%) compared with placebo (-40 ± 9%) (Tanabe *et al.*, 2015). The peak in CK following MD was also smaller in the curcumin than placebo condition. However, there were no differences in other indirect markers of EIMD including range of motion (ROM), arm circumference, muscle soreness, IL-6 and TNF-α.

Curcumin supplementation (400mg/d) resulted in significantly lower concentrations of CK (-48%), IL-8 (-21%) and TNF-α (-25%) following EIMD compared to placebo in young males and females (McFarlin *et al.*, 2016). There were no differences in IL-6, Interleukin-10 (IL-10) or muscle soreness. Conversely, Nicol *et al.* (2015) reported minor alterations in biological indices (CK and IL-6) of EIMD with curcumin supplementation and large improvements in subjective muscle pain 24 and 48 h post eccentric leg press. It is possible that the naturally occurring dose of curcumin provided by Nicol *et al.* (2015) did not provide enough free curcumin to elicit large changes in inflammatory biomarkers. A dietary supplement containing curcumin taken for 30 days also resulted in reduced measures of pain and tenderness post-eccentric exercise compared to a placebo group (Udani *et al.*, 2009). No differences in markers of inflammation (CRP, TNF-α, IL-1, IL-6), muscle damage (CK, Mb), ROM were found between groups and this may have been due to the very small sample size of n = 10.

There has been limited research to date on the potential applications of curcumin with inconsistent findings due to different populations studied, different exercise protocols and supplement dosing regimens. Natural curcumin has poor bioavailability in humans, with many studies showing very low, or even undetectable concentrations in blood due to its poor absorption, rapid metabolism, chemical instability, and rapid systemic elimination (Anand *et al.*, 2007). Many formulations are being created with improved bioavailability, however, most of the exercise-based literature does not present curcumin absorption data, making it difficult to attribute any effects directly on curcumin. There is evidence suggesting curcumin may

improve post-exercise recovery and subsequent performance through the modulation of oxidative stress, antioxidant capacity, inflammation and muscle damage but further work is needed to elucidate when it is most appropriately used (Campbell, Carlini and Fleenor, 2021).

2.9 Conclusion

Few studies have determined the effects of n-3 PUFA supplementation in human subjects on exercise performance and the associated changes in muscle damage and inflammation. In the limited studies that have been conducted, results have been equivocal. Variables such as the exercise protocol, participants recruited (trained, untrained, young, old, special populations), dosage, fatty acid composition and duration of supplementation, timing of measurement, and the selection of biomarkers are likely to have contributed to the discrepancies between studies. Another potentially mitigating factor is that humans are metabolically unique, with idiosyncrasies in digestion, absorption, tissue distribution and cellular metabolism. Consequently n-3 PUFA incorporation can be different within individuals consuming the same dosage of n-3 PUFAs. It is therefore important to quantify the levels of incorporation in the cells and as a minimum, have a measure of supplementation compliance for participants. There are relatively few published human studies that have data on the fatty acid composition of circulating lipids, mainly due to difficulties associated with collecting blood, the complexity and costs of conventional analysis and the application of time-consuming and relatively costly procedures (Marangoni, Colombo and Galli, 2004).

The primary aims of this thesis were to:

1) Investigate whether n-3 PUFA supplementation improves exercise performance and the associated physiological markers
2) Ascertain whether the negative impact when recovering from acute exercise can be modulated by n-3 PUFA supplementation

3) Determine whether the strength and power improvements associated with resistance exercise training can be enhanced by n-3 PUFA supplementation in untrained and trained males

The null hypotheses tested within this thesis:

1) Supplementation with n-3 PUFA does not improve exercise performance
2) The negative impact when recovering from acute exercise is not altered by n-3 PUFA supplementation
3) Supplementation with n-3 PUFA does not further modulate any improvements in strength and power associated with resistance training

Chapter 3: General Methods

The methods described in this chapter are those that were employed in the majority of the investigations in this thesis. Methods that were specific to individual studies are not included but are in the methods section in the relevant chapter. All studies were approved by Loughborough University Ethics Advisory Human Participants Sub-committee and conformed to the Declaration of Helsinki. All investigations were registered as clinical trials and the identifying numbers are given in each experimental chapter.

3.1 Participant recruitment

Healthy adult males were recruited for all studies (18 – 45 yrs). Studies were advertised using physical posters (Appendix 1) around Loughborough town centre and university and digital posters on social media. Participants completed a general health screen questionnaire (Appendix 2) to ensure their suitability to take part. Volunteers were excluded if they had a history of cardiovascular, metabolic or haematological disorder. They were also excluded if they regularly consumed NSAIDS, antioxidant or fish oil supplements, or ate more than 2 portions of oily fish per week. Females were also excluded due to the fluctuations in exercise performance over the menstrual cycle. Participants were given verbal and written participant information about each study prior to providing written informed consent (Appendix 3).

3.2 Pre-trial measures

3.2.1 Dietary and Exercise Control

With regards to exercise, in chapters 4 and 5 participants were instructed to limit their physical activity in the 48 hours prior to and for the duration of the data collection period (other than study requirements). In chapter 6, participants continued with their normal training regime which was determined by the rugby coaching staff.

In terms of diet, a 3-day food diary was collected on the first trial of each study and participants were asked to replicate this food diary prior to all trials. Participants were

requested not to consume alcohol for the 24 hours preceding and during the trial period. They were also asked to limit their fish intake to 2 portions per week. NetWISP (Tinuviel Software, Llanfechell, Anglesey, UK) dietary analysis software was used to check the nutritional composition of dietary intakes were similar between treatment groups. On the morning of experimental trials, participants arrived at the laboratory following a minimum 10 hours fast. The same time of day was used for each trial to minimise diurnal variation with the exception of chapter 6 game day trials, where the timing of the game determined when the trial took place. Participants were requested to maintain their habitual diet and physical activity patterns between trials.

3.2.2 Anthropometrics

A stadiometer (Seca 274 Stadiometer, Hamburg, Germany) was used to measure height to the nearest 0.1cm with socks and shoes removed. Body mass was measured with digital scales (Seca mBCA 515, Hamburg, Germany) to the nearest 0.1kg with participants wearing shorts and t-shirt, with socks and shoes removed.

3.3 Collection and preparation of blood

3.3.1. Blood Sampling

In chapter 4 venepuncture was used for the 24h post exercise blood sample, in chapter 5 venepuncture was used for the 48h post exercise blood sample and in chapter 6 all blood samples were collected using this venepuncture protocol. A 21-gauge needle was inserted into an antecubital vein.

In chapter 4 and 5 cannulation was used for blood sampling during the main testing sessions. A 21-gauge intravenous catheter was inserted into an antecubital vein. When a blood draw was required, 2ml of blood was initially taken and discarded to account for any saline in the catheter and connecting hose. To prevent cells from adhering to the inner surface of the catheter and clotting which may have led to occlusions a 5ml saline (0.9% sodium chloride) flush was used following all blood draws.

Blood was collected into the K_2 dipotassium ethylene diamine tetra-acetic acid (EDTA) vacutainers (Becton, Dickinson and Company, NJ, USA) for Peripheral Blood mononuclear cell (PBMC) isolation and whole blood cell counts. Blood was collected into silica coated vacutainers (Becton, Dickinson and Company, NJ, USA) for analysis of inflammatory and muscle damage markers.

3.3.2 Serum Isolation

Whole blood was collected into silica clot activator vacutainers and allowed to clot at room temperature for 30 mins before being centrifuged at 1500 g (4°C) for 15 mins according to the manufacturer recommendations (https://www.bd.com/resource.aspx?idx=30770, accessed on 26-08-2020). The resulting serum was aliquoted and frozen at -80°C for future analysis.

3.3.3 PBMC Isolation

PBMCs were isolated from whole blood using density gradient centrifugation according to manufacturer guidelines

(https://www.sigmaaldrich.com/technical-documents/protocols/biology/isolation-of-mononuclear-cells/recommended-standard-method.html , accessed on 26-08-2020). Briefly, whole blood (10ml) was mixed 1:1 with balanced salt solution and inverted. This solution was then layered onto 13.5ml of Ficoll-Paque Premium (GE Healthcare, Chicago, USA) and centrifuged at 400 x g for 35 mins at 20°C. The PBMCs were extracted and washed in Hanks balanced salt solution, using centrifugation at 100 x g for 10 mins at 4°C, the pellet was then resuspended and washed again. The final pellet was suspended in 200µl Phosphate Buffered Saline (PBS) and frozen at -80°C for future analysis of fatty acid methyl esters.

3.3.4 Sample storage

Whole blood, serum and PBMCs were stored in a -80°C freezer according to the Human Tissue Act (HTA) 2004 guidelines. Once used they were appropriately disposed of in accordance with the guidelines.

3.4 Blood Analysis

3.4.1 Cell Counting

Cell counts were performed on fresh whole blood using the COULTER® Ac·T™ 5diff (Beckman Coulter, Inc., London) (chapter 4) and the Horiba Yumizen H500 (Horiba Ltd, Kyoto, Japan) (chapter 5 and 6). The analyser was changed due to a breakdown of the COULTER® Ac·T™ 5diff prior to commencing testing for chapter 5. Haematocrit and haemoglobin were used to ascertain plasma volume changes that were used to adjust serum protein concentrations.

3.4.2 Analysis of n-3 PUFA capsules, PBMC and whole blood fatty acid composition

Fatty acid methyl esters (FAME) were prepared by incubation of the 100µl of oil from the n-3 PUFA capsules, PBMC or whole blood with 3.4ml BHT-methanol standard and 200µl of acetyl chloride at 70°C for 60 minutes. The reaction was cooled and 5 ml of 6% (K_2CO_3) was added to stop and neutralise the reaction. n-hexane (1.5 ml) was then added and centrifuged. The washing step was repeated, and the supernatant evaporated until dry using nitrogen gas and reconstituted in 100 µl of hexane with 20 µg/ml of internal standard. A 1 µl sample was injected on the GCMS (Gas Chromatography Mass Spectroscopy). The sample analysis involved the use of FAMEWAX column (30 m x 0.25 mm internal diameter (ID) x 0.25 µm film thickness) fitted in a Varian CP 3800/4000 GCMS (Agilent Technologies, CA, USA) equipped with an ion-trap mass analyser. The oven temperature program was set at initial temperature 130 ºC and then increased from 130 ºC to 208 ºC at a rate of 6 ºC /min. The temperature was programmed to increase at a rate of 2 ºC /min to 225 ºC where it was held for 10 min with a total analysis time of 31.5 min. The injector temperature was set at 220 ºC with a split ratio of 10:1. Helium was used as a carrier gas with a constant flow rate of 1.5 ml/min. Trap temperature was set at 180 ºC, manifold temperature 50 ºC, transfer line 230 ºC and ion source temperature was set at 200 ºC. FAMEs were characterised using electron ionisation in full scan mode (m/z 10 - 400) at a scan rate of 0.93 seconds per scan. The individual FAMEs were identified by comparing to the retention times of a Supelco 37 Mix FAME standard which has

been used to identify complex mixtures of saturated, monounsaturated and polyunsaturated complemented with the MS NIST library. The results were expressed as the relative percentages of the total identified fatty acids.

This method was carried out by a PhD student within the Chemistry department at Loughborough University for chapter 4. This student and GCMS were no longer available to conduct the analysis for chapters 5 and 6, therefore I conducted the analysis myself, using a different GCMS but the same method. However, the results were unreliable and therefore not included in the results of this thesis.

3.4.3 Expressing fatty acid data

Fatty acids are reported in two formats: relative and absolute. With relative reporting, known as fatty acid profiling, the units are expressed as percentage by weight of total fatty acids (abbreviated as %, wt:wt, or g/100g). Based upon the literature the apparent preferred method for presenting fatty acid data is as a relative weight percentage of the total fatty acids with 78% of the data reported as weight percentage (Stark *et al.*, 2016). The main advantage of this method is that it simplifies the comparisons of the complex interactions between competing fatty acids, however, it is important that a full fatty acid profile is provided if this is the method of presentation to allow for interpretation of changes in the profile. It is also useful to present total fatty acids 'identified' vs. total fatty acids as there is a wide range of fatty acids identified and these are not necessarily consistent between studies, making comparisons between studies difficult. Fatty acid profiling tends to have lower variability than absolute concentration and tends to be normally distributed (Brenna *et al.*, 2018).

Absolute expressing of fatty acids provides a concentration of total fatty acid within a fluid or tissue with the units of expression being mg fatty acids/mL plasma, for example. Measuring in this way means an average mass per unit of fluid or tissue can be defined and this can be restricted to one single fatty acid or a range of fatty acids. Therefore, changes in a single fatty acid can be measured without being influenced by the changes in other fatty acids, which may be important when investigating specific fatty acids. There tends to be greater intersubject variability

when measuring absolute concentrations and generally non parametric approaches to statistics must be performed (Brenna *et al.*, 2018).

There is a lack of a 'gold standard' for measuring fatty acid status in human blood (Stark *et al.*, 2016). Using a relative approach allows total fatty acids to be normalised to the total fat amount, which is often reflective of sampling (e.g., volume of plasma, number of cells), but it does not capture changes in the total fatty acid pool or take into account which or how many fatty acids were identified. If lipemia occurs and increases the total fatty acid pool, then decreases in a particular target fatty acid (e.g. EPA) could be misleading but the absolute concentration may be unchanged. Ideally, all reports of fatty acids should include sufficient data to convert relative to absolute concentrations and vice versa (Brenna *et al.*, 2018).

For the purpose of this thesis, fatty acid profiling will be used as we are unable to identify the exact number of PBMCs in the sample analysed for fatty acids. Although the same volume of the PBMC pellet suspended in 200µl PBS will be used for all analysis the extraction of PBMCs from the 10ml blood sample is unlikely to have been consistent due to experimenter inconsistencies and due to the baseline leukocyte count being variable between trials.

3.4.4 Muscle Damage Markers

Serum concentrations of CK, LDH and Mb were determined spectrophotometrically using ABX Pentra assays on a Pentra C400 analyser (Horiba Medical, Japan). All samples for a participant were performed within a single run to minimise run-to-run variation. Haematocrit and haemoglobin values were used to ascertain plasma volume changes that were used to adjust serum CK, LDH and Mb concentrations (Dill and Costill, 1974). The CV for this analysis is typically <3%.

Table 1 – Assay parameters for the CK, LDH and Mb on a Pentra C400

Assay	Assay range	Calculated Linear assay (R^2)	Limit of detection
CK	8 – 4500 U/L	0.9930	8 U/L
LDH	10 – 3600 U/L	0.991	10 U/L
Mb	9.3 – 2500 ng/ml	0.9994	9.3 ng/ml

3.4.5 Cytokines

Circulating levels of IL-6 (Chapter 4 and 5) were measured in the serum. In chapter 4 high sensitivity enzyme immunoassay kits (R & D Systems, Minneapolis, USA) were used according to manufacturer's guidelines. All samples for a participant were performed within a plate to minimise plate-to-plate variation and were performed in duplicate. In chapter 5 circulating levels of IL-6 were determined using BD™ Cytometric Bead Array (CBA) Enhanced Sensitivity Flex Sets (BD Bioscience, UK) on a flow cytometry platform (BD Accuri™ C6 Flow Cytometer, BD Bioscience, UK). All samples were diluted 1:3 with CBA buffer. All samples for a participant were performed within a single run to minimise run-to-run variation. Haematocrit and haemoglobin values were used to ascertain plasma volume changes that were used to adjust serum IL-6 (Dill and Costill, 1974).

Table 2 – Assay parameters for the R & D Systems high sensitivity enzyme immunoassay kits

Assay	Assay range (pg/ml)	Calculated Linear assay (R^2)	Limit of detection (pg/ml)	CV (Intra-assay)
IL-6	0.2 – 10	0.9839	0.031	< 5%

Taken from
https://resources.rndsystems.com/pdfs/datasheets/hs600c.pdf?v=20210308
accessed on 08-03-2021

Table 3 – Assay parameters for the BD Cytometric Bead Array Enhanced Sensitivity Flex Sets on an Accuri™ C6 Flow Cytometer

Assay	Assay ranges (pg/ml)	Linear assay (R^2)	Limit of detection (pg/ml)	CV
IL-6	0.3 - 200	1.0000	0.68	< 6%

3.5 Statistical Analysis

IBM SPSS Statistics 24 (IBM, New York, USA) was used to analyse data from all investigations. A Shapiro-Wilk test was used to determine normal distribution and Mauchly's test of sphericity was used to assess homogeneity of variance. When Mauchly's test of sphericity was violated, Greenhouse-Geisser estimates of sphericity were used. T-tests (where appropriate) or repeated measures analysis of variance were used to determine the impact of n-3 PUFA supplementation on performance, muscle damage markers and inflammatory cytokines. If findings were significant, the Bonferroni post-hoc tests were used to ascertain where the differences occurred. All data are presented as mean ± SD, unless otherwise stated. The level of significance for all statistical analysis was accepted at $p < 0.05$. P values are given to 3 decimal places. If the p value is lower than 3 decimal places, then it will be presented as 0.001.

Chapter 4: The effects of fish oil supplementation on cycling performance, muscle damage and inflammation in healthy male cyclists: a randomised, placebo controlled, crossover study

4.1 Introduction

There is a lack of consensus in the literature regarding the effects of n-3 PUFA supplementation on endurance performance, particularly in cycling. Despite this n-3 PUFAs are one of the most popular dietary supplements used by elite athletes (Shaw, Slater and Burke, 2016). Studies that have used cycling time trials as an indicator of cycling performance have failed to find an effect of daily n-3 PUFA supplementation (Oostenbrug *et al.*, 1997; Poprzecki *et al.*, 2009; Lewis *et al.*, 2015). The performance tests used in the studies identified were a mixture of set workload trials (Oostenbrug *et al.*, 1997; Lewis *et al.*, 2015) and set time trials (Poprzecki *et al.*, 2009), however, only the study by Oostenbrug *et al.*, (1997) had cyclists as participants. It is unclear whether the lack of effect of supplementation is due to the type of performance test employed (i.e. predetermined workload or time) or the dosing strategy (quantity, timing, and composition) of n-3 PUFA used. The doses of n-3 PUFA did not exceed 1.81g/d, which could be an explanation for the lack of effect as Calder (2015) stated a dose of at least 2g/d is necessary to achieve an anti-inflammatory effect.

Interestingly, despite the lack of effect of n-3 PUFAs directly on performance, the literature indicates the potential for improvements in physiological markers associated with endurance performance. Whilst $\dot{V}O_2$ max appears to be unaffected by n-3 PUFA supplementation (Bloomer *et al.*, 2009; Boss *et al.*, 2010; Kawabata *et al.*, 2014; Delodder *et al.*, 2015), there is a trend for increased time to fatigue (Huffman *et al.*, 2004) and a reduction in submaximal $\dot{V}O_2$ following n-3 PUFA supplementation (Peoples *et al.*, 2008; Kawabata *et al.*, 2014). Hingley *et al.*, (2017)

found a decrease in the mean oxygen consumption expressed relative to workload over a 5-minute time trial following 8 weeks of docosahexaenoic acid (DHA) rich tuna oil suggesting an improved cycling economy during a physiologically demanding time trial. However, cycling performance was not improved possibly due to the short nature of the 5-minute test, not allowing time for the lower oxygen cost to directly improve performance. The effects of n-3 PUFAs on heart rate (HR) are also equivocal, with some studies showing a reduction in submaximal HR (Peoples *et al.*, 2008; Buckley *et al.*, 2009; Boss *et al.*, 2010) and others reporting no effect on submaximal (Huffman *et al.*, 2004; Bloomer *et al.*, 2009; Kawabata *et al.*, 2014) or maximal HR (Raastad, Hastmark and Strømme, 2007; Buckley *et al.*, 2009; Macartney *et al.*, 2014). An important factor to note is that except for the study by Bloomer *et al.* (2009), all of the studies highlighted are parallel groups design, not repeated measures.

Supplementing with n-3 PUFA attenuates post-exercise increases in plasma inflammatory markers such as tumour necrosis factor (TNF)-α, IL-6 and IL-1ra in endurance exercise that elicits high levels of inflammation and muscle damage (Marques *et al.*, 2015; Mickleborough *et al.*, 2015). Conversely, no changes in plasma IL-6, and IL-1ra concentration have been reported in male cyclists following a cycling time trial (Nieman *et al.*, 2009) or in runners following a marathon (Toft *et al.*, 2000) after n-3 PUFA supplementation. There appears to be a link between the level of muscle damage and inflammation that occurs and the effectiveness of n-3 PUFA supplementation.

Collectively, previous studies investigating the effects of n-3 PUFA supplementation on cycling performance and inflammation have produced mixed results. However, an important limitation of most of the aforementioned studies is that they have not utilised a crossover, double-blinded and placebo-controlled design. Burke and Peeling (2018) suggest conducting repeated trials in the same individual to confirm the robustness of a measured response to a supplement is important. The one previous study that did use this approach utilised an exercise stimulus that didn't sufficiently induce inflammation in the placebo trial alone (Bloomer *et al.*, 2009). Previous studies have also used relatively low dosages of n-3 PUFAs (≤ 3g/day) that may not have sufficiently increased circulating levels of EPA and DHA to observe an

impact on performance (Oostenbrug *et al.*, 1997; Poprzecki *et al.*, 2009; Lewis *et al.*, 2015) or exercise induced inflammation (Nieman *et al.*, 2009). In addition, evidence has highlighted a large degree of inter-individual variation in the capacity to metabolise n-3 PUFAs (Nording *et al.*, 2013), however, previous research has neglected to measure incorporation of n-3 PUFA (Huffman *et al.*, 2004; Poprzecki *et al.*, 2009). In the few previous studies that have measured incorporation of fatty acids, the incorporation has been analysed in the plasma (Delarue, Labarthe and Cohen, 2003; Bortolotti, Tappy and Schneiter, 2007; Bloomer *et al.*, 2009). Whilst this approach allows a measure of incorporation it does not distinguish between the incorporation of fatty acids into different cell types. As this study will be investigating inflammation and muscle damage, we feel it is prudent to measure the incorporation into the PBMCs.

The aim of the present randomised, double-blind, placebo-controlled, crossover design study is to determine whether 4 weeks of n-3 PUFA supplementation could improve cycling performance and exercise-induced changes in markers of muscle damage and inflammation. We hypothesised that a high dose of n-3 PUFA supplementation would lower the oxygen cost of exercise, reduce heart rate and RPE and accordingly improve cycling performance in a time trial scenario. In addition, exercise-induced increases in markers of muscle damage and inflammation will be attenuated following n-3 PUFA supplementation.

4.2 Method

The experimental protocol followed the Declaration of Helsinki principles and was approved by the Loughborough University Ethics Human Participants sub-committee (Study ID: R14-P72) and registered as a clinical trial on www.clinicaltrial.gov (Study ID: NCT03205241). G*Power version 3.1.9.2 (Faul *et al.*, 2007) was used to perform an *a priori* power analysis for a repeated-measures ANOVA comparing n-3 PUFA supplementation with olive oil supplementation. The effect size for this analysis was estimated from a recent study (Buckley *et al.*, 2009) investigating the effect of n-3 PUFA supplementation on submaximal heart rate and found a reduction of -7.8 ± 2.3 beats·min^{-1} in the fish oil group compared with a decrease of -1.9 ± 1.9

beats·min^{-1} in the placebo group. This indicated that the best estimate of the true population standardised mean difference was δ = 2.80, meaning the olive oil condition will have a higher submaximal heart rate compared to the n-3 PUFA condition. The effect size estimate was entered into the power analysis with the following input parameters: α (two-way) = 0.05, power = 0.80. The power analysis suggested that N = 10 are required in this study to detect a difference between the two supplements with 80% probability. Ten trained male cyclists participated in the study.

Participants

Physiological characteristics of the participants are presented in Table 4. Participants were recruited and excluded according to the criteria in section 3.1 (general methods). A 3-day food diary was completed the day prior to, day of, and day after the first performance test and this was replicated in their subsequent 3 trials (section 3.2.1). All testing took place during the competition phase of the cycling season and all participants were regularly competing in British Cycling category 3/4 races.

Table 4 – General characteristics of 10 trained male cyclists

Variable	Mean ± SD
Age (years)	38 ± 7
Body mass (kg)	73.5 ± 7.9
Stature (m)	1.76 ± 0.08
Sum of 8 Skinfolds (mm)	79.6 ± 20.6
BMI	24 ± 2
Wmax (W)	327 ± 29
Wmax (W·kg^{-1})	4.5 ± 0.3
$\dot{V}O_2$max (ml·kg·min^{-1})	54 ± 5

Wmax, maximal work rate

Experimental Design

The duration of the study was 14 weeks, including a pre-test and familiarisation 5 - 10 days before the initial performance trial and three subsequent performance trials. Four cycling performance trials were performed: pre- and post-supplementation of

both n-3 PUFA and olive oil, for four weeks in a double-blind, randomised crossover design (Figure 3). Simple randomisation using an Excel spreadsheet was used to determine which supplement was given in the first 4 weeks of the study. Double blinding was achieved by the two supplements being given a label that was unknown to the investigator.
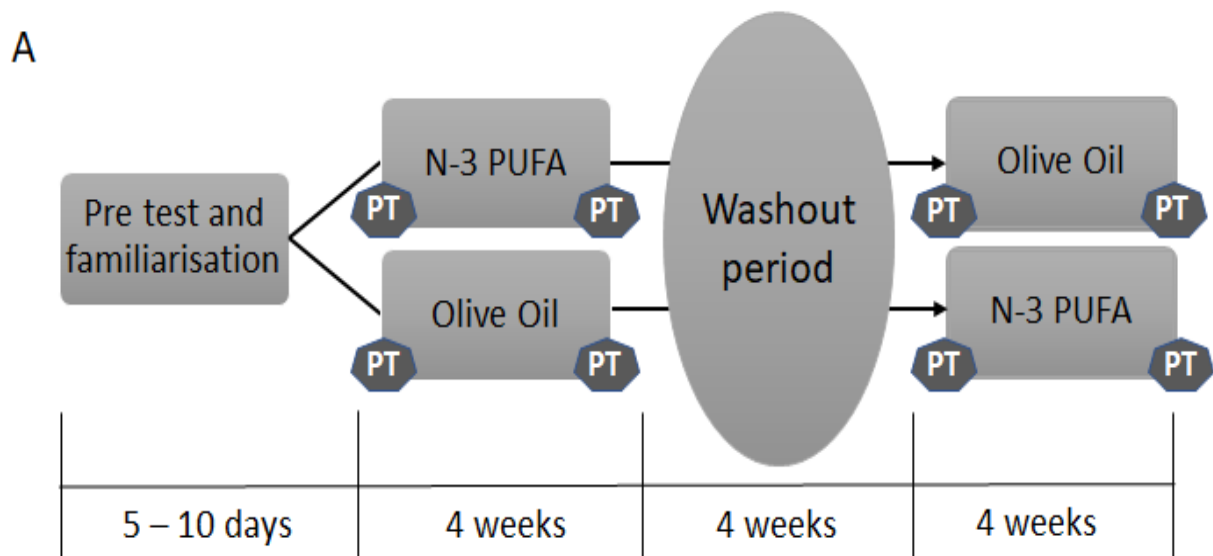
Figure 3 – Schematic Representation of Study Design. PT: Performance Test

The supplementation periods were separated by a four-week washout period. During the testing period, participants were asked to maintain and keep a record of their physical activity, and this was analysed to ascertain whether training load was consistent across the two trials and to ensure participants were rested the day prior to the performance test's. The record logged the type of activity, duration, distance, and session Rating of Perceived Exertion (sRPE).

Supplementation

Both placebo (Olive Oil, Puritan's Pride, New York, USA) and n-3 PUFA (Holland and Barrett, Warwickshire, UK) were provided in capsule form. Participants were instructed to take six capsules per day (two with breakfast, lunch and dinner) providing 5.7 g of n-3 PUFA and 0.01 g per day of α-Tocopherol or 6 g per day of olive oil. The dose of α-Tocopherol used to stabilise the PUFAs within the supplement is substantially lower than in studies demonstrating a potent antioxidant

effect in the context of blunting training adaptations (Ristow *et al.*, 2009). The composition of the capsules is reported in Table 5. The contents of the capsules were independently verified according to the method detailed in 3.4.2. The n-3 PUFA dose was chosen based on previous findings (Metherel *et al.*, 2009) showing a similar dose in males can induce significant changes over 4 weeks in the lipid profile of human blood. Many previous studies investigating the effects of n-3 PUFAs on sports performance have typically used olive oil as a placebo (Gray *et al.*, 2014; Lewis *et al.*, 2015; Mickleborough *et al.*, 2015) as olive oil does not appear to have an effect on performance and the associated physiological variables (Peoples *et al.*, 2008; Lewis *et al.*, 2015; Jannas-Vela *et al.*, 2017). Compliance for taking the capsules was monitored by capsule counting upon return of supplement pots by participants and verified with blood sampling and analysis of whole blood fatty acid incorporation.

Table 5 – Composition of n-3 PUFA capsules and Olive Oil capsules as stated by manufacturer and EPA and DHA average values from random batch sampling independently verified by GC-MS

	n-3 PUFA as stated by the manufacturer	Direct determination of n-3 PUFA through the in-house GC-MS method	Olive Oil
EPA (mg)	680 mg	601 mg	0
DHA (mg)	272 mg	253 mg	0
Oleic Acid (mg)	0 mg	0 mg	1000 mg
α-Tocopherol (mg)	1.85 mg	Not measured	0

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; GC-MS, gas chromatography-mass spectrometry

Pre-test and Familiarisation

Participants underwent an anthropometric assessment including height, body mass (section 3.2.2) and 8 skinfold measurements (triceps, subscapular, biceps, iliac crest, supraspinale, abdominal, thigh and calf) according to the International Society for the Advancement of Kinanthropometry (ISAK), by a level 1 ISAK accredited anthropometrist.

Maximal work rate (Wmax) and $\dot{V}O_2$ max were determined using a graded exercise test according to Amann, Subudhi, & Foster (2004). Following a warm-up period of 5 min at 100 W, workload was increased by 50 W every 3 min until volitional exhaustion. Participants pedalled at a self-selected pedal cadence between 80 and 120 rpm and were given verbal encouragement throughout to maintain their preferred pedal cadence. The Wmax was determined using the formula:

Wmax = Wout + [(t/180) x 50]

Wout is the workload of the last completed stage and t is the time in seconds in the final stage. The Lode Excalibur Sport electromagnetically braked ergometer (Lode B.V, Groningen, Netherlands) was used in hyperbolic mode for the Wmax test. Hyperbolic mode allows a constant pre-set work rate to be imposed on the participant, independent of the cadence. Volitional exhaustion was deemed to have occurred when participants dropped 20 rev·min^{-1} below their self-selected pedal cadence, at which point they were instructed to stop pedalling. VO_2 max was determined using primary criteria of a plateau in oxygen uptake despite an increase in workload and secondary criteria of heart rate ≥ 90% of age-predicted maximum, blood lactate ≥ 8mmol·L^{-1} and respiratory exchange ratio ≥ 1.10 (Howley, Bassett and Welch, 1995).

On completion of the graded exercise test, participants were given a 10min rest period before performing a 15-min familiarisation session. This was conducted to improve the reliability of the Performance Tests, and involved the participant cycling at their required workload (70% Wmax) in linear mode.

Performance Trial

All performance tests were conducted in the morning (7 – 9am) following a 10 hour overnight fast. Participants' subsequent tests were performed at the same time of day to minimise diurnal variation, with stable climatic conditions (20 - 22°C and humidity between 45 – 55%). Participants were asked to refrain from strenuous exercise and the consumption of alcohol and caffeine (<24 h) and to arrive at each trial in a fully rested and hydrated state. Participants were advised to stay hydrated by drinking water *ad-libitum* throughout the tests.
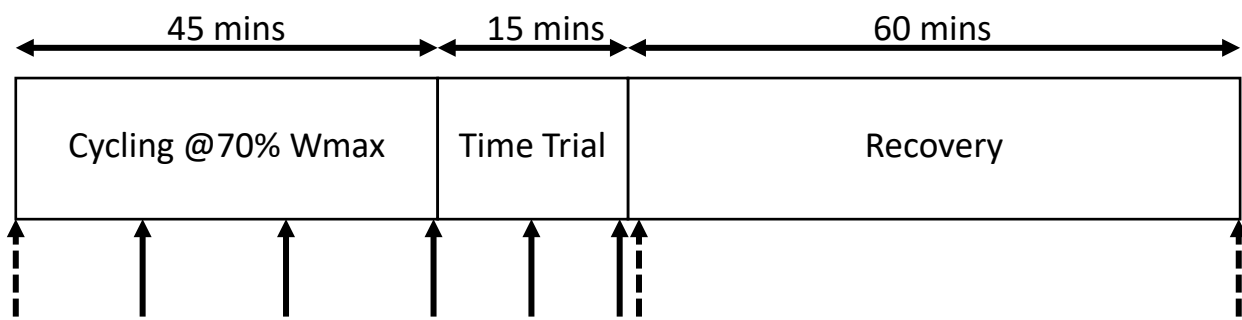
Figure 4 – Performance Trial Protocol. Blood sample is represented by dashed arrow expired air collection, HR and RPE are represented by full arrow.

The performance trial consisted of 45 min submaximal cycling at 70% Wmax, followed by 15 min time trial (Figure 4). This test was chosen as it is reliable, has a low CV (3.49%) (Jeukendrup *et al.*, 1996) and allows for physiological variables to be analysed during steady state exercise and during time trial performance. During the 70% Wmax phase the electromagnetically braked ergometer was in the hyperbolic mode, so that work rate (70% Wmax) was independent of pedalling rate. The ergometer was changed to linear mode for the 15-min time trial so that with increasing pedalling rate the work rate increased. This was to allow the participant to pace themselves and try to maintain a high cadence over the time trial to maximise power output. The linear factor was set so workload was 70% of Wmax if they pedalled at the mean pedalling rate observed in the $\dot{V}O_{2max}$ test using the following formula:

$W = [L \times (RPM)^2]$

where the RPM is the mean RPM calculated from the $\dot{V}O_{2max}$ test.

The participants were given verbal encouragement to perform as much work as possible in the time trial. The same investigator provided encouragement to the participant across all tests. The cycle ergometer was connected to a computer that measured work rate every second and the mean power performed over the 15-min time trial was calculated as a measure of performance. The participants received no feedback on the power output, heart rate or cadence, and were only provided feedback through elapsed time, to avoid test-retest influence.

HR, RPE and 1-minute expired air samples (into douglas bags) were collected at time points depicted by solid arrows denoted on Figure 4. Minute ventilation (V_{ESTPD}), $\dot{V}O_2$ and Carbon dioxide production ($\dot{V}CO_2$) and Respiratory Exchange Ratio (RER) were determined using a Servomex 1500 (Servomex, UK) and Harvard Dry Gas Meter (Harvard Apparatus, Massachusetts, USA).

Blood Sampling

Prior to performance trials, an intravenous cannula was inserted into an antecubital vein of the non-dominant arm for the collection of blood samples (section 3.3.1). Blood samples were taken prior to the start of exercise, immediately following the performance test and 60 min following exercise cessation (Figure 4). Serum was prepared (section 3.3.2) for analysis of markers of muscle damage and inflammatory cytokines at all time points. In the baseline sample, an extra 2 x 10ml EDTA vacutainers were collected for the isolation of PBMCs (section 3.3.3).

Blood Analysis

Determination of serum CK and Mb is described in section 3.4.4. Serum IL-6 and was determined according to section 3.4.5. Determination of fatty acids in the PBMCs and whole blood is described in section 3.4.2. Cell counts were measured according to section 3.4.1.

Data Analysis

Statistical analysis was performed according to section 3.5. For the main analysis, all dependent variables (HR, RPE, $\dot{V}O_2$, RER) were analysed using a 2 (supplement) x 2 (trial) x 2 (submaximal) or 3 (TT) (time) repeated-measures ANOVA. Time trial mean power, PBMC and whole blood total fatty acids were analysed using a 2 (supplement) x 2 (trial) ANOVA. Dietary and training data were analysed using a paired samples t-test.

4.3 Results

No differences were found in participants' dietary intake for total kilocalories, total grams of protein, carbohydrate and fat between all phases of the study in the 3-day food diaries completed prior to each trial. No differences were found in mean training duration (1303 ± 899 vs 1398 ± 617 mins), training distance covered (369 ± 270 vs 467 ± 285 km) or sRPE (14 ± 1 vs 13 ± 2) for n-3 PUFA trial vs olive oil trial, respectively.

Fatty acid composition of Whole Blood and PBMCs

Compliance, as estimated from mean return capsule count, was 89% for both n-3 PUFA and olive oil, with no difference between the two supplements (p = 0.955). Three participants experienced the adverse effect of fishy burps during the n-3 PUFA supplementation phase. There was no difference in saturated, monounsaturated, n-6 or n-3 PUFAs expressed as % total fatty acids in whole blood or in PBMCs, pre- n-3 PUFA or olive oil supplementation. Following 4 weeks of n-3 PUFA supplementation, there was an increase in % total n-3 PUFAs in the whole blood (p = 0.043), however, there was no difference in % total n-3 PUFAs in the PBMCs (Table 6). Four weeks of olive oil supplementation did not alter the % total n-3 PUFAs in the whole blood or PBMCs. Interestingly, following n-3 PUFA supplementation, EPA increased significantly in PBMCs (*p* = 0.005, Table 6) and approached significance in DHA (*p* = 0.056). In the whole blood there was a significant increase in DHA after n-3 PUFA supplementation (*p* = 0.001), but not EPA (p = 0.104) (Table 6).

*Table 6 – Whole blood and peripheral blood mononuclear cell (PBMC) fatty acid profile changes following 4 weeks dietary supplementation with n-3 PUFA and olive oil (n = 10). Data expressed as % total fatty acids, mean ± SD. *Significantly different to before (p < 0.05). EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.*

	Whole Blood		PBMC	
	Before	After	Before	After
Saturated fatty acids				
n-3 PUFA	63.58 ± 2.42	63.01 ± 3.11	70.81 ± 21.76	62.29 ± 19.75
olive oil	65.26 ± 2.67	62.97 ± 2.11	82.47 ± 19.94	77.52 ± 21.62
Monounsaturated fatty acids				
n-3 PUFA	19.93 ± 2.95	18.50 ± 1.83	5.48 ± 3.58	7.64 ± 2.90
olive oil	18.24 ± 3.26	19.72 ± 1.95	4.98 ± 3.68	4.84 ± 4.05
n-6 polyunsaturated fatty acids				
n-3 PUFA	14.83 ± 2.37	14.77 ± 2.41	19.41 ± 15.46	20.40 ± 12.52
olive oil	15.03 ± 2.25	15.48 ± 1.67	9.85 ± 12.61	13.35 ± 14.90
n-3 polyunsaturated fatty acids				
n-3 PUFA	1.67 ± 0.99	3.72 ± 1.22*	3.84 ± 2.83	10.58 ± 4.64
olive oil	1.46 ± 0.84	1.83 ± 1.23	3.00 ± 2.33	4.45 ± 3.01
EPA				
n-3 PUFA	1.34 ± 0.97	2.93 ± 1.13	0.66 ± 0.55	3.32 ± 1.59*
olive oil	1.09 ± 0.87	1.41 ± 1.15	0.72 ± 0.24	1.12 ± 0.37
DHA				
n-3 PUFA	0.32 ± 0.17	0.79 ± 0.18*	1.64 ± 1.37	3.00 ± 1.27
olive oil	0.38 ± 0.09	0.42 ± 0.15	1.15 ± 0.96	1.73 ± 1.32

Performance Trial

Participants were able to maintain a mean power output of 70% Wmax during the 45 min submaximal part of the performance test in all trials, this equated to an oxygen consumption of 74 ± 8% $\dot{V}O_2$max. There was no difference (p = 0.07) in the mean power after taking n-3 PUFA supplementation (pre 239 ± 34 W vs post 243 ± 33 W) compared with olive oil supplementation (pre 246 ± 38 W vs post 235 ± 36 W, Figure 5). No differences were observed for $\dot{V}O_2$, HR, RER or RPE during the 45-min steady-state cycling or during the 15 min time trial prior to or following either n-3 PUFA or olive oil supplementation (Table 7). There were no correlations between the

change in n-3 PUFA levels and time trial performance, V̇O$_2$ or HR following 4 weeks n-3 PUFA supplementation.
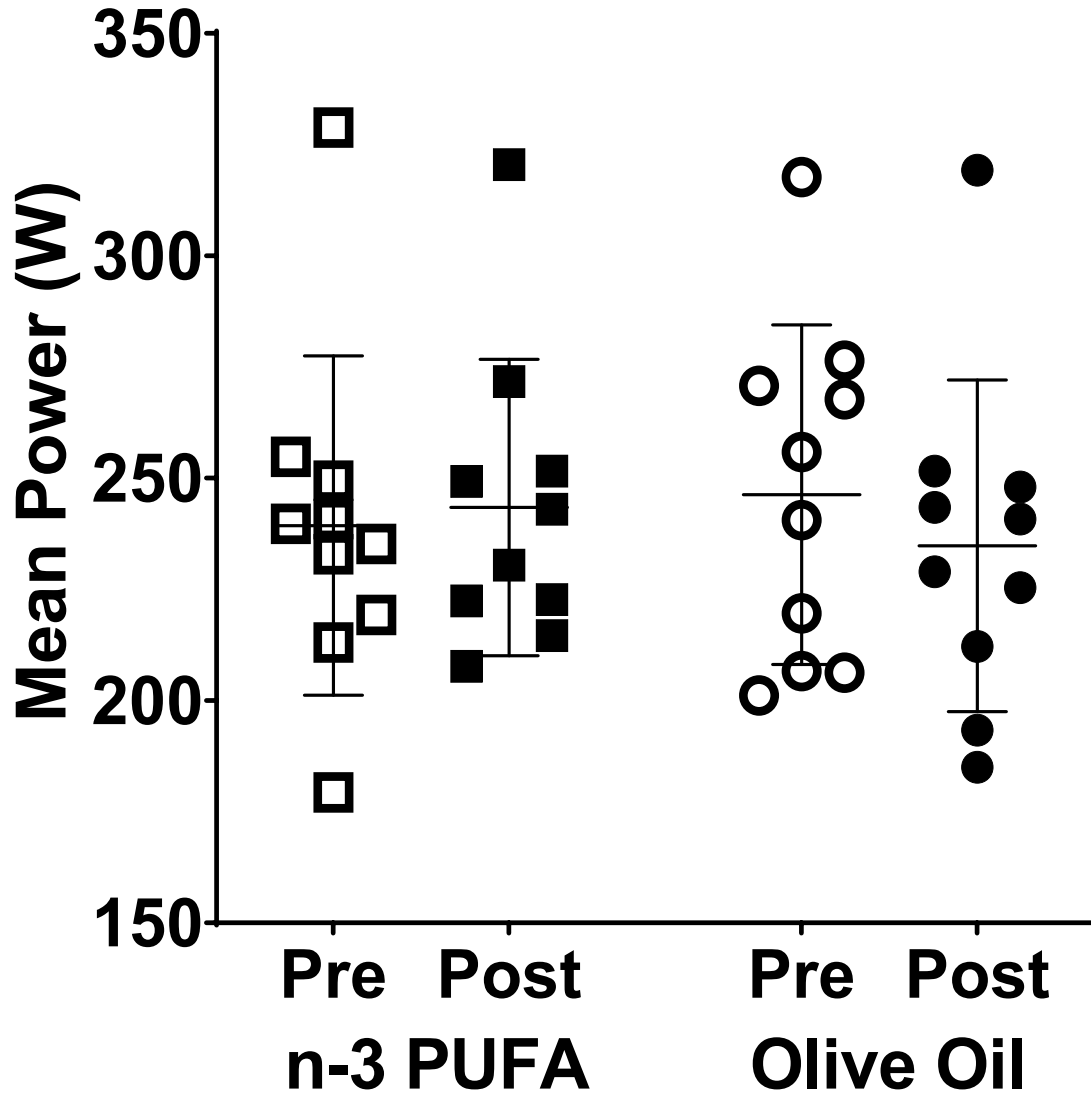
Figure 5 – Individual and mean (±SD) power (W) during a 15-minute time trial, pre- and post-4 weeks n-3 PUFA and olive oil supplementation (n = 10).

Table 7 – Physiological parameters during 45 min steady state cycling and 15-minute time trial before and after 4 weeks of n-3 PUFA or olive oil supplementation (n = 10). Values represent means ± SD. P values represent repeated measures ANOVA analysis. 14, 39, 44, 7 and 14 represent timings of $\dot{V}O_2$, HR, RER and RPE collections.

Timepoint	$\dot{V}O_2$ (L·min⁻¹)		Heart Rate (b·min⁻¹)		RER		RPE	
Supplement	Before	After	Before	After	Before	After	Before	After
Steady State								
14								
n-3 PUFA	2.82 ± 0.43	2.77 ± 0.36	146 ± 13	147 ± 10	0.97 ± 0.06	0.98 ± 0.08	12 ± 1	12 ± 1
olive oil	2.91 ± 0.44	2.90 ± 0.46	150 ± 9	149 ± 14	0.98 ± 0.05	1.00 ± 0.06	12 ± 1	12 ± 1
29								
n-3 PUFA	2.86 ± 0.46	2.79 ± 0.44	153 ± 13	153 ± 11	0.93 ± 0.06	0.98 ± 0.12	14 ± 2	13 ± 1
olive oil	2.97 ± 0.46	2.88 ± 0.47	155 ± 11	156 ± 15	0.96 ± 0.07	0.98 ± 0.07	14 ± 2	14 ± 1
44								
n-3 PUFA	2.91 ± 0.44	2.82 ± 0.42	153 ± 15	155 ± 11	0.92 ± 0.05	0.98 ± 0.11	15 ± 2	14 ± 1
olive oil	3.02 ± 0.48	2.92 ± 0.43	158 ± 13	154 ± 14	0.95 ± 0.06	0.97 ± 0.08	14 ± 2	14 ± 2
P value	0.73		0.06		0.27		0.58	
Time Trial								
7								
n-3 PUFA	3.20 ± 0.53	3.26 ± 0.68	159 ± 11	165 ± 8	0.95 ± 0.04	0.99 ± 0.06	16 ± 2	16 ± 2
olive oil	3.35 ± 0.48	3.15 ± 0.54	161 ± 12	159 ± 10	0.98 ± 0.06	0.97 ± 0.05	16 ± 2	16 ± 2
14								
n-3 PUFA	3.43 ± 0.59	3.26 ± 0.68	164 ± 10	176 ± 7	0.96 ± 0.05	0.97 ± 0.05	18 ± 1	18 ± 2
olive oil	3.61 ± 0.48	3.40 ± 0.67	168 ± 11	173 ± 9	0.95 ± 0.06	0.98 ± 0.03	18 ± 2	18 ± 1
P value	0.76		0.58		0.10		0.84	

Markers of Inflammation and Muscle Damage

There was a significant increase in plasma IL-6 in response to exercise ($p < 0.001$) with values immediately post time trial higher compared to both baseline ($p < 0.001$) and 1-hour post time trial, additionally, 1-hour post time trial was significantly higher than baseline ($p < 0.001$). No supplementation or interaction effects were noted (Figure 6). No correlations were found between the change in n-3 PUFA incoporation and exercise-induced alterations in IL-6. There was a significant increase in CK ($p = 0.002$) and Mb ($p < 0.001$) in response to exercise (Figure 7 and Figure 8), however, this was not associated with supplementation.
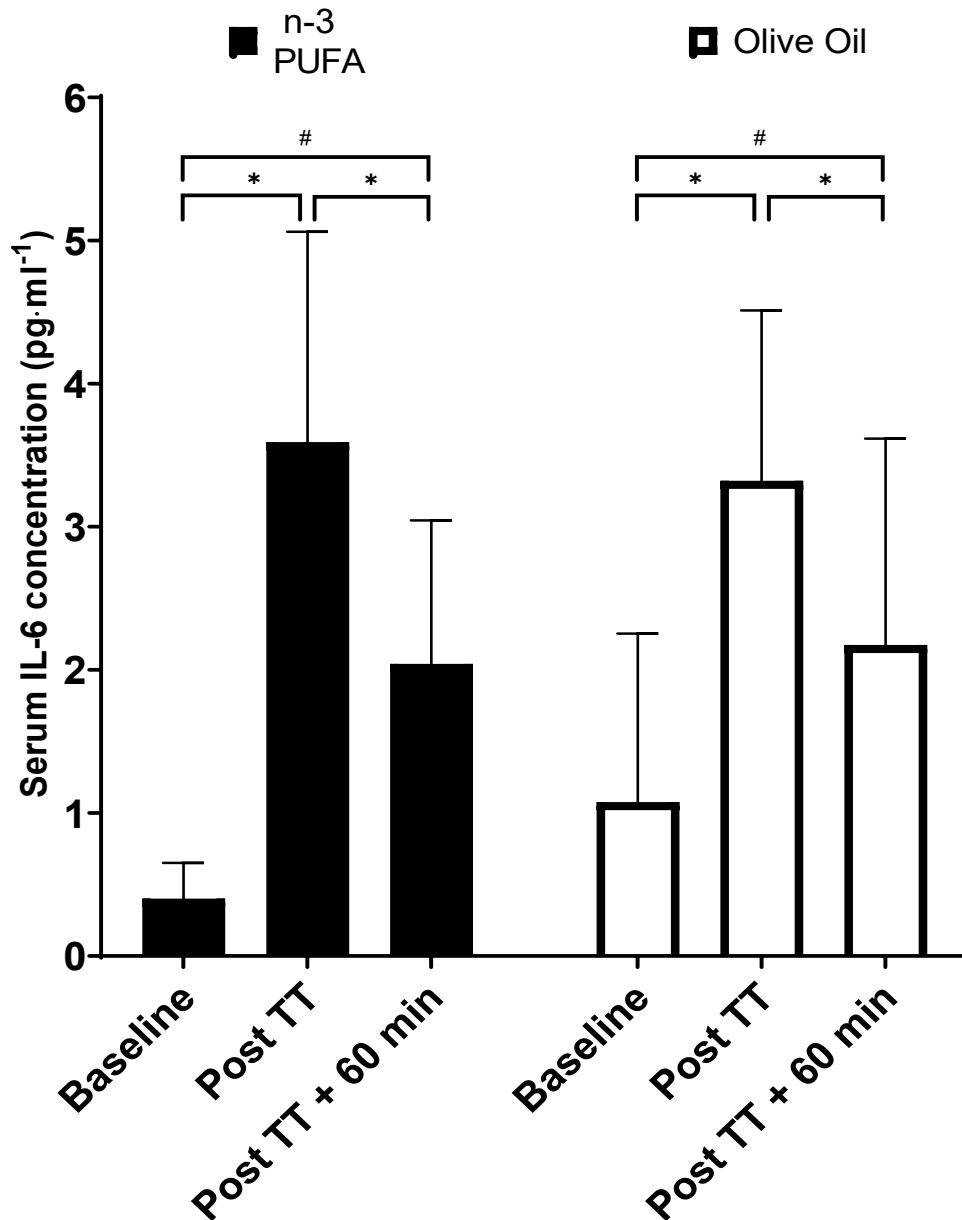
Figure 6 – Plasma volume-adjusted serum interleukin-6 (IL-6) levels at baseline, immediately post time trial (Post TT) and 1-hour post time trial (Post TT + 60min) following 4 weeks n-3 PUFA (filled bars) and olive oil (unfilled bars) supplementation (n = 10). Values represent means ± SD. #Significantly (P < 0.001) different from baseline and *Significantly different (P < 0.001) different from immediately post time trial.
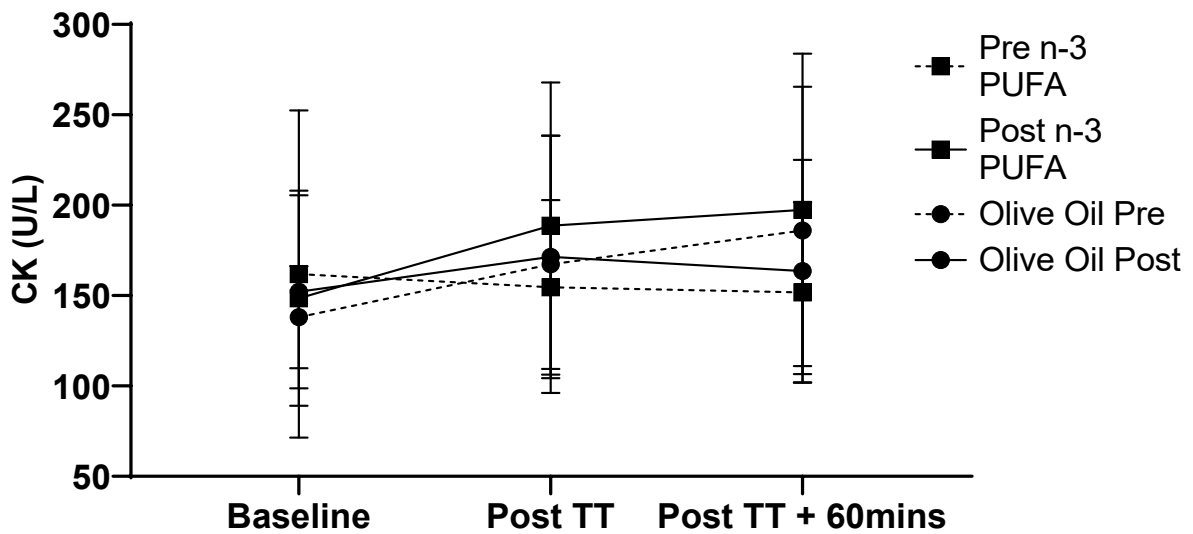
Figure 7 – Plasma volume-adjusted serum creatine kinase (CK) levels at baseline, immediately post time trial (Post TT) and 1-hour post time trial (Post TT + 60min) pre (dashed line) and post (solid line) 4 weeks n-3 PUFA (squares) and olive oil (circles) supplementation (n = 10). Values represent means ± SD.
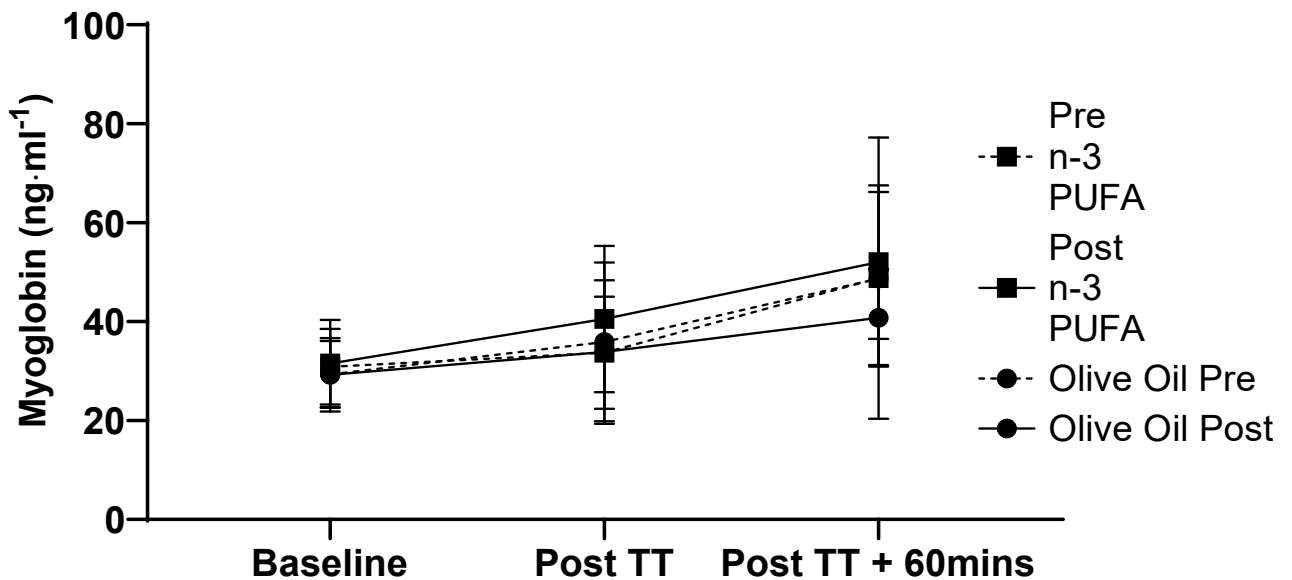
Figure 8 – Plasma volume-adjusted serum Myoglobin levels at baseline, immediately post time trial (Post TT) and 1-hour post time trial (Post TT + 60min) pre

(dashed line) and post (solid line) 4 weeks n-3 PUFA (squares) and olive oil (circles) supplementation (n = 10). Values represent means ± SD.

4.4 Discussion

This study investigated the effects of n-3 PUFAs on cycling performance and changes in markers of both inflammation and muscle damage using a randomised, double-blind, placebo-controlled, crossover design. Our results extend and confirm the conclusions of Nieman *et al.* (2009) and Toft *et al.* (2000) who both demonstrated incorporation of n-3 PUFAs into plasma and peripheral blood mononuclear cells, respectively, but did not find an improvement in performance in trained individuals. The present study had a stronger research design, using a higher dosage of n-3 PUFA supplementation and an exercise intensity more likely to be employed in the training programs of trained cyclists. Four weeks of n-3 PUFA supplementation at a daily dose of 5.7 g (EPA: 4.1 g, DHA: 1.6 g) significantly increased the percentage of n-3 PUFAs in the whole blood and increased EPA levels in the PBMCs and DHA levels in whole blood (Table 6). However, this had no impact on cycling performance or exercise-induced changes in markers of inflammation and muscle damage in trained male cyclists. n-3 PUFA supplementation did not affect HR, RPE, RER or $\dot{V}O_2$ during either submaximal or maximal exercise during a time trial. Markers of exercise-induced inflammation and muscle damage were also unaffected by n-3 PUFA supplementation.

Reductions in submaximal HR (Peoples *et al.*, 2008; Buckley *et al.*, 2009; Boss *et al.*, 2010) and $\dot{V}O_2$ (Peoples *et al.*, 2008; Kawabata *et al.*, 2014) in previous n-3 PUFA supplementation studies have been shown, which would suggest an effect on performance, although this was not identified in the present study. A potential rationale for the lack of effect on the physiological parameters measured during submaximal exercise is that in previous studies exercise intensity has generally been lower (i.e. 55% peak workload) than the 70% peak workload used in the present study. Despite finding reductions in submaximal heart rate following n-3 PUFA supplementation, Macartney *et al.* (2014) found no differences in heart rate between conditions during repeated sprints. Together with findings from the present study, this data suggests that n-3 PUFA supplementation is only effective at decreasing the

heart rate response during submaximal exercise, not at higher exercise intensities such as the present study. It is possible that n-3 PUFA supplementation increases fat oxidation during lower intensity exercise, reducing carbohydrate oxidation (Delarue *et al.*, 1996), therefore allowing individuals to use predominantly fats for energy at a higher absolute work rate, meaning they could essentially go for longer due to the greater fat stores than carbohydrate (Achten and Jeukendrup, 2004) and consequently heart rate is lowered along with perceived effort. However, only increases in basal fat oxidation, not fat oxidation during exercise, have been previously reported following 3 weeks of n-3 PUFA supplementation (Couet *et al.*, 1997). Moreover, there was no difference in the RER in the present study, suggesting fat and carbohydrate oxidation were unaffected by supplementation.

Calder (2015) suggested that the anti-inflammatory properties of n-3 PUFAs may ameliorate exercise-induced elevations in IL-6. Despite an increase in IL-6 observed immediately after the cycling performance test (Figure 6), the responses were not altered following 4 weeks of n-3 PUFA or placebo supplementation. Nieman *et al.* (2009) also reported no effect of 2.4g/d n-3 PUFA supplementation for 6 weeks on IL-6. Bloomer *et al.* (2009) found reduced resting levels of inflammatory biomarkers following n-3 PUFA supplementation for 6 weeks, but no effect on markers of inflammation or oxidative stress after a 60 minute treadmill climb using a weighted backpack. It is possible that the exercise stimulus used in the present study was insufficient to cause debilitating increases in IL-6 making the effects of n-3 PUFA supplementation too small to detect. The muscle damage markers CK and Mb followed the same pattern displaying a small increase in response to exercise, but this was unaffected by n-3 PUFA supplementation. Previous studies employing much longer durations of exercise such as marathons (Toft *et al.*, 2000) and ultra-endurance (Goussetis *et al.*, 2009; Comassi *et al.*, 2015) events have demonstrated much greater increases in levels of IL-6. It is likely that n-3 PUFA is more effective at reducing inflammation in these exercise protocols (Santos *et al.*, 2013).

Studies have indicated that long-term exercise can influence host defense in response to acute exercise (Rhind *et al.*, 1996). The participants in the present study were trained ($\dot{V}O_2$ max = 54 ± 5 ml·kg·min^{-1}), which may explain why markers of both muscle damage and inflammation only increased minimally following exercise. It is

likely that they were accustomed to the exercise intensity employed in this study. Their bodies have adapted to overcome the exercise-induced inflammation through years of training (Peake *et al.*, 2017), and it is likely that a small modification, such as 4 weeks n-3 PUFA supplementation, is unable to further overcome the inflammation. Importantly, it is difficult within a laboratory-based setting to replicate the overreached and over-trained states that athletes often experience because of their training. Previous work has identified that short-term intensified training can depress immune cell function at rest (Gleeson, Nieman and Pedersen, 2004). In the current study, cyclists were rested (to ensure standardisation) prior to commencing the performance tests, therefore likely to have lower levels of circulating markers of muscle damage inflammation. Trained cyclists may rarely be in this situation and therefore the potential benefit of n-3 PUFA on performance could be context dependent. Furthermore, the lack of effect of n-3 PUFA supplementation on performance and acute phase markers may be due to the large inter-individual variability in the capacity to incorporate n-3 PUFAs into the cell membrane, as discussed above. Monitoring individual incorporation rates, as well as analyzing a wider array of systemic inflammatory markers to identify patterns or clusters that provide an inflammatory signature may enable a greater insight into the exercise-induced inflammatory response (Calder *et al.*, 2013).

To monitor incorporation of n-3 PUFAs, we monitored the fatty acid composition of whole blood and cells with a specialised function: PBMCs, whereas previous studies have predominantly measured incorporation in plasma (Andrade *et al.*, 2007; Gray *et al.*, 2012; Allaire *et al.*, 2016; Tsuchiya *et al.*, 2016) and erythrocytes (Peoples *et al.*, 2008; Kawabata *et al.*, 2014; Da Boit, Sibson, Sivasubramaniam, *et al.*, 2016). Altering fatty acid composition of the diet (Faber *et al.*, 2011) or consuming n-3 PUFA supplements can effect PBMC phospholipid composition (Thies *et al.*, 2001; Rees *et al.*, 2006) and total lipid composition in humans (Yaqoob *et al.*, 2000; Browning *et al.*, 2012, 2014; Walker *et al.*, 2014). The present results support previous literature that collectively suggests a time and dose dependent effect of n-3 PUFA supplementation on cell membrane incorporation rates (Yaqoob *et al.*, 2000; Rees *et al.*, 2006).

PBMC lipid composition is closely controlled by absorption and metabolism rather than simply a reflection of dietary intake, and incorporation of PUFAs into the PBMCs strongly influences the immune response (Kew *et al.*, 2003). Despite an identical dose of n-3 PUFA being given to all participants, there was a large variability in the incorporation of n-3 PUFAs after supplementation indicated by the large standard deviations in (Table 6). In the present study, the repeated-measures design controlled for the inter-individual variability in incorporation of n-3 PUFAs, which is not accounted for in previous studies using a parallel groups design. However, it was not possible to provide participants with an n-3 PUFA supplementation based on their baseline n-3 PUFA levels due to the lack of rapid GC-MS testing available on the day of their initial blood sample. Moreover, the rate of incorporation within a participant was unknown and could not be established until the individuals had taken supplementation.

Classic crossover design studies with extended supplementation washout periods are prohibitive, particularly in exercise studies where training over a lengthy washout period could result in improved performance, confounding the effect of the supplementation. In the present study, a 4 week washout period was chosen in order to allow time for n-3 PUFA to return to baseline levels (Metherel *et al.*, 2009), whilst minimizing the impact of training effects due to study duration. However, erythrocyte EPA has been shown to only return to baseline after 18 weeks washout period and DHA was still elevated above baseline at 18 weeks following a fish-based diet supplemented with 5g fish oil (Brown, Pang and Roberts, 1991). Data from the current study are in accordance with Thies *et al* (2001), suggesting the washout of n-3 PUFA from PBMCs is quicker than from erythrocytes, as baseline levels of both EPA and DHA prior to n-3 PUFA and placebo supplementation were almost identical in the five participants that were randomised to n-3 supplementation first.

Whilst it may be suggested that a sample size of ten is small and may lack statistical power, this was mitigated by performing a power calculation to determine the sample size for the investigation. However, the calculation was based on submaximal heart rate and not on the primary outcome measure of this study; mean power, due to a lack of consensus in the literature. It was therefore difficult to detect a meaningful difference in the primary outcome based on the sample size suggested. The results

from the power analysis suggested that the study was only powered to detect large, not small effects in submaximal heart rate and given the variability of heart rate and the numerous variables that influence heart rate it is unsurprising that no difference in heart rate was detected.

A repeated measures crossover design was employed in this study and a performance test was completed prior to any supplementation period, giving a true baseline to compare with the post-supplementation data. Burke and Peeling (2018) stated that crossover designs require fewer athletes than a parallel groups design when comparing methodologies for investigating performance changes with supplement use and that fully controlled studies with a baseline and treatment measurement for both experimental and placebo conditions are the best design. Previous repeated measures investigations into n-3 PUFA supplementation and cycling performance have generally given participants either an n-3 PUFA or placebo supplement for some time and then assessed the difference between supplements rather than comparing to a performance test conducted before any supplementation (Delarue, Labarthe and Cohen, 2003), which provides little indication of whether the supplementation affected individual performance. Alternatively, they have used an independents groups design (Nieman *et al.*, 2009; Kawabata *et al.*, 2014; Macartney *et al.*, 2014; Lewis *et al.*, 2017), which does not account for individual differences in incorporation of n-3 PUFA.

In conclusion, this study extends the literature using a higher dose of n-3 PUFA and a stronger research design, despite this there was no improvement in cycling performance, or the physiological variables associated with improved cycling performance. Whilst it is known there is a threshold dosage for n-3 PUFA of at least 2g/d to observe an anti-inflammatory effect (Calder, 2015) and we used a dose in excess of this, with the incorporation being confirmed by a significant increase in the % of n-3 PUFAs in the PBMCs and whole blood. There may also be a threshold for levels of inflammation and muscle damage caused by exercise, below which n-3 PUFA may be ineffective.

Chapter 5: Fish oil supplementation and eccentric training does not improve exercise performance or recovery

5.1 Introduction

The beneficial effects of resistance training, including improving strength, power and overall athletic performance, have led to it becoming an integral part of an athletes training (Faigenbaum *et al.*, 2016). Eccentric training, in particular, is a potent stimulus for enhancements in muscle mechanical function and skeletal muscle adaptation and subsequent improvements in performance (Douglas *et al.*, 2017). Whilst it is normal for athletes to undertake eccentric exercise, it is becoming more popular amongst the general population due to the greater levels of muscle hypertrophy compared with concentric training (Schoenfeld *et al.*, 2017), however, this is associated with greater exercise-induced muscle damage (EIMD) (Clarkson and Hubal, 2002; Peake, Nosaka and Suzuki, 2005). Muscle damage is greater, indicated by a greater loss of force, following eccentric contractions of a longer length (Newham *et al.*, 1988). Consequently, eccentric contractions of the quadriceps in this chapter were performed through a distance of 80 degrees to ensure a large proportion of the contraction was performed when the muscle was at a longer length. Furthermore, this study was conducted on non-resistance trained individuals as muscle damage should be more pronounced in these individuals.

Lowe *et al.* (1995) found that the presence of leukocytes in muscle tissue was associated with increased protein degradation after eccentric exercise in mice and despite the degradation of damaged structures being well regulated, it is likely that some intact structures within the muscle are also affected by scavenger phagocytes, and the repair process may have caused some delayed and additional muscle damage. In humans, this same inflammatory response to eccentric exercise has been indicated (Peake, Nosaka and Suzuki, 2005). Current practice in athletes is generally to avoid anti-inflammatory medication due to the requirement of the inflammatory process to induce skeletal muscle adaptation (Lilja *et al.*, 2018),

making recovery from exercise (particularly eccentric exercise) longer and more painful. An alternative solution would be to supplement with n-3 PUFAs due to their anti-inflammatory properties (Calder, 2015). The incorporation of n-3 PUFAs into the muscle tissue could potentially reduce this infiltration of leukocytes and speed up the recovery following muscle damage, whilst not interfering with skeletal muscle adaptation.

There is conflicting evidence in the literature evaluating whether n-3 PUFA supplementation can reduce the degree of EIMD and inflammation (section 2.8.1 and 2.8.2). Whilst their appears to be a sound rationale for the reduction, this is not translated into all studies that have investigated n-3 PUFA supplementation. A further issue with the majority of the previous literature, detailed in chapter 2, is that baseline data (i.e. prior to supplementation) was not collected to allow for the comparison from pre to post supplementation. Therefore, differences between supplements are identified post supplementation without accounting for any differences in the response to muscle damaging exercise there may have been between groups prior to supplementation. Whilst markers of muscle damage such as MVCs, visual analogue scales (VAS) for soreness and levels of myofibril proteins are shown to be reliable within person, there are large differences in these markers between individuals, indicating a pre-supplementation trial is pertinent.

The overarching aim of this study is to investigate the effects of n-3 PUFA supplementation and the effects of n-3 PUFA combined with eccentric training on exercise performance and recovery in untrained men. Indirect markers of muscle damage, inflammation and quadriceps fatigue will be measured before and up to 48 hours after muscle damaging exercise, before and after a supplementation period and before and after a period of eccentric training combined with supplementation. It is hypothesised that supplementation with n-3 PUFA compared to a placebo, would significantly reduce markers of muscle damage, inflammation, quadriceps fatigue and result in a smaller drop in exercise performance following eccentric muscle damaging exercise. Given evidence suggests interfering with muscle damage and inflammation may attenuate adaptation to training particularly when NSAIDs are taken by young adults (Trappe *et al.*, 2002; Lilja *et al.*, 2018), this chapter also addresses whether n-3 PUFA supplementation affects training adaptations indicated

by changes in performance. Whilst NSAIDs are cyclooxygenase (COX) inhibitors (Trappe and Liu, 2013) and can suppress MPS after eccentric resistance training (Trappe *et al.*, 2002), n-3 PUFAs are thought to dampen inflammation rather than blocking the COX pathway, potentially allowing greater MPS after resistance exercise. Therefore, the second hypothesis of this chapter is that n-3 PUFA supplementation and eccentric training will result in greater improvements in performance indicated by improved strength and power compared with a placebo and eccentric training.

5.2 Method

The experimental protocol followed the Declaration of Helsinki principles and was approved by Loughborough University Ethics Human Participants sub-committee (Study ID: R15-P124) and registered as a clinical trial on www.clinicaltrial.gov (Study ID: NCT03259412). Prior to study entry, participants were informed that the study was investigating the effects of fatty acid supplementation, therefore they were unaware that the aim of the study was to specifically examine n-3 PUFAs as it is recognised that n-3 PUFA supplementation is difficult to blind due to the fishy taste often experienced and this was a limitation of chapter 4.

Experimental Design

A repeated measures design, with parallel pair matched groups for isometric and eccentric quadricep strength was used. The study consisted of a familiarisation session and three experimental trials. The study design is shown in Figure 9. Participants attended their first formal testing session (Trial A) 7 ± 3 days following familiarisation. This testing session was repeated after 21 days supplementation (Trial B) and again after 8 weeks eccentric training and supplementation (Trial C). Details of what this encompassed are shown in Figure 10.
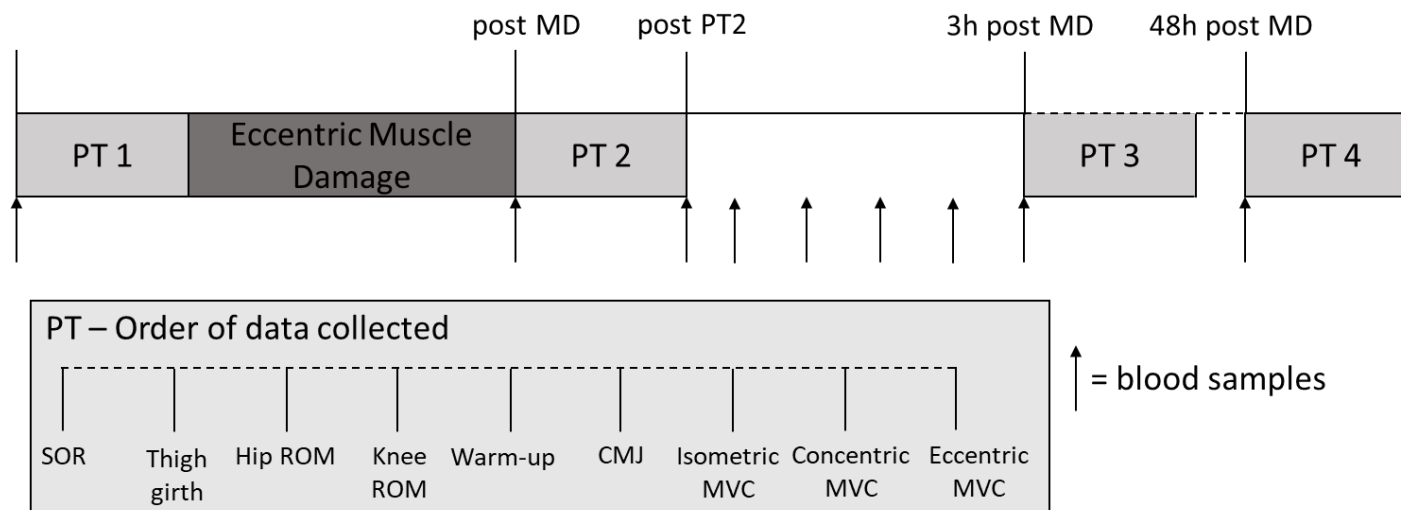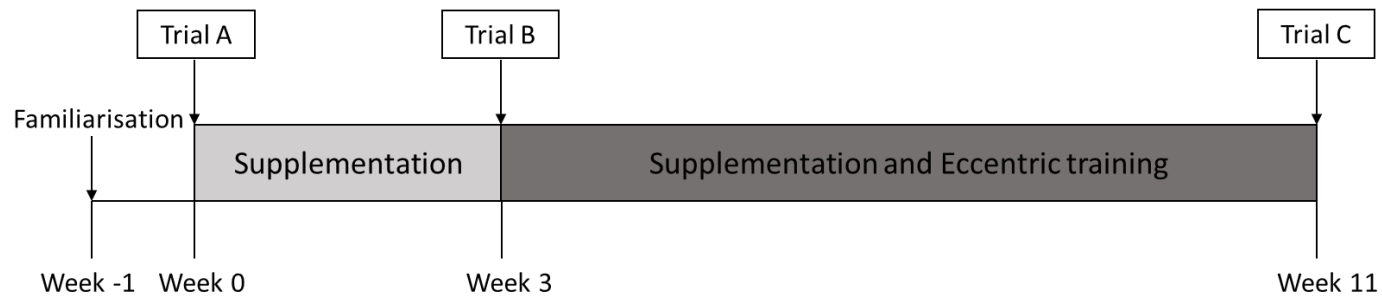
CMJ, Countermovement jump; MD, Muscle-damaging exercise; MVC, Maximal Voluntary Contraction PT, Performance Test; ROM, Range of motion; SOR, muscle soreness

Participants

A power analysis was not conducted to determine sample size for this study due to the limited time and resources available to complete the study. Instead, a time period was set and as many participants were recruited and tested during this period. Eighteen physically active but non-resistance trained males volunteered for the study and provided written informed consent. Prior to participation a health screen questionnaire was used to determine the suitability to participate in the study. Participants were recruited and excluded according to the criteria in section 3.1 (general methods). They also completed an International Physical Activity Questionnaire – Short Form (IPAQ-SF) (Craig *et al.*, 2003) and were asked whether they regularly took part in regular resistance exercise to ensure they fit the non-resistance trained in the previous six months criteria. The participants were instructed to avoid any form of therapeutic intervention throughout the study including antioxidants, anti-inflammatory drugs, stretching, ice and massage therapy. Participants were assigned to one of two groups, supplement, or placebo, in a stratified (according to isometric and eccentric strength), double blind fashion. A flow diagram of participant interaction is provided in Figure 11. Participant characteristics taken at baseline Trial A are shown in Table 9.
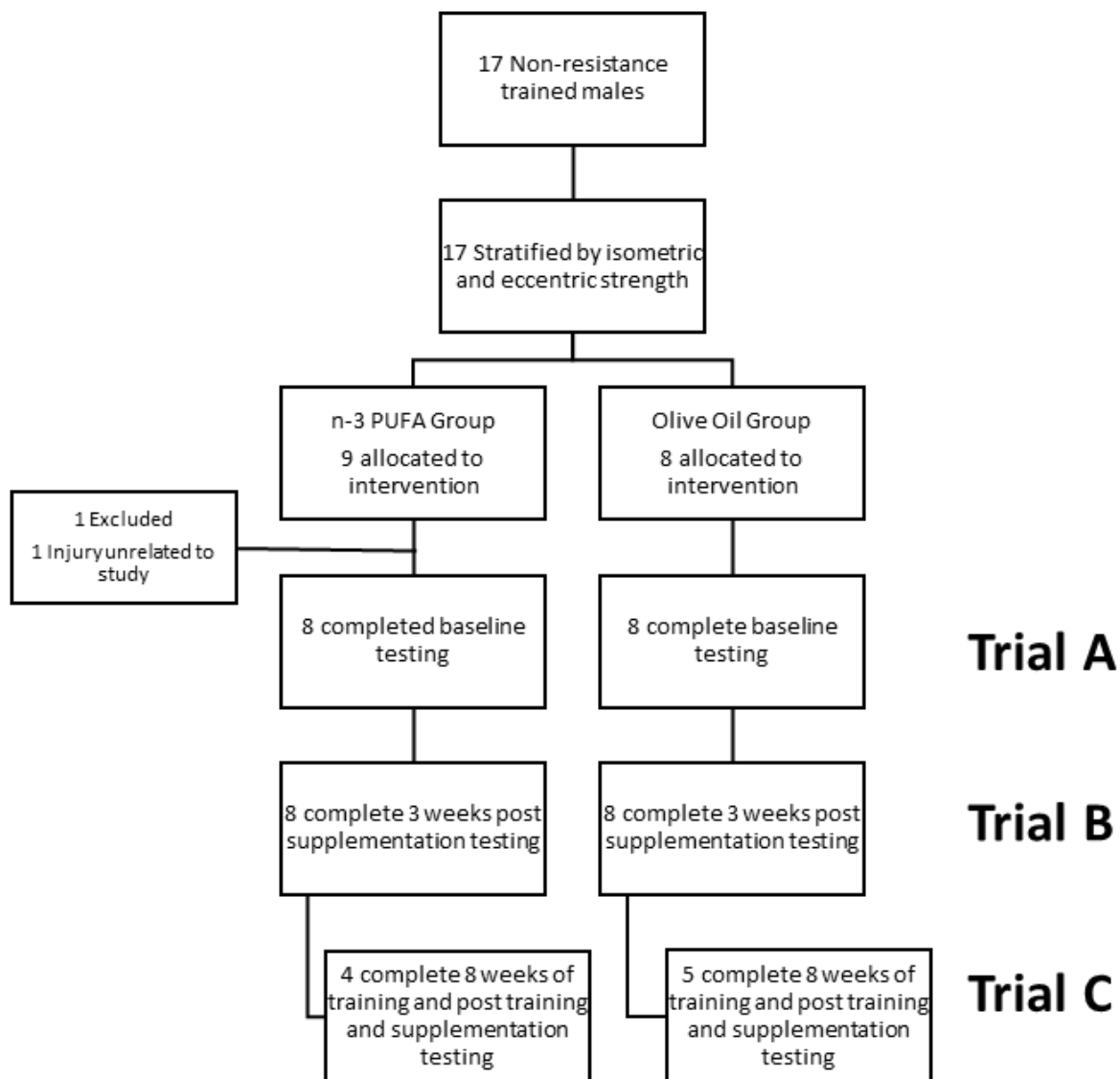
Figure 11 – Participants Flow Diagram

On completion of Trial A, participants were required to consume either 6 capsules per day of n-3 PUFAs (5.1 g/d n-3 PUFA: 3 g/d EPA, 1.2 g/d DHA, 0.9 g/d DPA, Premium Omega-3, Norwegian Pure-3, Oslo, Norway) or 6 capsules per day of placebo (6 g/d olive oil, Norwegian Pure-3, Oslo, Norway). They were advised to have 2 capsules per meal; however, they were informed that if they had forgotten to take their capsules during the day, they could all be taken that evening. The dose was chosen based on previous findings showing a similar dose was sufficient to induce changes to the fatty acid profile of both blood and skeletal muscle (McGlory *et al.*, 2014). Returned capsules were counted to determine the compliance of

supplementation. To help improve the reliability of the capsule count, participants were given a known small excess number of capsules and asked to return them after the initial 3-week period of supplementation and then every two weeks during the training phase and they were replenished with more supplements.

Participants completed food diaries the day prior to the trial and continued to record until the 48-hour post muscle damage blood sample during trial A, this was then replicated for trial B and C (section 3.2.1).

Familiarisation

An anthropometric assessment of height and body mass was conducted (section 3.2.2). Participants warmed up on a cycle ergometer for 5 minutes at 100 W. The participant was seated on the Humac Norm dynamometer (CSMI, Massachusetts, USA) and the lever arm was setup ensuring alignment with the lateral epicondyle of the femur with a hip angle of 85°. The lever arm pad was secured proximal to the malleolus and straps were used to secure the participant to the seat and to avoid compensatory movements during the test (Figure 12). The dynamometer settings were recorded, and the same position was used for all subsequent performance tests. Participants were familiarised to the exercise protocols in the performance test and muscle damage protocol.

Experimental Trials

Figure 9 provides a schematic view of the experimental trials. Participants attended the laboratory according to the pre-trial standardisation (section 3.2.1) Participants' subsequent tests was performed at the same time of day to minimise diurnal variation, with stable climatic conditions (18 - 20°C and humidity between 45 – 55%). Participants were advised to stay hydrated by drinking water *ad-libitum* throughout all trials, with water continuously made available by the investigator.

All testing sessions began with anthropometric measurements (section 3.2.2). A measure of muscle soreness was recorded, then thigh girth and knee and hip ROM

before Performance Test 1, after Performance Test 2 and immediately before Performance Test 3 and 4 (Figure 10).

Muscle Soreness (SOR)

Quadriceps muscle soreness with the knee extended, whilst seated, was rated using a VAS. The VAS consisted of a 100 mm line with anchor points of 'no pain' on the left and 'worse pain imaginable' on the right. Participants marked a point along the line representing the pain level in their quadriceps. The value was recorded by measuring the distance from the left anchor point to the mark made by the participant. It has been shown that 90% of pain ratings can be repeated within 9 mm using the VAS (Bijur, Silver and Gallagher, 2001).

Thigh Girth

Swelling of the thigh was assessed by locating the mid-point of the linear distance between the inguinal fold and the posterior superior border of the patella with a spring-loaded anthropometric tape measure (Seca 201, Hamburg, Germany). Participants were standing fully relaxed in the anatomical position with weight distributed evenly between both legs. Measurements were performed in triplicate to reduce experimenter error and the mean reported (Mickleborough *et al.*, 2015). Measurement marks were maintained throughout each trial using a semi-permanent ink marker.

Range of Motion (ROM)

To examine the ROM of the hip joint, two hip joint angles (extended and flexed) were measured with a goniometer (True-Angle Goniometer, Gaiam Pro, Boulder, Colorado, USA) whilst the participant was in the prone (extension) and supine (flexed) position. Participants flexed or extended both of their hips to their maximal range, one at a time, with no assistance from the investigator. ROM was calculated by adding the flexed joint angle to the extended joint angle. Knee joint ROM was measured in the flexed position whilst the participant lay prone with both knees fully extended. Subjects flexed both knees, one at a time, with no assistance from the investigator. ROM was measured as active knee flexion from full extension. The angle was measured with a goniometer using universal landmarks (lateral

epicondyle of the femur, lateral malleolus and greater trochanter) that were marked with a semi-permanent marker to ensure consistency on future measurements within trial (Watkins *et al.*, 1991). For all goniometer measurements three values were taken and the mean was reported in degrees.

To correspond with leg strength measurements on the strongest leg reported in the results section, ROM and thigh girth were only reported for the strongest leg.

Performance Test (PT)

Participants warmed up on a cycle ergometer for 5 minutes at 100 W (Lode B.V, Netherlands). There was no warm-up performed prior to Performance Test 2, immediately post-muscle damaging exercise as the participants had already completed prior exercise at this stage.

Countermovement Jump (CMJ)

Countermovement jump (CMJ) performance was measured on a portable performance analysis system (Quattro Jump 9290AD, Kistler, Switzerland). To perform the jumps, participants stood with feet shoulder width apart, performed a rapid descent into a squat followed by a rapid vertical jump executed with maximal force. Participants were instructed to keep their hands on their hips for the full movement to minimise assistance from arm swinging and to land in the same position as take-off. The reliability for this test has been shown to be greatest amongst all jumping tests (Cronbach's α = 0.98) (Markovic *et al.*, 2004). Participants performed up to 5 maximal efforts interspersed by 60 s passive recovery with the maximum height of the jumps used for analysis.

Maximal Voluntary Contractions (MVC)

Bilateral Maximal Voluntary Contraction (MVC) of the knee flexors and extensors were measured on the same Humac Norm dynamometer as the familiarisation. A specific warm-up was conducted on the dynamometer prior to each set of MVCs consisting of submaximal contractions (2 x 50%, 1 x 75% and 1 x 90% or perceived MVC) of the upcoming exercise. Participants performed three 3-second Maximal Voluntary Isometric Contractions (MIVCs) of the knee extensors at 75° knee joint

angle with 30 seconds rest between contractions. Maximal Voluntary Concentric Contraction (MVCC) and Maximal Voluntary Eccentric Contraction (MVEC) of the knee flexors and extensors were measured on the same apparatus. The dynamometer lever arm was programmed to flex the participant's knee from a start position of 10° of flexion (where 0° is full extension, leg straight in seated position) to 90° of flexion, thus allowing a ROM of 80° and the velocity of movement was set at $60°·s^{-1}$ (Figure 12) (Chena *et al.,* 1991). Participants performed three maximal contractions with a 30 second rest period. Verbal encouragement and visual feedback (torque output) were provided during each MVC. Peak torque of the strongest leg was used in the data treatment, with the highest value used for analysis.

Eccentric Muscle Damage Protocol

The eccentric muscle damage protocol was performed on the same apparatus as the MVCs and was bilateral, although performed one leg at a time. The protocol used was a modified version of Byrne, Eston and Edwards, (2001), which induced strength losses and increases in CK. The participant began with their leg at the start position (10° flexion) and were asked to maximally contract their quadriceps against a resistance while the lever arm moved to the finish position (90° knee flexion). Once at the finish position, they were advised to relax their leg and the dynamometer moved them back to the start position to avoid a concentric contraction being performed. The lever arm moved at a set speed of $60°·s^{-1}$. The bout consisted of 20 sets of 10 repetitions with each set being separated by 1 minute's rest. Rating of Perceived Exertion (RPE, Borg, 1982) was assessed at the end of each set. Visual feedback and verbal encouragement were provided to all participants to maximise torque output for each contraction. The total work completed was used for analysis.

Blood Sampling and Analysis

Venous blood samples were obtained (section 3.3.1) at baseline, post muscle damage (MD), post PT2, post MD+3h and post MD+48h for whole blood cell counts and the analysis of serum (section 3.3.2) CK, LDH, Mb and IL-6 (Figure 11). Additional samples were obtained 1, 1.5, 2 and 2.5h post MD for whole blood cell

counts and these were analysed according to section 3.4.1. Determination of serum CK, LDH and Mb is described in section 3.4.4. Serum IL-6 was determined according to 3.4.5.

Eccentric Training

Training was performed on the same apparatus as the strength testing and muscle damaging exercise. In accordance with the muscle damaging exercise, only the eccentric component of the knee extension was performed. All sets were composed of 10 maximal repetitions and 2 minutes rest was allowed between each set. Table 8 details the number of sets within the 14 sessions completed over the 8 weeks. The training schedule was adapted from (Baroni, Geremia, *et al.*, 2013). All sessions were scheduled allowing for a minimum of 48 hours recovery from their previous session. The first training session was conducted 3 days after Trial B and the final session was completed 3 days prior to Trial C. Subjects were supervised and verbal encouragement and visual feedback (torque output) were provided during each contraction. The peak torque and mean work done were recorded for analysis.

Table 8 – Training Schedule

	Session 1 – 2	Session 3 – 6	Session 7 – 10	Session 11 - 14
Sets	3	4	5	6

Data Analysis

Statistical analysis was performed according to section 3.5. To account for inter-individual variability, data analysis for CMJ, sprints, MVCs and thigh girth were conducted as percentage change from pre-muscle damage scores (Absolute data is provided in Appendix 6). For Part 1 (Difference between Trial A and B) of the results the change between trial A and B was calculated for the baseline variables CMJ height, isometric, concentric and eccentric strength, and an independent samples t-test was used to determine any differences. A mixed model ANOVA was then used to evaluate the muscle damage on all dependent variables; 2 group (olive oil vs n-3 PUFA) by 2 trials (pre-supplementation vs post-supplementation), by 4–9-time levels. For Part 2 (Difference between Trial B and C) of the results the change between trial B and trial C was calculated for the baseline dependent variables CMJ

height, isometric, concentric and eccentric strength, and an independent samples t-test was used to determine any differences. A mixed model ANOVA was then used to evaluate the muscle damage on all dependent variables; 2 group (olive oil vs n-3 PUFA) by 2 trials (pre-training vs post-training), by 4–9-time levels. If findings were significant, the Bonferroni post-hoc tests were used to ascertain where the differences occurred.

5.3 Results and Discussion Part 1

As shown in the Participants Flow Diagram (Figure 11), not all participants completed the training element of the study or Trial C, therefore this results section will be presented in two parts. Firstly, the findings of 3 weeks n-3 PUFA supplementation (Part 1) will be reported in section 5.3.1 (n = 16) and the discussion related to this will be presented in section 5.3.2, next the results of n-3 PUFA and eccentric training (Part 2) will be described in section 5.4.1 (n = 9) and the discussion related to this will be presented in 5.4.2. Part 1 (section 5.3) and part 2 (section 5.4) are independent in terms of the findings as they could have been two distinct studies, however due to the similarities in introduction and method it was decided to present the data in one chapter but with two sections that are interlinked.

5.3.1 Results Part 1 - Three weeks n-3 PUFA supplementation

5.3.1.1 Baseline Characteristics

Compliance, as estimated from mean return capsule count, was 97% for the n-3 PUFA group and 95% for the olive oil group, with no difference between the two supplements. There were no significant differences in physical characteristics (age, height, body mass and body fat %), baseline strength or IPAQ between treatment groups ($p > 0.05$, Table 9).

Table 9 – Participant characteristics at baseline Trial A

	Olive Oil (n = 8)	n-3 PUFA (n = 8)	P value
Age (years)	29 ± 11	27 ± 7	0.703
Height (cm)	179.5 ± 3.8	180.6 ± 6.5	0.699
Body Mass (kg)	80.5 ± 10.7	83.5 ± 19.5	0.707
Body Fat (%)	19.8 ± 6.9	21.7 ± 9.9	0.657
IPAQ (MET-minutes/week)	2577 ± 1874	2892 ± 2289	0.768
Isometric Peak Torque Strongest Leg (Nm)	274 ± 38	258 ± 34	0.395
Concentric Peak Torque Strongest Leg (Nm)	217 ± 29	223 ± 42	0.744
Eccentric Peak Torque Strongest Leg (Nm)	292 ± 46	284 ± 61	0.769

IPAQ: International Physical Activity Questionnaire

5.3.1.2 Exercise Performance

There were no differences between groups in CMJ height ($p = 0.638$), isometric ($p = 0.184$), eccentric ($p = 0.422$) or concentric ($p = 0.660$) pre- to post-supplementation.

5.3.1.3 Muscle Damage

The total work done (J) during the MD protocol did not change between Trial A and B for either treatment group ($p > 0.05$, Figure 13)
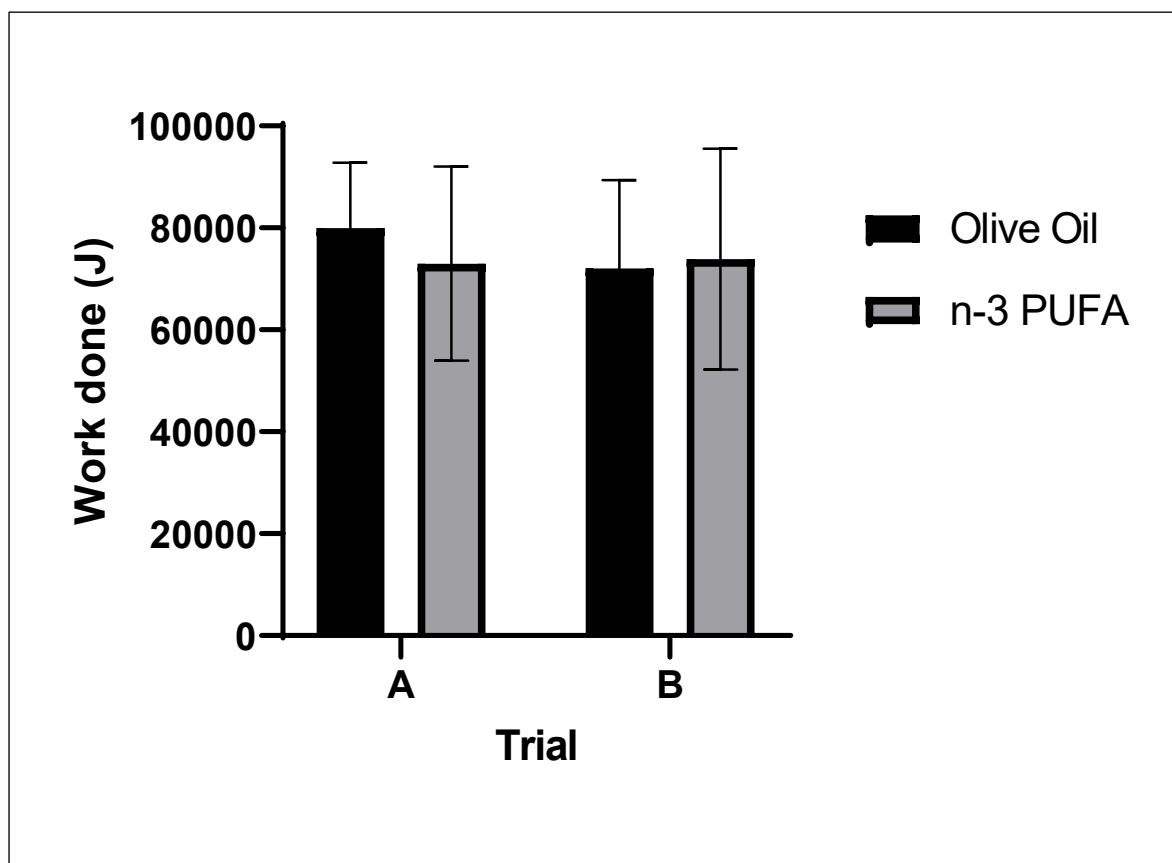
Figure 13 – Impact of supplement on total work done during muscle damage protocol in Trial A and Trial B (n = 16)

5.3.1.4 Effects of Muscle Damage on CMJ and Strength

CMJ and MVC torque values were indicated by relative change compared with baseline value as 100% within trial. CMJ height and quadriceps isometric, eccentric and concentric torque following MD are presented in Figure 14. CMJ height significantly decreased following MD and remained below baseline levels at 48 hours post MD in all trials ($p < 0.001$). There was a main effect of trial on CMJ height, with the drop in jump height following muscle damage being attenuated in the post-supplementation trial ($p = 0.046$), this was not different between groups. There was an interaction effect for trial x timepoint ($p = 0.018$) with the jump height as a percentage of the baseline jump height being higher at all timepoint post supplementation, this was not different between groups. There was no main effect of group ($p = 0.711$) on CMJ height or any group x trial x time effect ($p = 0.973$).

There was a main effect of time on isometric, eccentric and concentric peak torque ($p < 0.001$) across all trials, with peak torque decreasing after muscle damage (Figure 14). There was a main effect of trial on isometric ($p = 0.001$), eccentric ($p = 0.013$) and concentric ($p = 0.001$) peak torque, with the decline in peak torque following muscle damage in the pre-supplementation trial being significantly larger than in the post-supplementation trial. There was no main effect of group on isometric ($p = 0.862$), eccentric ($p = 0.862$) or concentric ($p = 0.515$) peak torque. There were no group x trial x time effects on isometric ($p = 0.164$), eccentric ($p = 0.625$) or concentric ($p = 0.552$) peak torque.
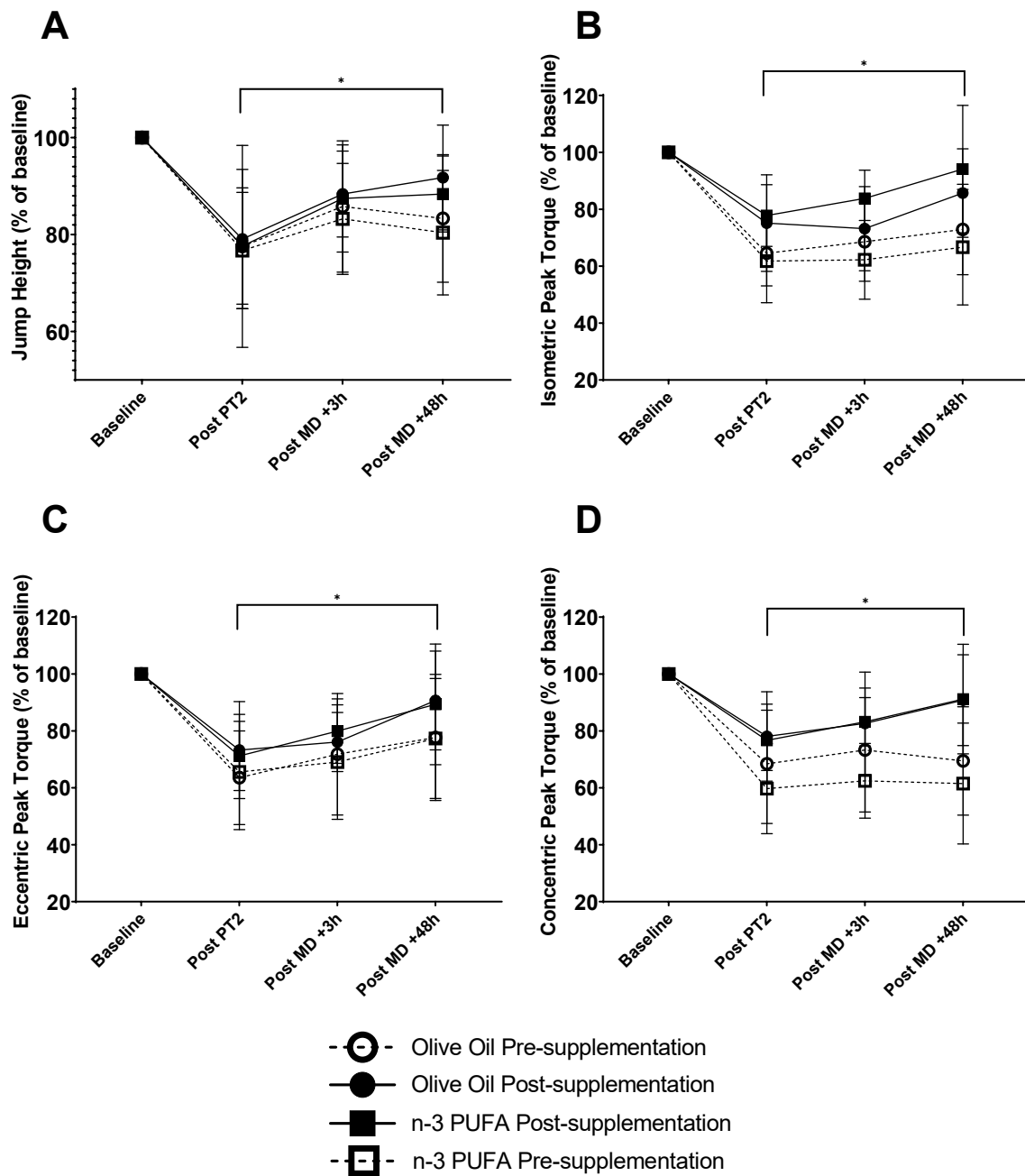
*Figure 14 – Percentage change from baseline (mean ± SD) in countermovement jump (CMJ) height (A), isometric peak torque (B), eccentric peak torque (C), and concentric peak torque (D) after performance test 2 (Post PT2), and 3 and 48h after muscle damaging exercise (post MD) pre- and post-supplementation in the olive oil and n-3 PUFA group (n = 16). *significantly different from baseline in olive oil and n-3 PUFA groups (p < 0.05)*

5.3.1.5 ROM, Swelling and Soreness

There was a main effect of time on hip and knee flexion ROM (p < 0.001, Table 10) with a decrease in the ROM following MD that did not return to baseline values 48-hours post MD. This was consistent between n-3 PUFA and olive oil groups, pre- and post-supplementation Thigh girth in the strongest leg also showed a main effect of time (p = 0.002), however there was a slight drop in thigh girth at 3- hours post MD and then an increase at 48- hours post MD to above baseline values. A VAS scale was used to assess the participant's perception of soreness in both legs. There was a significant increase in soreness across all groups following MD and this remained elevated until 48-hours post MD (p < 0.001, Table 10). There were no main effects of trial or group and no interaction effect for any of these functional measures of MD.

Table 10 – Effect of 3 weeks supplementation on functional measures of muscle damage following eccentric exercise

Variable/ Trial/ Group	Baseline	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
Hip ROM (°)					
Pre-supplementation					
Olive Oil	90.0 ± 12.6	77.3 ± 15.1	73.4 ± 13.6	72.7 ± 17.6	0.749; 0.111;
n-3 PUFA	87.7 ± 12.4	67.2 ± 13.7	74.3 ± 17.0	74.2 ± 19.9	<0.001; 0.186
Post-supplementation					
Olive Oil	82.8 ± 15.3	70.6 ± 22.0	72.5 ± 15.1	76.5 ± 21.1	
n-3 PUFA	81.4 ± 14.7	66.0 ± 19.4	67.6 ± 17.9	77.4 ± 17.6	
Knee Flexion ROM (°)					
Pre-supplementation					
Olive Oil	122.2 ± 6.0	118.6 ± 5.1	116.3 ± 4.5	116.2 ± 6.5	0.724; 0.962;
n-3 PUFA	120.1 ± 7.8	114.8 ± 8.7	115.9 ± 5.0	117.1 ± 8.8	<0.001; 0.398
Post-supplementation					
Olive Oil	121.2 ± 6.0	115.5 ± 6.7	117.3 ± 6.4	118.4 ± 7.2	
n-3 PUFA	118.3 ± 6.9	114.8 ± 5.6	117.3 ± 5.9	118.9 ± 7.1	
Thigh Girth (% of baseline)					
Pre-supplementation					
Olive Oil	-	99.7 ± 0.8	99.8 ± 0.5	100.3 ± 0.8	0.379; 0.500;
n-3 PUFA	-	100.1 ± 0.3	99.9 ± 0.4	100.6 ± 0.6	0.002; 0.794
Post-supplementation					
Olive Oil	-	100.1 ± 0.4	100.0 ± 0.6	100.4 ± 0.5	
n-3 PUFA	-	100.3 ± 0.5	99.8 ± 0.5	100.4 ± 1.1	
VAS (mm)					
Pre-supplementation					
Olive Oil	10.1 ± 5.4	48.7 ± 13.8	39.6 ± 15.0	60.4 ± 20.7	0.321; 0.483;
n-3 PUFA	9.9 ± 6.7	60.4 ± 20.0	47.5 ± 23.3	54.7 ± 35.2	<0.001; 0.057
Post-supplementation					

Olive Oil	8.3 ± 6.1	48.7 ± 29.4	33.8 ± 18.4	42.2 ± 22.7
n-3 PUFA	7.8 ± 5.7	63.7 ± 15.5	51.7 ± 18.8	58.2 ± 26.5

Values are mean ± SD (n = 16). MD, muscle damage; ROM, range of motion; VAS, visual analogue scale. Raw data for the % change data is provided in Appendix 6.

5.3.1.6 Systemic markers of muscle damage

Table 11 shows the results for serum MD markers; LDH, CK and Mb. There was a significant effect of time on LDH levels with LDH increasing post MD, peaking at 3-hours post MD (p = 0.002). CK activity increased between baseline and post MD and continued to rise to 48-hours post MD. The differences between all timepoints were significant for both groups pre- and post-supplementation (p = 0.008). Pre-supplementation, the levels of Mb increased in response to MD and remained elevated up to 48 hours post MD pre-supplementation, however, following supplementation, Mb peaked at 3-hours post and then declined almost back to baseline levels by 48-hours post (Figure 15).

Table 11 – Effect of 3 weeks supplementation on systemic markers of muscle damage and inflammation following eccentric exercise

Variable/ Trial/ Group	Baseline	Post MD	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
LDH (% increase from baseline)						
Pre-supplementation						
Olive Oil	-	10.7 ± 25.2	12.0 ± 27.5	38.6 ± 57.2	31.6 ± 52.3	0.941; 0.211;
n-3 PUFA	-	8.0 ± 5.4	17.7 ± 12.4	26.1 ± 12.8	43.4 ± 42.2	0.002; 0.445
Post-supplementation						
Olive Oil	-	-0.7 ± 27.4	13.5 ± 37.1	18.2 ± 34.4	14.6 ± 31.6	
n-3 PUFA	-	4.3 ± 12.6	6.9 ± 13.1	19.6 ± 8.3	18.4 ± 13.1	
CK (% increase from baseline)						
Pre-supplementation						
Olive Oil	-	42.3 ± 28.9	80.7 ± 54.6	276.2 ± 247.7	307.1 ± 215.6	0.339; 0.054;
n-3 PUFA	-	61.5 ± 38.6	98.5 ± 65.9	285.0 ± 228.2	745.1 ± 1012.9	0.008; 0.346
Post-supplementation						
Olive Oil	-	28.1 ± 36.1	52.2 ± 55.1	122.6 ± 109.1	139.2 ± 163.6	
n-3 PUFA	-	41.6 ± 25.5	68.2 ± 35.4	145.7 ± 65.1	212.1 ± 112.4	
Mb (% increase from baseline)						
Pre-supplementation						
Olive Oil	-	399.1 ± 167.3	774.7 ± 519.2	1070.7 ± 869.3	125.6 ± 272.85	0.473; <0.001;
n-3 PUFA	-	482.7 ± 175.8	762.9 ± 249.9	908.0 ± 392.3	777.7 ± 948.5	0.002; 0.105
Post-supplementation						
Olive Oil	-	203.2 ± 131.5	391.7 ± 208.1	417.9 ± 217.1	19.11 ± 55.7	
n-3 PUFA	-	225.1 ± 83.1	433.2 ± 152.2	483.7 ± 182.4	11.5 ± 16.0	
IL-6 (pg/mL)						
Pre-supplementation						

Olive Oil	1.48 ± 1.62	1.97 ± 1.52	3.13 ± 2.50	3.82 ± 6.03	1.39 ± 1.47	0.931; 0.038;
n-3 PUFA	0.33 ± 0.21	3.06 ± 2.23	3.26 ± 1.55	2.40 ± 1.99	0.96 ± 1.11	0.003; 0.811
Post-supplementation						
Olive Oil	0.54 ± 0.28	1.76 ± 1.66	1.27 ± 1.66	2.07 ± 1.61	0.84 ± 0.87	
n-3 PUFA	0.31 ± 0.35	2.91 ± 3.25	1.90 ± 0.99	1.99 ± 1.20	0.66 ± 0.70	

Values are mean ± SD (n = 16). MD, muscle damage; LDH, lactate dehydrogenase; CK, creatine kinase; Mb, myoglobin; IL-6, interleukin-6. Raw data for % change data is provided in Appendix 6
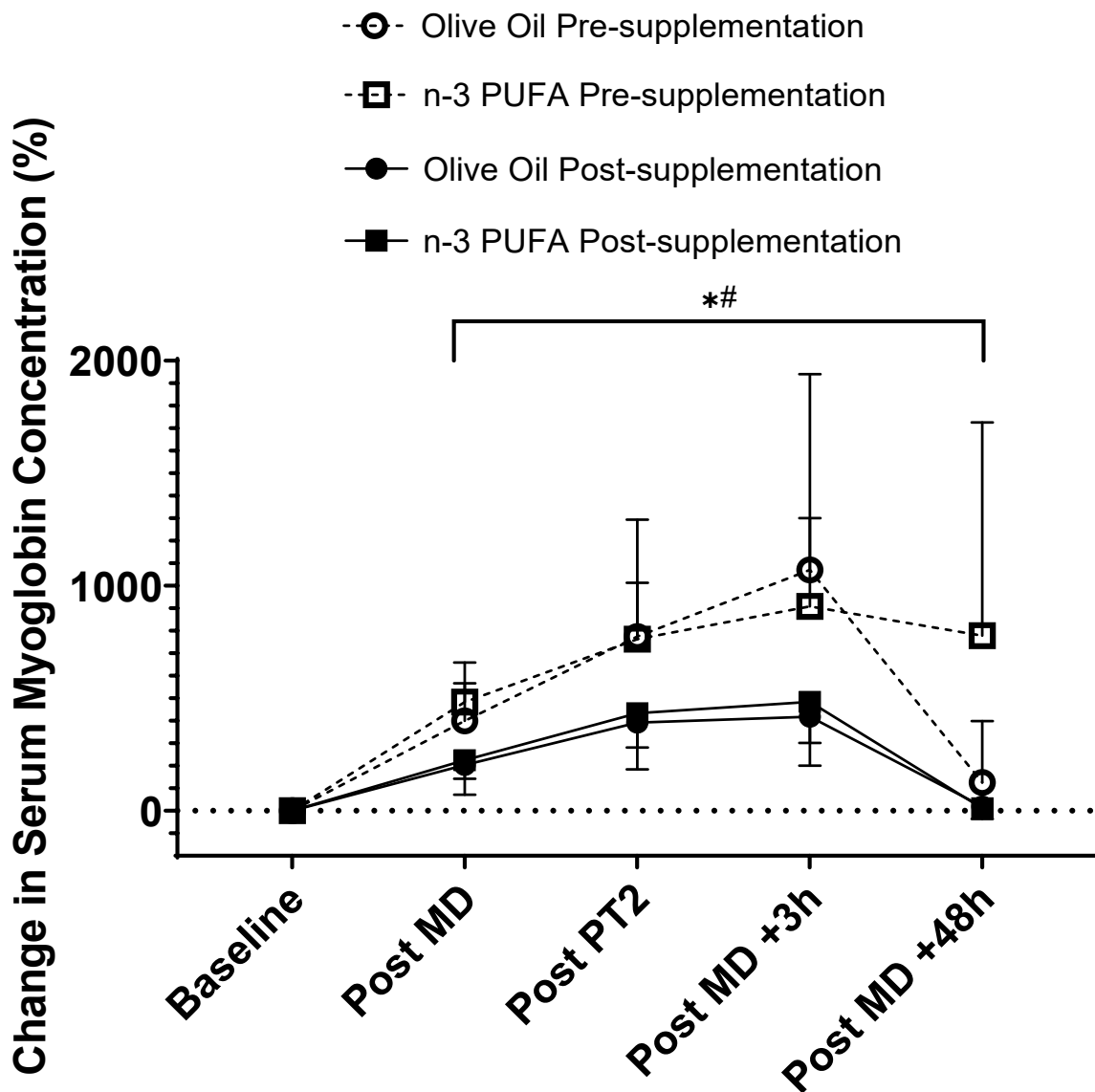
Figure 15 – Change in serum myoglobin concentration (%) from baseline within trial to immediately post muscle damaging exercise (post MD), after performance test 2 (Post PT2), 3 and 48h after muscle damaging exercise (post MD) pre-o and post- supplementation in the olive oil and n-3 PUFA group (n = 16). *n-3 PUFA Post-supplementation significantly different from pre-supplementation trial (p < 0.05). #Olive Oil Post-supplementation significantly different from pre-supplementation trial (p < 0.05).

5.3.1.7 Systemic markers of inflammation

Serum IL-6 results are shown in Table 11. There was a significant main effect of time (p = 0.003) with IL-6 increasing post MD and peaking at 3-hours post in the olive oil group both pre- and post-supplementation. In the n-3 PUFA group IL-6 peaked post performance test 2 pre-supplementation and immediately post- muscle damage post-supplementation. There was no interaction effect between groups (p = 0.811). There was a main effect of time (p < 0.001) with white blood cell count increasing rapidly immediately post MD and then steadily increasing to a peak at 3h post MD, returning to baseline by 48h post MD. Whilst Figure 16 shows a trend for white blood cell count pre-supplementation to be higher than post 3 weeks n-3 PUFA or olive oil supplementation, there was no interaction effect detected. Paired samples T-tests revealed a significant difference between pre- and post- olive oil supplementation two hours post muscle damage and 48 hours post muscle damage with post-supplementation levels lower than pre-supplementation levels at these timepoints.
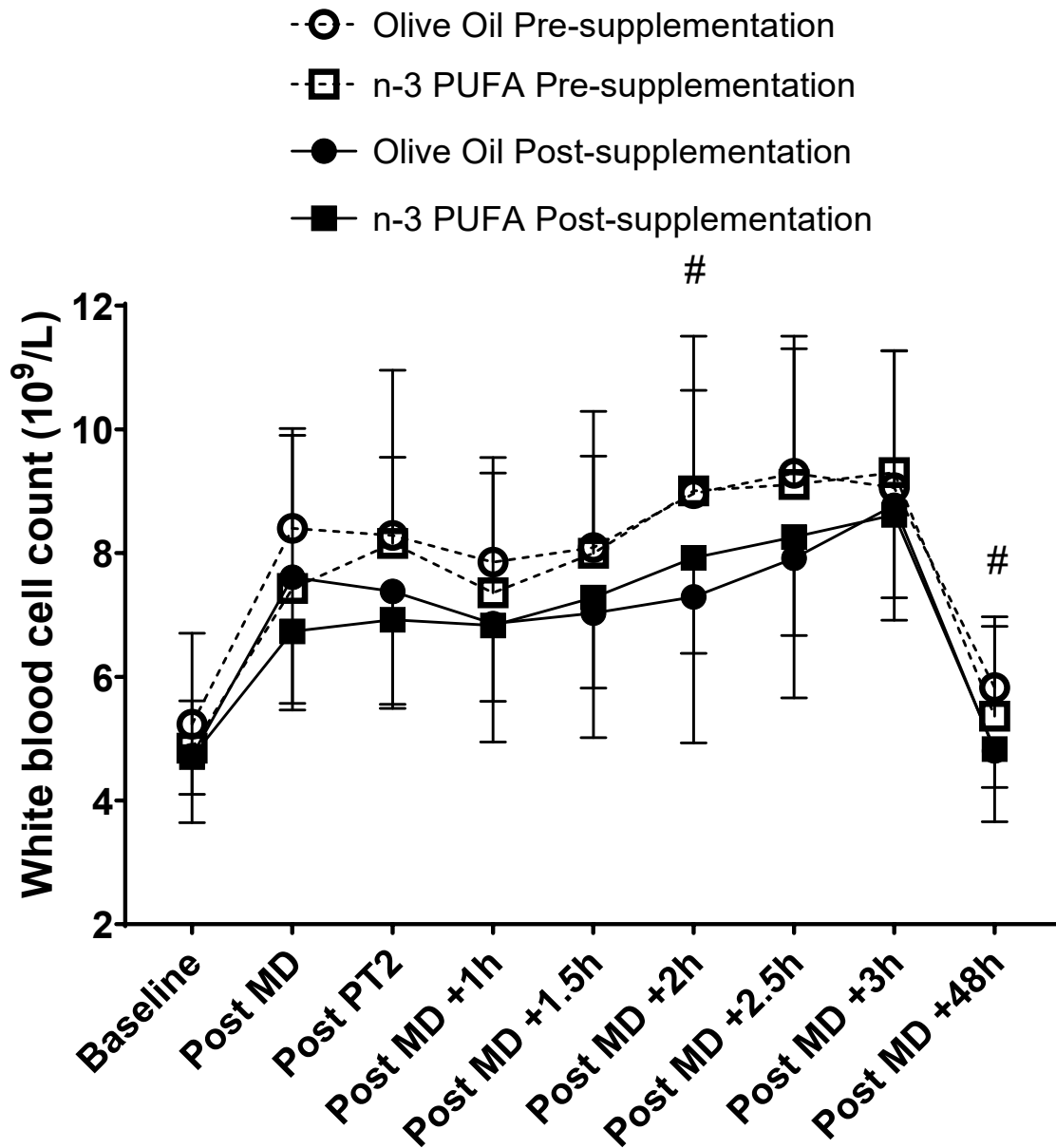
Figure 16 – White blood cell count (10^9/L) before (baseline), immediately post muscle damaging exercise (post MD), after performance test 2 (Post PT2), 1, 1.5, 2, 2.5, 3 and 48h after muscle damaging exercise (post MD) pre- and post- supplementation in the olive oil and n-3 PUFA group (n = 16). #Olive Oil Pre-supplementation significantly different (p < 0.05) to Olive Oil Post-supplementation.

5.3.2 Discussion - Part 1 - Three weeks n-3 PUFA supplementation

5.3.2.1 Exercise Performance

Whilst it was not hypothesised that three weeks of supplementation would affect exercise performance, these measurements were recorded to provide results to compare with post training data and to form the baseline results within trial A and B to determine whether muscle damage had occurred at subsequent time points. The findings indicate that three weeks of olive oil or n-3 PUFA supplementation decreased CMJ height. The mechanism for this is not fully known, although it could have simply been due to participants effort levels, however, Mike *et al.*, (2017) suggested that longer eccentric contractions may negatively impact explosive movements such as vertical jump. It is possible that the eccentric contractions performed in trial A negatively impacted CMJ performance in trial B. Interestingly both isometric and concentric peak torque also slightly decreased following 3 weeks supplementation. This possibly occurred due to lower self-motivation to perform maximal contractions as the participants may have been preserving energy for the eccentric muscle damage protocol that they were now more familiar with having previously completed it in trial A. However, eccentric peak torque did not change between trial A and B in either group. This finding is consistent with the majority of the literature showing that peak torque is unchanged by n-3 PUFA supplementation in males (Gray *et al.*, 2014; Lewis *et al.*, 2015, 2017; Hingley *et al.*, 2017) and females (Lenn *et al.*, 2002; Lembke *et al.*, 2014; McKinley-Barnard *et al.*, 2018).

5.3.2.2 Muscle Damage and Inflammation

This study evaluated the efficacy of 3 weeks of n-3 PUFA supplementation in reducing muscle damage following eccentric exercise. Individuals that are unaccustomed to eccentric exercise experience varying degrees of muscle damage after performing eccentric contractions. Peak muscle damage occurs when force is applied to the muscle during the lengthening phase (Peake *et al.*, 2005). It appears from the significant time effects and magnitude of responses that the eccentric muscle damage protocol adequately induced muscle damage, as indirectly assessed by the increases in intramuscular proteins (CK, LDH, Mb), increases in perceived

soreness and reductions in muscular performance. Increases in circulating white blood cells and IL-6 also suggest that the muscle damage stimulus caused the infiltration of leukocytes to the damaged muscle and effectively stimulated an inflammatory response.

The total work done during the eccentric muscle damaging protocol in trial A and B did not differ and there was no difference between groups. This is in contrast to Jouris, Mcdaniel and Weiss, (2011) who found that healthy men and women were able to complete a greater total eccentric exercise volume after 7 days of 3 g/d n-3 PUFA supplementation. The main difference between the work by Jouris, Mcdaniel and Weiss, (2011) and the present study is the MD protocol was targeted at the elbow flexors and only 2 sets performed compared with the knee extensors and 20 reps per leg being performed in the present study. There are very few EIMD studies that quantify the MD volume prior to supplementation, or in fact have a pre-supplementation trial in the study design, therefore it is not possible at this stage to conclude whether short term n-3 PUFA supplementation alone increases eccentric exercise capacity.

Despite participants performing the same amount of work in trial A and B in the eccentric exercise protocol, the muscle damage indicated by the reductions in torque following MD were lower in trial B compared to trial A in both groups. Previous research has demonstrated significantly lower isometric strength loss 1 day post eccentric exercise in an n-3 PUFA group (91%) compared with a control group (77%) (Ochi, Tsuchiya and Yanagimoto, 2017). Tsuchiya *et al.*, (2016) also found significantly higher peak torque in the n-3 PUFA group compared with the placebo group at 2, 3 and 5 days after maximal eccentric contractions of the elbow flexors. In the present study isometric strength loss 48 hours post MD after n-3 PUFA supplementation (91%) was lower than before n-3 PUFA supplementation (67%), with the changes in peak torque of similar magnitude to previous literature (Tsuchiya *et al.*, 2016; Ochi, Tsuchiya and Yanagimoto, 2017). However, contrasting previous literature (Rajabi *et al.*, 2013; Mickleborough *et al.*, 2015; Ochi, Tsuchiya and Yanagimoto, 2017) there was no difference in peak torque following MD between the n-3 PUFA group and the olive oil group.

Consistent with the strength results, the increases in serum Mb, IL-6 levels and white blood cell counts were lower in trial B than trial A in both groups. The lower serum Mb and the attenuated drop in peak torque in trial B compared with trial A suggest a repeated bout effect as the participants were no longer unaccustomed to eccentric exercise. Whilst a short familiarisation session took place before trial A it is clear this was insufficient to remove the repeated bout effect. The repeated bout effect refers to the skeletal muscle adaptation and reduced susceptibility to muscle damage following subsequent bouts of the same exercise (McHugh, 2003; Hyldahl, Chen and Nosaka, 2016). The repeated bout effect only attenuates the extent of the muscle damage rather than preventing it from occurring as muscle biopsies revealed significant fibre disruption 21 hours post eccentric exercise in strength trained men (Gibala *et al.*, 2000). Hirose et al. (2004) found an increase in CK and Mb only after the first bout of eccentric exercise of the elbow flexors. The repeated bout effect makes it difficult to delineate whether the lower Mb, IL-6 and white blood cells and attenuated decreases in peak torque after MD seen in both groups following supplementation were a result of the repeated exercise or the supplementation.

There were no differences in the other systemic markers of MD (CK and LDH). It is likely that the large standard deviations due to large differences in individual responses prevented significant effects of trial in serum CK and LDH. It is also worth noting that some inflammatory markers, particularly CK, relate poorly to the magnitude or time course of EIMD as indicated by other markers such as Mb (Warren, Lowe and Armstrong, 1999).

Moreover, there were no differences between the groups or trials in the functional measures of (knee flexion, hip ROM, thigh girth). If n-3 PUFAs do have a role in the protection of muscle cell membrane, then it is possible that n-3 PUFA supplementation would attenuate the reductions in ROM following eccentric exercise. This finding has been demonstrated in previous research (Tartibian, Maleki and Abbasi, 2009; Rajabi *et al.*, 2013; Mickleborough *et al.*, 2015; Tsuchiya *et al.*, 2016; Ochi, Tsuchiya and Yanagimoto, 2017). However, much of the previous literature has also found no effect of n-3 PUFA supplementation on ROM (Lenn *et al.*, 2002; Phillips *et al.*, 2003; DiLorenzo, Drager and Ankin, 2014; Lembke *et al.*, 2014; Corder *et al.*, 2016). The present study and previous research have used

goniometers to assess ROM in the damaged limb and whilst care is taken to identify and mark bony landmarks to ensure the measurement is reliable, if there are different experimenters then reliability can be very poor (Youdas, Bogard and Suman, 1993). This could be a reason for the difference in findings between studies. The reliability of thigh girth measurements has similar issues with a lack of intertester reliability and the fact that within the area of measurement the effects of fat, muscle and skin, etc, cannot be excluded. Changes in thigh girth in the present study were so small that it is likely that the experimenter error of measurement was greater than any changes induced by the eccentric exercise. It is expected this is also the reason for no effect of n-3 PUFA supplementation in previous research (Lenn *et al.*, 2002; Jouris, Mcdaniel and Weiss, 2011; Mickleborough *et al.*, 2015; Corder *et al.*, 2016; Tsuchiya *et al.*, 2016; Tinsley *et al.*, 2017). Future research should use MRI to measure muscle swelling following eccentric exercise.

In contrast to previous research (Jouris, Mcdaniel and Weiss, 2011; Rajabi *et al.*, 2013; Lembke *et al.*, 2014; Mickleborough *et al.*, 2015; Corder *et al.*, 2016; Ochi, Tsuchiya and Yanagimoto, 2017; Philpott *et al.*, 2018; Ramos-Campo *et al.*, 2020; VanDusseldorp *et al.*, 2020) there was no effect of 3 weeks n-3 PUFA supplementation on muscle soreness following eccentric exercise in the present study. Interestingly Tinsley *et al.*, (2017) also found no effect of 1 week of n-3 PUFA supplementation on muscle soreness in untrained females, but when soreness was analysed using effect sizes functional soreness was between 33 - 42% lower in the n-3 PUFA group compared with the placebo group. It is possible that the small sample sizes used in this previous study and the present study make it difficult to obtain any significant results, although two of the previous studies that did find an effect had smaller sample sizes than the present study (Jouris, Mcdaniel and Weiss, 2011; Ramos-Campo *et al.*, 2020). The present study is in accordance with previous research suggesting no effect of n-3 PUFAs on muscle soreness (Lenn *et al.*, 2002; Phillips *et al.*, 2003; Pumpa *et al.*, 2011; Gray *et al.*, 2014; Hayward *et al.*, 2016; Jakeman *et al.*, 2017). The discrepancy between findings is likely due to differences in the MD protocol, training status and sex of the participants.

5.4 Results and Discussion Part 2

5.4.1 Results - Part 2 - Eight weeks n-3 PUFA supplementation and eccentric training

It is important to note that a total of nine participants completed the eccentric training and supplementation part of the study, five in the n-3 PUFA group and four in the olive oil group (Figure 11). Therefore, analysis for this section has only been conducted on the nine participants that completed the 8 weeks of eccentric training. Both the n-3 PUFA and olive oil group completed all exercise sessions, however one participant in the olive oil group missed four training sessions with the right leg and one participant in the n-3 PUFA group missed two training sessions with the right leg due to musculoskeletal injuries sustained during the training. It is common to sustain musculoskeletal injuries in untrained individuals when taking part in this type of eccentric training regime (Baroni, Rodrigues, *et al.*, 2013).

5.4.1.1 Exercise Performance

Following 8 weeks of eccentric training CMJ height improved in the olive oil group by 1.1 ± 2.8 cm and n-3 PUFA group by 1.0 ± 1.9 cm, with no difference between groups (p = 0.965). Isometric peak torque increased by 23 ± 30 Nm in the olive oil group and increased by 45 ± 54 Nm in the n-3 PUFA group, with no difference between groups (p = 0.462). Eccentric peak torque increased by 69 ± 33 Nm in the olive oil group and by 27 ± 67 Nm in the n-3 PUFA group, with no difference between groups (p = 0.315). Concentric peak torque increased by 11 ± 23 Nm in the olive oil group and by 24 ± 62 Nm in the n-3 PUFA group, with no difference between groups (p = 0.701).

5.4.1.2 Muscle Damage

The total work done (J) during the muscle damage protocol did not change between Trial A, B and C for either treatment group (p = 0.172, Figure 17). However, one individual in the n-3 PUFA group and one in the olive oil group sustained an injury to their weakest leg during the training period and were unable to complete the muscle

damage protocol in that leg for trial C, effectively halving the total work done. Figure 17 shows the mean work done in trial C was considerably greater than in Trial A and B for 7 participants, but it was not considered significant due to the large standard deviation as the 2 injured individuals' work done dropped in trial C, but their data were still analysed. A sensitivity analysis was performed removing the 2 injured participants revealing a significant main effect of trial ($p < 0.001$). Bonferroni post-hoc showed no difference between trial A and B ($p = 1.000$) and significant differences between trial A and C ($p < 0.001$) and B and C ($p = 0.004$), with the mean work done in trial C (101559 ± 5706 J) much higher than in trial A (74387 ± 5812 J) or trial B (68275 ± 7493). There was no supplement x trial interaction effect with or without the 2 participants removed.
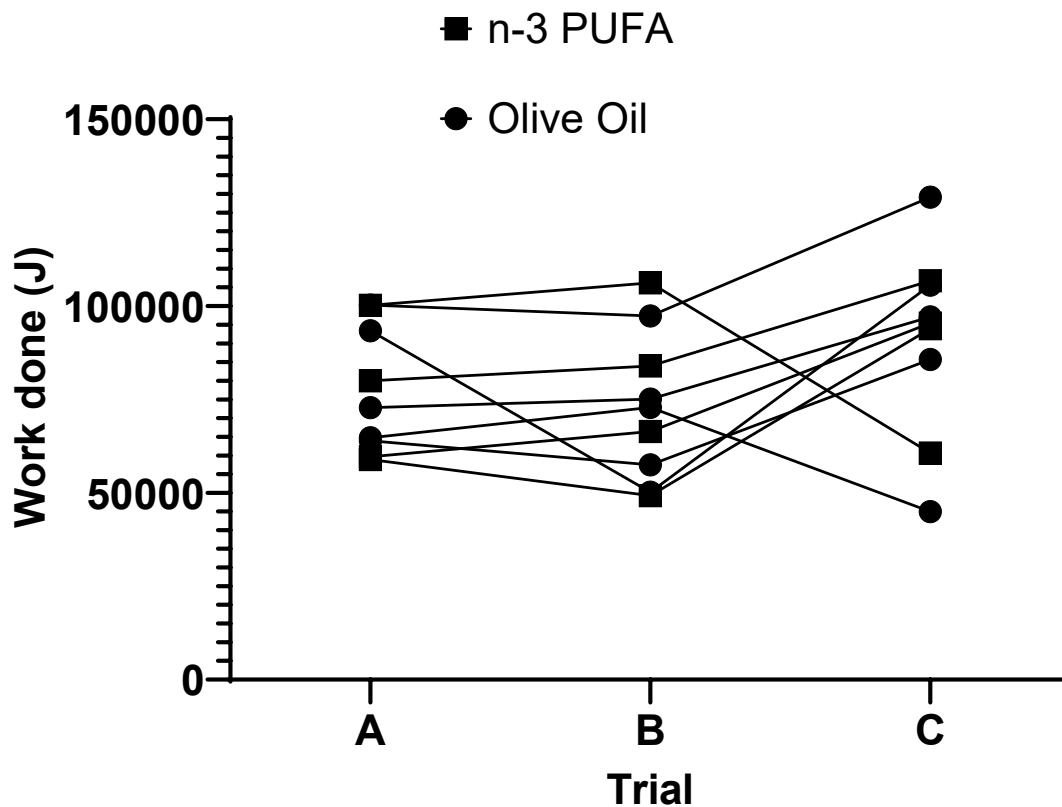
Figure 17 – Impact of supplement on total work done during muscle damage protocol in Trial A, B and C (n = 9)

5.4.1.3 Effects of Muscle Damage on CMJ and Strength

CMJ height and quadriceps isometric, eccentric and concentric torque following MD are presented in Figure 18. CMJ height significantly decreased following MD and remained below baseline levels at 48 hours post MD in all trials ($p < 0.001$). There was a trend for post-training CMJ height decrements to be reduced compared with pre-training ($p = 0.061$), but this was not different between supplements ($p = 0.991$). Isometric, eccentric and concentric peak torque decreased significantly after MD (Post PT2) and remained below baseline 3 hours post MD and returned to baseline levels at 48 hours post MD. There was no difference between supplements or between pre- and post-training (Figure 18).
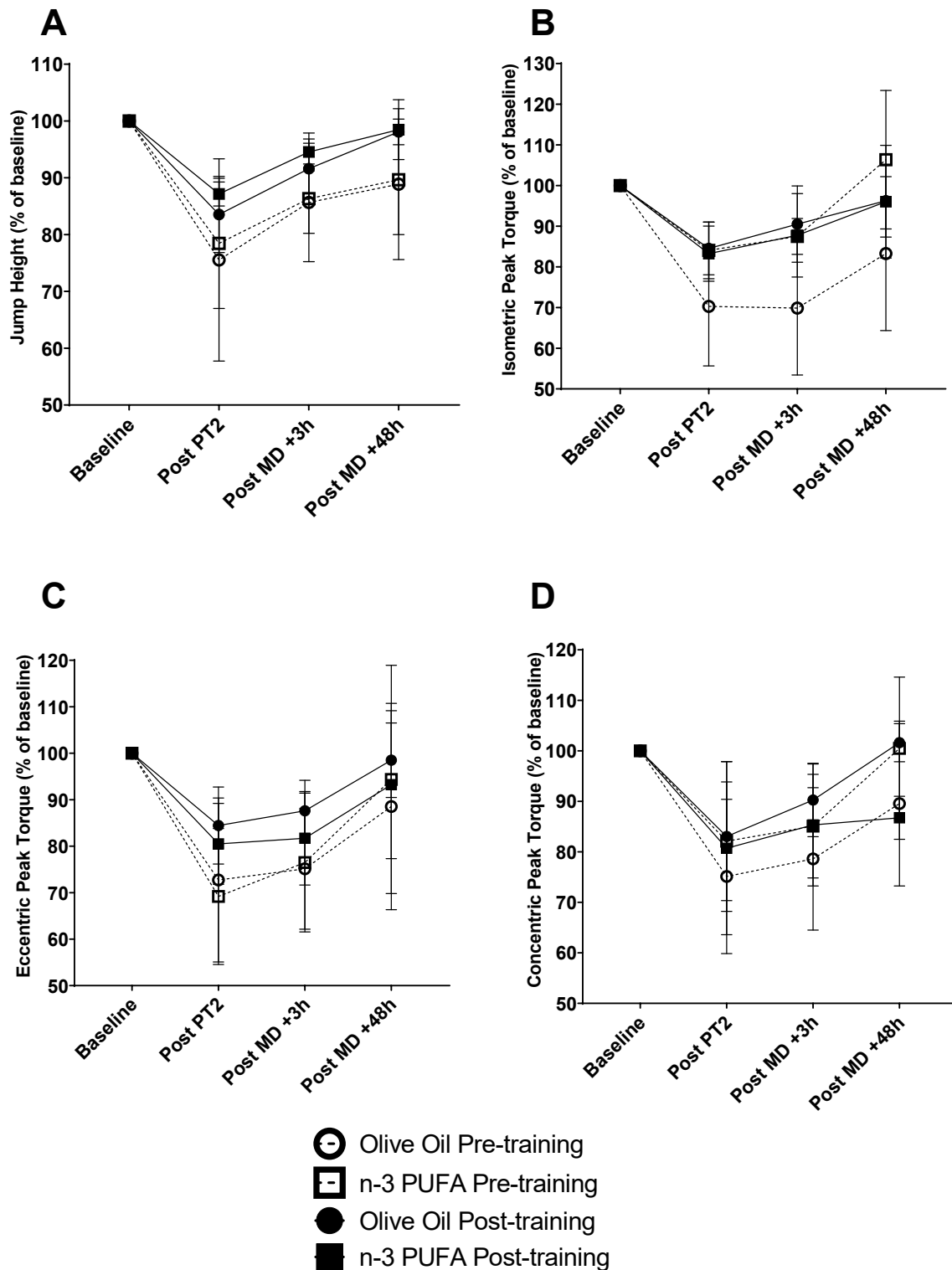
Figure 18 – Percentage change from baseline within trial (mean ± SD) in countermovement jump (CMJ) height (A), isometric peak torque (B), eccentric peak torque (C), and concentric peak torque (D) after performance test 2 (Post PT2), 3 and 48h after muscle damaging exercise (post MD) pre- and post-training in the olive oil and n-3 PUFA group (n = 9).

5.4.1.4 ROM, Swelling and Soreness

Knee flexion and hip ROM displayed a main effect of time ($p < 0.001$, Table 12) with a decrease in the ROM following MD that did not return to within trial baseline values 48h post MD. This was consistent across n-3 PUFA and olive oil groups, pre- and post-training. Thigh girth (% of baseline within trial) remained consistent between baseline and 3h post MD and there was a significant increase ($p = 0.055$) from 3h post MD to 48h post MD, this was not different between groups or trials. There was a significant increase in soreness across both groups following MD and this remained elevated at 48h post MD ($p < 0.001$, Table 12). There was also a trial x time interaction effect ($p = 0.046$) with soreness 3h and 48h after MD in both groups, lower in the post-training trial compared with the pre-training trial. There was no supplement x trial x time interaction effect for any of these functional measures of muscle damage.

Table 12 – Effect of 8 weeks training and supplementation on functional measures of muscle damage following eccentric exercise

Variables/ Groups	Baseline	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
Knee Flexion ROM (°)					
Pre-training					
n-3 PUFA	116.1 ± 4.5	114.5 ± 4.5	116.2 ± 2.2	117.4 ± 6.2	0.428; 0.078;
Olive Oil	122.1 ± 7.1	115.7 ± 8.4	118.2 ± 6.7	118.4 ± 8.2	0.008; 0.092
Post-training					
n-3 PUFA	115.3 ± 3.7	111.7 ± 5.5	112.4 ±6.6	112.5 ± 2.5	
Olive Oil	120.5 ± 9.4	115.3 ± 11.0	116.4 ± 9.1	120.3 ± 9.8	
Hip ROM (°)					
Pre-training					
n-3 PUFA	88.3 ± 13.4	74.4 ± 16.3	78.1 ± 13.7	88.7 ± 9.3	0.454; 0.238;
Olive Oil	80.2 ± 17.7	62.9 ± 23.5	67.1 ± 15.4	67.8 ± 19.4	<0.001; 0.338
Post-training					
n-3 PUFA	84.0 ± 17.6	78.9 ± 16.7	73.8 ± 11.3	80.6 ±8.5	
Olive Oil	81.1 ± 12.9	70.1 ± 18.7	74.7 ±18.8	75.4 ± 22.5	
Thigh Girth (% of baseline within trial)					
Pre-training					
n-3 PUFA	-	100.3 ± 0.2	100.2 ± 0.2	101.2 ± 0.6	0.411; 0.224;
Olive Oil	-	100.1 ± 0.3	99.8 ± 0.6	100.5 ± 0.5	0.004; 0.814
Post-training					
n-3 PUFA	-	99.9 ± 0.8	99.9 ± 0.6	100.2 ± 0.7	
Olive Oil	-	99.9 ± 0.5	99.9 ± 0.4	100.1 ± 0.8	
VAS (mm)					
Pre-training					
n-3 PUFA	6.5 ± 6.8	56.4 ± 9.6	48.2 ± 21.8	62.6 ± 34.0	0.198; 0.369;
Olive Oil	5.3 ± 5.9	50.7 ± 31.3	32.1 ± 20.5	40.3 ± 22.9	<0.001; 0.998
Post-training					
n-3 PUFA	11.2 ± 13.6	57.1 ± 19.2	41.8 ± 30.5	39.6 ± 24.0	
Olive Oil	7.5 ± 12.9	49.7 ± 28.8	23.6 ± 17.9	17.9 ± 22.1	

Values are mean ± SD (n = 9). MD, muscle damage; ROM, range of motion; VAS, visual analogue scale.

5.4.1.5 Systemic Markers of Muscle Damage

Table 13 displays the results for serum MD markers; CK, LDH and Mb. Serum CK levels increased between baseline and post MD and continued to rise up to 48-hours post MD (p = 0.009). This pattern was observed in all trials for both supplements. Serum LDH increased up to 3h post MD and then dropped slightly at 48h post MD but did not return to baseline levels. Again, this was seen in all trials for both supplements, with no difference between supplements. The levels of Mb increased from baseline, peaking after performance test 2 in the n-3 PUFA trials and peaking at 3h post MD in the olive oil trials with no difference between pre- and post-training.

Table 13 – Effect of 8 weeks training and supplementation on systemic markers of muscle damage and inflammation following eccentric exercise

Variables/ Trial / Groups	Baseline	Post MD	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
LDH (% increase from baseline within trial)						
Pre-training						
n-3 PUFA	-	3.2 ± 17.5	6.9 ± 18.6	19.6 ± 10.8	13.0 ± 14.9	0.756; 0.722;
Olive Oil	-	-0.7 ± 21.1	10.3 ± 19.4	21.4 ± 28.6	19.4 ± 25.2	0.006; 0.851
Post-training						
n-3 PUFA	-	14.1 ± 18.2	15.8 ± 20.1	32.4 ± 23.3	20.6 ± 14.4	
Olive Oil	-	5.1 ± 32.9	13.5 ± 37.5	17.9 ± 36.1	11.5 ± 33.0	
CK (% increase from baseline within trial)						
Pre-training						
n-3 PUFA	-	32.4 ± 14.8	64.3 ± 29.7	146.4 ± 72.1	139.6 ± 69.6	0.312; 0.132;
Olive Oil	-	8.0 ± 12.6	20.0 ± 15.2	71.1 ± 43.4	65.6 ± 36.0	0.009; 0.703
Post-training						
n-3 PUFA	-	71.1 ± 52.3	120.1 ± 88.3	227.4 ± 150.1	186.6 ± 130.3	
Olive Oil	-	32.8 ± 31.6	57.5 ± 60.3	126.3 ± 127.4	166.4 ± 182.9	
Mb (% increase from baseline within trial)						
Pre-training						
n-3 PUFA	-	235.6 ± 96.1	474.6 ± 192.2	472.0 ± 242.0	0.0 ± 9.2	0.155; 0.957;
Olive Oil	-	192.9 ± 152.7	381.8 ± 251.0	461.8 ± 238.9	-1.7 ± 30.6	<0.001; 0.244
Post-training						
n-3 PUFA	-	331.8 ± 187.8	635.6 ± 380.6	565.0 ± 305.5	17.2 ± 31.6	
Olive Oil	-	110.2 ± 93.9	217.6 ± 170.5	336.4 ± 288.2	-28.4 ± 40.3	

IL-6 (pg·mL^{-1})
Pre-training

n-3 PUFA	0.33 ± 0.48	3.40 ± 3.70	2.48 ± 0.75	2.82 ± 0.68	0.26 ± 0.10	0.377; 0.627;
Olive Oil	0.72 ± 0.15	2.51 ± 1.94	1.83 ± 1.85	2.72 ± 1.89	1.10 ± 1.05	<0.001; 0.283
Post-training						
n-3 PUFA	0.59 ± 0.24	2.54 ± 2.07	2.22 ± 2.35	1.80 ± 0.91	0.41 ± 0.23	
Olive Oil	0.52 ± 0.16	2.60 ± 2.20	5.37 ± 4.66	3.37 ± 1.07	1.05 ± 0.86	

Values are mean ± SD (n = 9). MD, muscle damage; LDH, lactate dehydrogenase; CK, creatine kinase; Mb, myoglobin; IL-6, interleukin-6.

5.4.2.6 Systemic Markers of Inflammation

Serum IL-6 results are shown in Table 13. There was a significant main effect of time (p = 0.001) with IL-6 increasing post MD and peaking post MD in the n-3 PUFA group in both trials. In the olive oil group pre-training, serum IL-6 peaked 3h after MD and post-training it peaked immediately following performance test 2. Serum IL-6 had almost returned to baseline values in all groups and trials by 48h post MD. There was no interaction effect between groups or trials (p = 0.283). White blood cell count increased (p < 0.001), peaking at 3h post MD and then declined back to baseline by 48h post MD in both groups and trials (Figure 19). There was no supplement x trial x time interaction effect (p = 0.721).
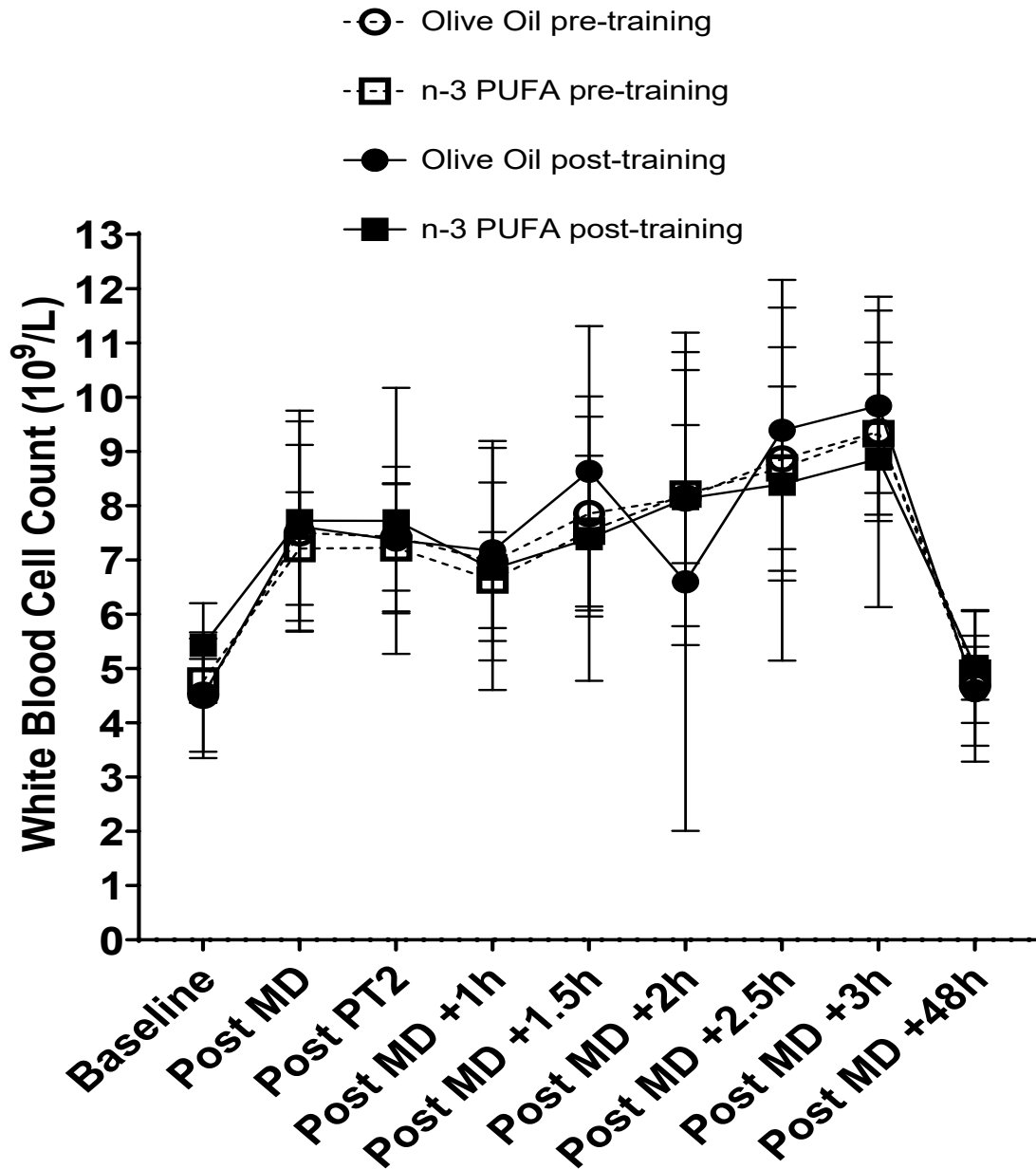
Figure 19 – White blood cell count (10^9/L) before (baseline), immediately post muscle damaging exercise (post MD), after performance test 2 (Post PT2), 1, 1.5, 2, 2.5, 3 and 48h after muscle damaging exercise (post MD) pre- and post-training in the olive oil and n-3 PUFA group (n = 9).

5.4.2 Discussion - Part 2 - Eight weeks n-3 PUFA supplementation and eccentric training

5.4.2.1 Exercise Performance

The key finding in terms of exercise performance was that the increases in CMJ height and peak torque were consistent between groups and of similar magnitude to the increases in peak isometric, concentric and eccentric torque following a 12-week eccentric training programme (Baroni, Rodrigues, *et al.*, 2013). This suggests that adaptations to eccentric training are not blunted by n-3 PUFA supplementation, although they were also not enhanced by n-3 PUFA supplementation when compared with the placebo, olive oil. Therefore, athletes could be advised to take n-3 PUFA supplements to reduce EIMD and inflammation rather than NSAIDS which can blunt muscle protein synthesis (Trappe et al., 2002) and attenuate strength and muscle hypertrophic adaptations from resistance training (Lilja et al., 2018), negating the effects of the exercise.

Contrary to the hypothesis, n-3 PUFA supplementation together with 8 weeks eccentric training did not result in greater improvements in strength and power than olive oil combined with eccentric training. Following two weeks of a 60% energy-restricted diet young resistance trained males had a greater 1RM leg extension when they supplemented with 4 g/d n-3 PUFA compared with a placebo suggesting increases in strength due to n-3 PUFA supplementation is most evident under catabolic conditions (Smith *et al.*, 2011a; Lalia *et al.*, 2017). In the present study, despite increases in CMJ height and peak torque in both supplemented groups following 8 weeks of eccentric quadriceps training, no differences were found between n-3 PUFA and placebo groups. McGlory *et al.*, (2014) previously demonstrated that 4 weeks of n-3 PUFA supplementation significantly increased the n-3 PUFA concentrations in the muscle cell with a slightly lower dose (5g/d) than the one used in this study. The n-3 PUFA supplementation used in the present study was a higher dose and taken for a longer duration so n-3 PUFA muscle concentrations should have increased. The uptake of n-3 PUFA into the muscle cell has been suggested to prime the muscle to respond to anabolic stimuli in both young

(Smith *et al.*, 2011b) and older (Smith *et al.*, 2011a) adults, thus it is surprising to have not seen a greater training improvement with n-3 PUFA supplementation.

Following 8 weeks of n-3 PUFA supplementation there was a potentiated response of MPS to the intravenous infusion of amino acids (Smith *et al.*, 2011b). In the present study it is possible that the dietary protein intake was insufficient for the n-3 PUFA supplementation to potentiate the MPS response, which may be why the performance measure of strength was not different between n-3 PUFA and placebo groups. Protein intake was not recorded during the 8-week training period, so it is impossible to assess whether the dietary protein intake was sufficient for a potentiated MPS response. Our findings were in accordance with the two other previous studies where training was prescribed and monitored whilst participants supplemented with n-3 PUFA, although these were conducted in older adults. Da Boit *et al.*, (2016) found that n-3 PUFA supplementation enhanced the increases in maximal isometric torque and muscle quality after 18 weeks of resistance exercise training in older women but not in men and Cornish *et al.*, (2018) found skeletal muscle strength following 12 weeks resistance training was not enhanced by n-3 PUFA supplementation in men. However, Strandberg *et al.*, (2019) did find that a diet enriched with n-3 PUFA combined with 24 weeks resistance training induced a significant hypertrophy of type IIA muscle fibres together with down-regulation of IL-1β and upregulation of mTOR in skeletal muscle of recreationally active older women. Furthermore, the decreases in IL-1β were associated with gains in leg lean mass and 1RM leg extension. It is important to note that the n-3 PUFA enriched diet also prescribed a protein intake of 20% of the total energy intake, whereas the diets analysed by Cornish *et al.*, (2018) only provided 17% of energy intake and there was no dietary assessment in the study by Da Boit *et al.*, (2016). Given the evidence provided above regarding the potentiated response of MPS to amino acids infusion (Smith *et al.*, 2011b), future research should evaluate combined supplementation protocols including protein and n-3 PUFA in conjunction with resistance training. There also appears to be a difference between males and females in the adaptations to strength training when supplemented with n-3 PUFAs, future research should aim to further explore this in a young population.

5.4.2.2 Muscle Damage and Inflammation

To our knowledge, this is the first study to examine the effects of EIMD and inflammation following prescribed eccentric training combined with n-3 PUFA supplementation in a well-designed, double-blind, placebo-controlled trial. There was no difference following training or between supplements on the effects of the MD on CMJ height, peak torque, knee and hip ROM, thigh swelling, muscle soreness, systemic levels of intramuscular proteins, white blood cell count or IL-6 concentration. Conversely, eight weeks of eccentric training did reduce muscle soreness 3 and 48h post MD, but this was not different between groups. Previous work found that 8 weeks of n-3 PUFA supplementation reduced the increases in IL-6 following muscle damaging exercise and this was associated with an attenuated strength loss, muscle soreness and reduction in ROM (Tsuchiya *et al.*, 2016), however there was no training element to this study. The present study and previous training studies (Da Boit, Sibson, Sivasubramaniam, *et al.*, 2016; Cornish *et al.*, 2018) have shown that n-3 PUFA supplementation does not augment the strength gains resulting from resistance training in males, however, these studies have not investigated EIMD. It is possible that the training alone improved the recovery from MD and there was no capacity for further benefit of n-3 PUFAs, although only the results of perceived muscle soreness in this study support this theory.

The total work done during the eccentric muscle damaging protocol in trial B and C did not differ and there were no differences between groups, however, this was due to one participant in each group sustaining an injury during the training period and being unable to complete the muscle damage protocol on the injured leg. All other participants completed more work in trial C than trial A and B (Figure 17). As a result, it is difficult to directly compare the effects of the muscle damage between trial B and C because potentially more damage was caused by the extra work done in trial C. However, this was expected due to the nature of the training study, as the participants got stronger, they were able to perform more work. This could be the reason for the lack of effect of either training or supplementation on the recovery from MD, as participants were more damaged in trial C.

5.5 Conclusion and Limitations

This study investigated the effects of n-3 PUFA and eccentric training on strength and power related indices of performance and determined whether n-3 PUFA reduced EIMD. Importantly this study included a trial prior to participants taking supplements enabling a within-subject measure in addition to the between groups analysis. To determine whether the eccentric exercise induced EIMD, the participants' perception of soreness, their ROM, muscle swelling, intramuscular proteins (CK, LDH and Mb) in the serum were measured. The perception of soreness and levels of serum intramuscular proteins both increased following the eccentric exercise and ROM was reduced suggesting the eccentric exercise had the desired effect of damaging muscle fibres and stimulating an inflammatory response in all three trials. No differences between olive oil and n-3 PUFA supplementation were detected.

A repeated bout effect was apparent between trial A and B following MD, demonstrated by the attenuated reductions in strength and power following MD in trial B and a lower perceived muscle soreness together with lower concentrations of Mb following MD in trial B. Despite a familiarisation session of the eccentric protocol on both legs, removing the repeated bout effect was difficult in this study due to the fact that there were three trials. Some studies have performed the damaging exercise unilaterally and had 2 trials, but this was not possible with the research design employed in this study. Even when this method has been used, there is still the possibility of a contralateral repeated bout effect (Xin *et al.*, 2014). It was also necessary in this study to induce a large amount of MD and this was easier to induce when using double the muscle mass in the EIMD protocol.

The only cytokine assessed in this study was IL-6, which is a limitation of the study as it does not provide a comprehensive assessment of the cytokine response. Funding limited the number of cytokines assessed in this study and rather than analysing using ELISAs, like in chapter 4, flow cytometry was used. Initially three cytokines were analysed (IL-6, TNF-α, and IL-1β) based upon previous research in this area (DiLorenzo, Drager and Ankin, 2014; Mickleborough *et al.*, 2015). However, there were too many missing data points due to low sensitivity of the flex sets used

for TNF-α and IL-1β and it would have been inappropriate to perform statistical analysis. Future research should aim to investigate several cytokines to gain a wider interpretation of the effect of n-3 PUFAs on inflammation. There is also a possibility that n-3 PUFA modulate local tissue inflammation without affecting systemic inflammatory markers (Lalia *et al.*, 2017). Future work could include muscle biopsies to determine the local tissue inflammation, although there are potential implications with the actual muscle biopsy procedure also causing inflammation which could be mistaken for exercise-induced inflammation (Malm *et al.*, 2000).

Unfortunately, due to technical difficulties incorporation of n-3 PUFAs were not measured in this study, however, compliance was used as a secondary measure to check supplements were consumed. Additional measures such as giving participants more supplements than they needed and giving them out in 2 weekly periods during the training phase should have helped to mitigate any non-compliance. Also as previously mentioned, a similar dose was sufficient to induce changes to the fatty acid profile of both blood and skeletal muscle (McGlory *et al.*, 2014), so it is expected that the n-3 PUFAs were incorporated in the present study.

A sample size calculation was not performed to determine the participant number required for the study. A major limitation of this study is the small sample size of 16 participants for part 1 and only 9 participants for part 2. Training studies are notoriously difficult to recruit for, especially when participants are required to be untrained. There was a limited timeframe to complete this study, due to the nature of the part-time PhD, and the study required a large time commitment from the participants at their own expense, which is why I was unable to recruit any more participants. Therefore, it must be acknowledged that a type II error may have occurred due to the small sample size.

To our knowledge, this is the first study to investigate the effects of eccentric training in association with n-3 PUFA supplementation on muscle damage following an eccentric muscle damaging protocol. Three weeks of n-3 PUFA supplementation and 8 weeks of n-3 PUFA supplementation combined with eccentric training did not improve exercise performance or reduce muscle damage and inflammation following eccentric muscle damaging exercise.

Chapter 6: Fish oil supplementation and recovery in elite male rugby union players following match play and resistance training

6.1 Introduction

A limited number of studies investigating the role of n-3 PUFA on performance and recovery have been performed on elite athletes (Heaton *et al.*, 2017). Evidence is often translated from studies in recreationally trained/untrained humans and it is likely there are different responses so they must be considered with caution (Thielecke and Blannin, 2020). The exercise protocols performed in chapters 4 and 5 were performed under well-controlled laboratory conditions in non-elite athletes, which potentially limit the applicability of findings to real-world elite performance. The exercise protocol used in chapter 4 aimed to mimic cycling time trial performance, however the lab setting created a more relaxed, less competitive environment for the cyclists meaning they may not have worked 100% maximally, evidenced by the mean RPE peak score of 18. The protocol used in chapter 5 clearly induced a large degree of muscle damage, but it was limited to the knee extensors and not reflective of damage that occurs from sporting activities such as rugby, additionally participants were untrained. Thus, to assess the effects of n-3 PUFA on sporting performance and recovery in a real sporting scenario, this study investigated the effects of n-3 PUFA supplementation on rugby union match play, resistance exercise performance and the recovery from these performances.

Rugby union is an intermittent, high-intensity contact team sport involving bouts of high-intensity activities such as running, kicking, passing, and tackling interspersed with low-intensity exercise (Roberts *et al.*, 2008). Rugby union match-play elicits markers of muscle damage such as CK and Mb (Roberts *et al.*, 2011). The combination of physical load and the metabolic and mechanical stresses associated with exercise induces post-match fatigue indicated by performance decrements, soreness and muscle damage (Takarada, 2003; Smart *et al.*, 2008; Cunniffe *et al.*, 2010; da Silva *et al.*, 2020). These decrements can last up to 72 hours post-game

and are associated with increased inflammation (Nunes *et al.*, 2019). It is common for rugby union players to have a game on a Saturday, rest day Sunday, followed by the heaviest loading gym session on the Monday morning, around 40 hours post-match. With the peak of muscle damage occurring between 24 – 72 hours post exercise (Close *et al.*, 2006) and acute inflammation being elevated from immediately post exercise up to 2 weeks post exercise (Peake, Nosaka and Suzuki, 2005), it is important to identify strategies to allow players to recover quickly to maintain physical and skill attributes throughout a season and allow ensuing training to take place with minimal soreness. Whilst previous studies have looked at a number of recovery strategies to reduce muscle damage post rugby match (see review by Calleja-González *et al.*, 2019), no previous studies have examined the effect of n-3 PUFAs on elite rugby union players post-match or post resistance-based training session. One study conducted during 5 weeks pre-season training in professional rugby union players observed that adding n-3 PUFAs to a protein-based supplement resulted in reduced muscle soreness and better maintenance of explosive power than the protein-based supplement alone (Black *et al.*, 2018). However, this study investigated the players over 5 weeks as opposed to the effect of one resistance training session or one match, so whilst it is expected that n-3 PUFA supplementation will have the same effect after a single session, it is not known as it has not previously been investigated.

The supplements for this study were provided by a commercial supplier who were interested in the effects of a supplement containing n-3 PUFA and curcumin, in addition to n-3 PUFA alone. Curcumin is a natural compound and bioactive polyphenol found in turmeric and has potential antioxidant and anti-inflammatory properties (Bongiovanni *et al.*, 2020). In elite rugby players supplemented with 6 g of curcumin and 60 mg piperine (48h before and 48h after exercise), the reduction in sprint mean power output 24h after muscle damaging exercise was lower in comparison to a placebo (glucose), however, there were no effects on muscle function, CMJ, CK or muscle soreness (Delecroix *et al.*, 2017). Post-eccentric exercise pain scores were lower in participants that ingested a multi-ingredient supplement containing curcumin for 30 days (Udani *et al.*, 2009). There is a lack of consensus within the literature regarding the benefits of curcumin which are likely due to its poor bioavailability. However, curcumin does have potential to attenuate

EIMD, particularly if metabolites are bio-available in a 24h window (Bongiovanni *et al.*, 2020). It is possible that multi-ingredient supplements containing curcumin enhance the bioavailability of curcumin and consequently improve its effectiveness in exercise recovery, as evidenced in the literature above. Curcumin and n-3 PUFA as a combined supplement have never previously been investigated in exercise recovery.

The aim of the present three arm, double-blind, placebo-controlled, study was to determine whether 6 weeks of n-3 PUFA supplementation could improve rugby match and resistance exercise-based performance and exercise-induced changes in markers of muscle damage and inflammation. Importantly this study investigated the effects of n-3 PUFA on recovery from actual competitive rugby union match play, which means the muscle damage may be caused by physical impacts as well as metabolic and mechanical stress, this is something that has never previously been investigated. It was hypothesised that n-3 PUFA supplementation would reduce the EIMD indicated by reduced muscle soreness and lower levels of the intramuscular proteins Mb, CK and LDH following a rugby game and a resistance-based training session compared with a placebo.

6.2 Method

The experimental protocol followed the Declaration of Helsinki principles and was approved by Loughborough University Ethics Human Participants sub-committee (Study ID: R17-P136). Prior to study entry, participants were informed that the study was investigating the effects of fatty acid supplementation in the form of a drink, therefore they were unaware that the aim of the study was to specifically examine n-3 PUFAs as it is recognised that n-3 PUFA supplementation is difficult to blind due to the fishy taste often experienced and this was a limitation of chapter 4.

Participants

Thirty-two elite level rugby union players from an English National League 1 performance squad volunteered for the study and provided written informed consent according to section 3.1 in the General Methods. A flow diagram of participant recruitment is provided in Figure 20. Participant characteristics recorded at their first

blood sample are shown in Table 14. The exclusion criteria consisted of vitamin or fish oil supplementation over past 6 months, habitual use of anti-inflammatory drugs, history of heart disease, coagulation/bleeding disorder, metabolic disease, serious allergy including food allergies, blood borne virus. The two participants excluded prior to randomisation was due to vitamin and fish oil consumption in the previous 6 months.
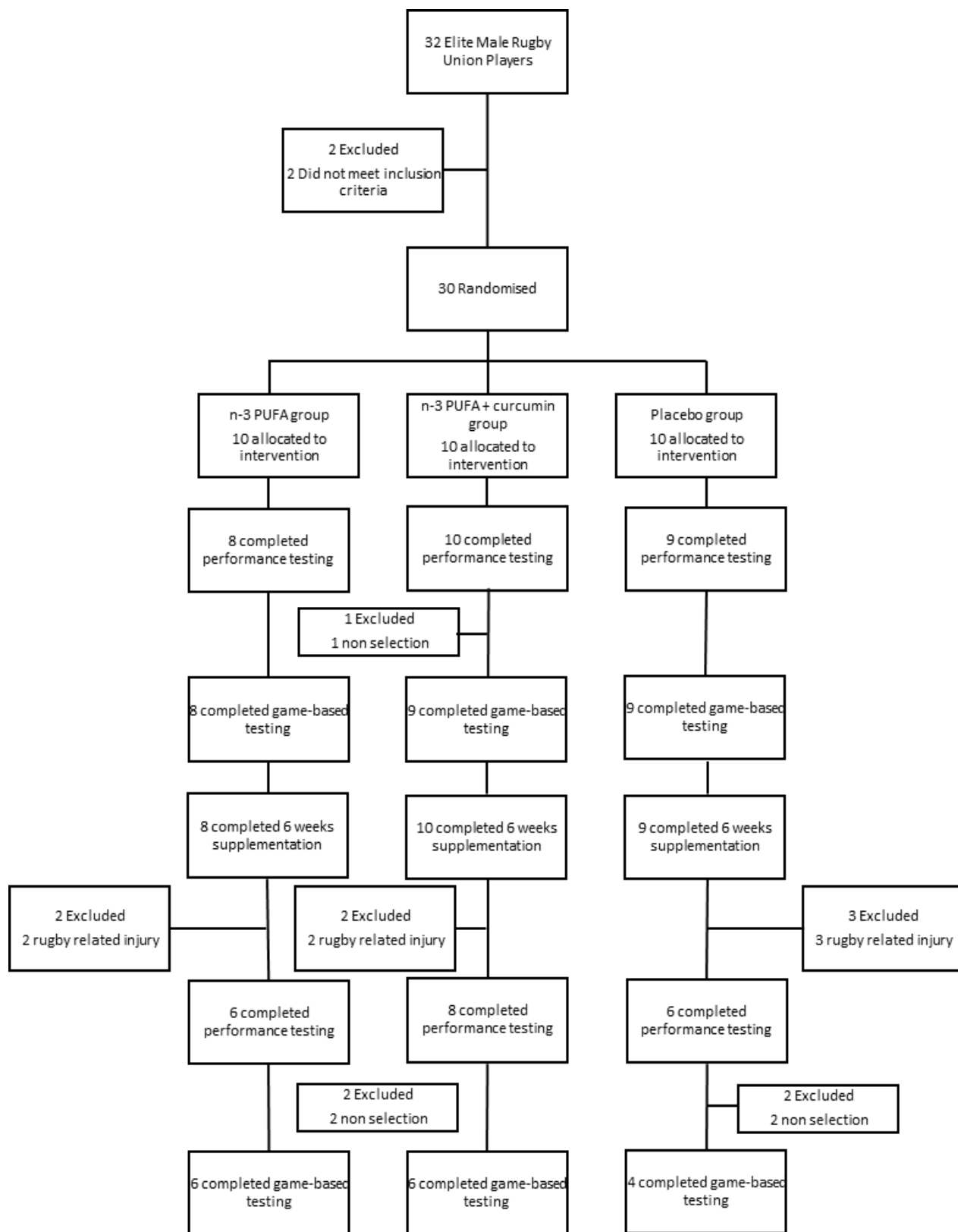
Figure 20 – Flow diagram of participant interaction

Experimental Design

A repeated measures design, with three parallel pair matched groups stratified when they commenced the study by playing position, body mass, 5RM box squat and 5RM hip bridge was used. Participants completed two bouts of performance testing and two bouts of game-based testing separated by a 6-week supplementation period (Figure 21). Four participants were supplemented for 8 weeks as they were not selected for the game that was scheduled for the end of the 6-week supplementation period, but they were selected for a game after 8 weeks of supplementation, so it allowed for a greater number of players to be included in the game-based analysis. For consistency, performance testing was still conducted after 6 weeks of supplementation in these four players.
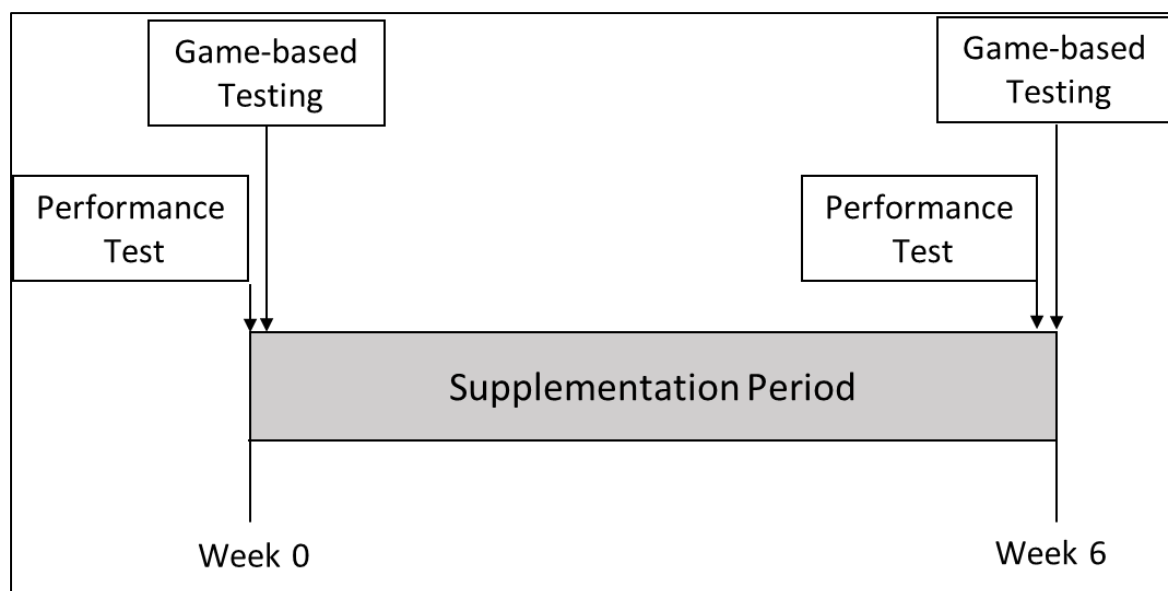
Figure 21 – Schematic Outline of the Study

Supplementation and Dietary Control

On completion of both the first performance and game testing, participants were required to drink two 200ml cartons, one with breakfast and one with dinner for six weeks. Participants were given one of three supplements: n-3 PUFA, which provided 1.8g EPA and 2.6g DHA, n-3 PUFA + Cu, which provided 1.2g EPA and 1.8g DHA and 1g curcumin, and placebo which contained a matched amount of carbohydrate juice but without the n-3 PUFA (Smartfish, Oslo, Norway). The participants and

investigators were blinded to the contents of the supplements and this was not revealed by the manufacturers until after data analysis was complete. Participants were given their cartons on a weekly basis and asked to return used cartons at the same time for compliance to be monitored.

Participants were asked to complete a food diary the day before and day of performance testing during the pre-supplementation trial and they were asked to replicate this post-supplementation. There were no dietary controls imposed for game-based testing.

Performance Testing

Performance testing was conducted at the same time of day pre- and post-supplementation to limit the effects of diurnal variation. Figure 22 provides a schematic of the performance testing. Firstly, anthropometric measurements (section 3.2.2) were taken, then a resting blood sample and a wellness questionnaire was completed. Players were already familiar with the wellness questionnaire, where they recorded their perceived soreness, energy levels, sleep hours and quality, along with details on any existing injuries. Whole body muscle soreness was measured on a scale from 1 (No soreness) to 10 (Unbearable Soreness). Energy levels and sleep quality were measured on a scale from 1 (Very poor) to 10 (Very good). A standardised 10-minute warm-up was conducted on a bike, followed by dynamic stretches, prior to 10m sprints, CMJs, squat jumps (SJ), 5RM box squat and 5RM hip bridge.
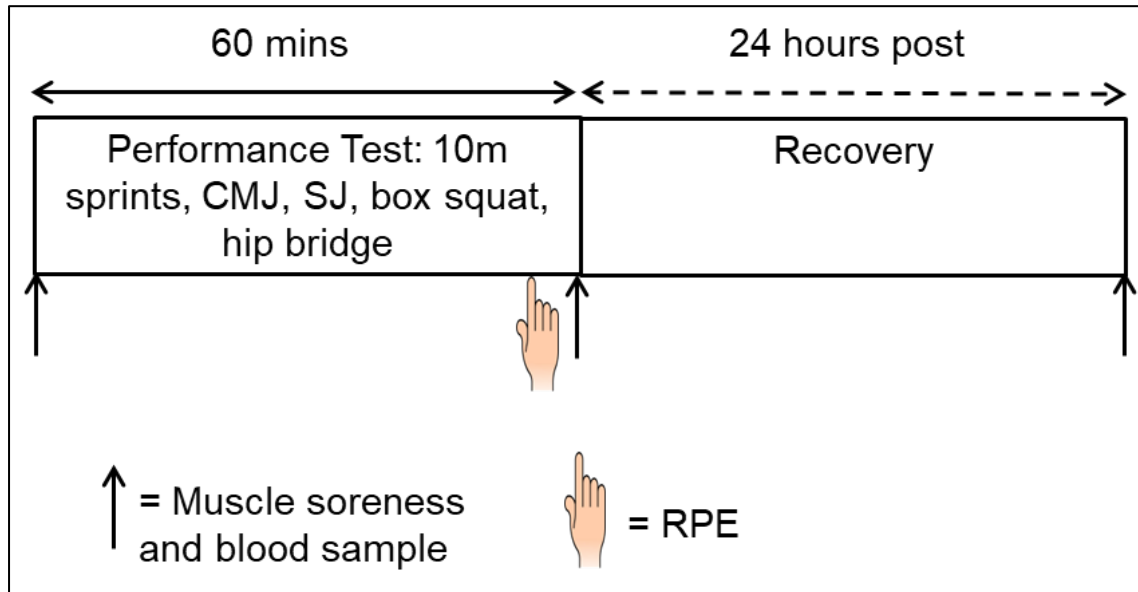
Figure 22 – Schematic of Performance Testing. CMJ, countermovement jump; SJ, squat jump; RPE, rating of perceived exertion.

Participants completed 3 maximal 10m sprints through timing gates (Brower timing systems, Draper, USA) with the fastest sprint being used for data analysis. Players were encouraged to sprint through the gates as fast as possible and were encouraged throughout by the same investigator. Three maximal CMJs and SJs were measured on a force plate (FD4000, Force Decks Ltd, London, UK) and power and jump height were recorded. For both jumps participants were instructed to keep their hands on hips throughout the whole movement. For the squat jump, they were instructed to hold a 90° bend at the knee joint for 3 seconds before jumping vertically as high and quickly as possible. If it was clear from the force trace that a countermovement had taken place, then that jump would be removed from the data analysis. The best attempt, determined by jump height, was used for data analysis. Five rep max tests were completed for box squats and hip bridges and the maximal scores were used for analysis. Players were familiar with all physical tasks involved in the Performance Testing as they were the same tasks as used in pre-season testing and periodically throughout the season, therefore no familiarisation was required.

Immediately after the testing session a venous blood sample was taken according to section 3.3.1, sRPE was recorded and another wellness questionnaire was

completed (minus sleep and sleep quality). A further venous blood sample and wellness questionnaire were completed 24h post performance testing.

Game-based Testing

Game-based testing took place immediately following the completion of 2 National League 1 rugby union fixtures separated by 6 weeks (Figure 23). The game time for each player was recorded for each game. Players were only included in the analysis if they completed at least 40 minutes of game play. A venous blood sample, game RPE and wellness questionnaire was taken from participants within 15 minutes of completion of the game. Forty hours post completion of the game, players provided another venous blood sample and completed a wellness questionnaire.
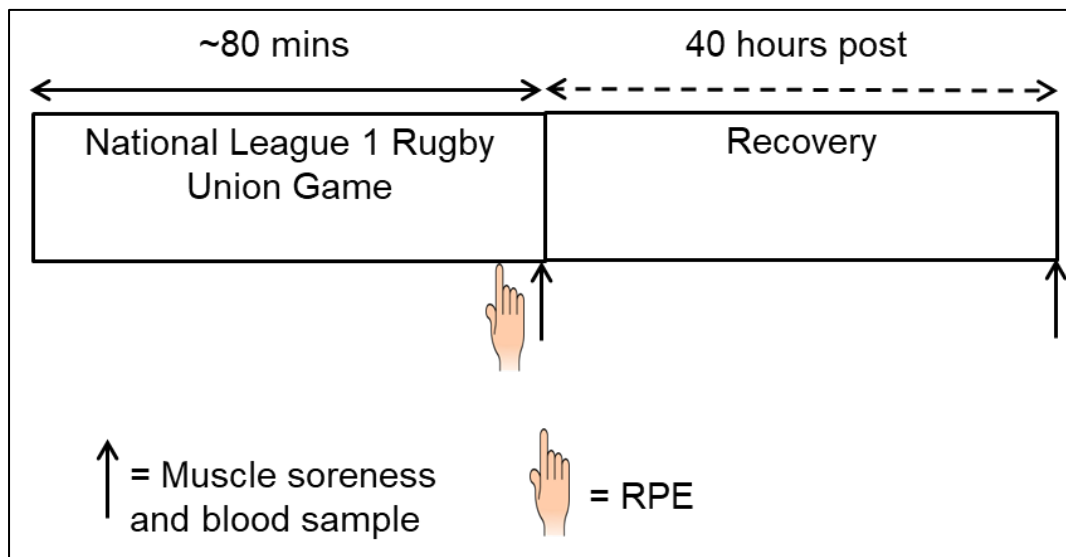
Figure 23 – Schematic of Game-based Testing. RPE, rating of perceived exertion.

Blood Sampling

Venous blood samples were obtained (section 3.3.1) before and after supplementation pre-performance testing, immediately post performance testing, 24 hours post performance testing, immediately post game-based testing and 40 hours post game-based testing. Serum was prepared (section 3.3.2) for analysis of markers of muscle damage at all time points. Whole blood was used for cell count analysis (section 3.4.1)

Blood Analysis

Determination of serum CK, LDH and Mb is described in section 3.4.4. White blood cell count analysis is described in section 3.4.1.

Data Analysis

Statistical analysis was performed according to section 3.5. Due to the nature of the game day, it was not possible to take pre-game measurements, therefore the pre-game measurements used in the analysis were the ones recorded for the equivalent pre-performance testing. For the performance testing (jumps, sprint, strength) a 3 group (n-3 PUFA, n-3 PUFA + Cu, placebo) by 2 trials (pre- and post-supplementation) mixed measures ANOVA was performed. Dependent variables in the recovery from performance testing and game-based testing were analysed using a 3 group (n-3 PUFA, n-3 PUFA + Cu, placebo) by 2 trials (pre- and post-supplementation) by 3 time mixed measures ANOVA.

6.3 Results

Compliance, as estimated from mean return carton count, was 100% for the n-3 PUFA group, 98% for the n-3 PUFA and curcumin group and 99% for the placebo group, with no difference between the two supplements.

There were no significant differences in physical characteristics (age, height, body mass) or strength between treatment groups ($p > 0.05$, Table 14). The total number of participants assigned to each group was 10 and there was an almost equal split of forwards and backs in each supplemented group (Table 14), however, this split was affected by dropouts in the study (see Figure 20).

Table 14 – Participant characteristics and playing position at pre-supplementation Performance Testing. RM, repetition maximum.

	n-3 PUFA (n = 10)	n-3 PUFA + curcumin (n = 10)	Placebo (n = 10)	P value
Age (years)	21 ± 1	21 ± 2	22 ± 1	0.14
Height (cm)	187.3 ± 4.2	187.8 ± 5.9	182.2 ± 6.3	0.06
Body Mass (kg)	98.20 ± 11.72	98.04 ± 8.24	96.33 ± 11.15	0.91
5RM Box Squat (kg)	155 ± 28	142 ± 15	146 ± 15	0.41
5RM Hip Bridge (kg)	166 ± 18	163 ± 32	167 ± 24	0.95
Forwards / Backs	5 / 5	6 / 4	5 / 5	-

Performance Testing

The sRPE was not different between pre- and post-supplementation for the performance test and there were no differences between supplements (Table 16). There was a significant training effect on the SJ, with participants in all groups jumping higher after 6 weeks of supplementation. There were no interaction effects in SJ, CMJ, maximum box squat or maximum hip bridge (Table 15). There was a tendency for those in the n-3 PUFA + Cu group to sprint faster post supplementation than pre-supplementation ($p = 0.060$, Figure 24). Two participants were unable to complete the sprint tests due to slight hamstring strains post supplementation.

Table 15 – Effect of six weeks supplementation on performance measures

Variables / Group	Pre-supplementation	Post-supplementation	P values (group; trial; interaction)
Squat Jump (cm)			
n-3 PUFA	35.5 ± 4.4	37.4 ± 5.5	
n-3 PUFA + Cu	37.0 ± 5.3	38.9 ± 4.8	0.777; 0.014; 0.931
Placebo	35.0 ± 7.1	36.4 ± 7.6	
CMJ (cm)			
n-3 PUFA	38.5 ± 5.7	38.8 ± 6.3	
n-3 PUFA + Cu	41.9 ± 5.3	42.7 ± 5.8	0.357; 0.497; 0.953
Placebo	36.9 ± 7.9	38.1 ± 7.9	
10m Sprint (s)			
n-3 PUFA	1.69 ± 0.09	1.70 ± 0.04	
n-3 PUFA + Cu	1.72 ± 0.08	1.67 ± 0.08	0.977; 0.983; 0.060
Placebo	1.67 ± 0.08	1.70 ± 0.04	
Max Box Squat (kg)			
n-3 PUFA	149 ± 25	150 ± 35	
n-3 PUFA + Cu	144 ± 14	146 ± 17	0.914; 0.905; 0.671
Placebo	150 ± 10	145 ± 9	
Max Hip Bridge (kg)			
n-3 PUFA	165 ± 21	179 ± 28	
n-3 PUFA + Cu	159 ± 33	169 ± 32	0.391; 0.069; 0.932
Placebo	177 ± 17	193 ± 40	

Values are mean ± SD (n = 20: n-3 PUFA n = 6, n-3 PUFA + Cu n = 8, Placebo n = 6). CMJ, countermovement jump.
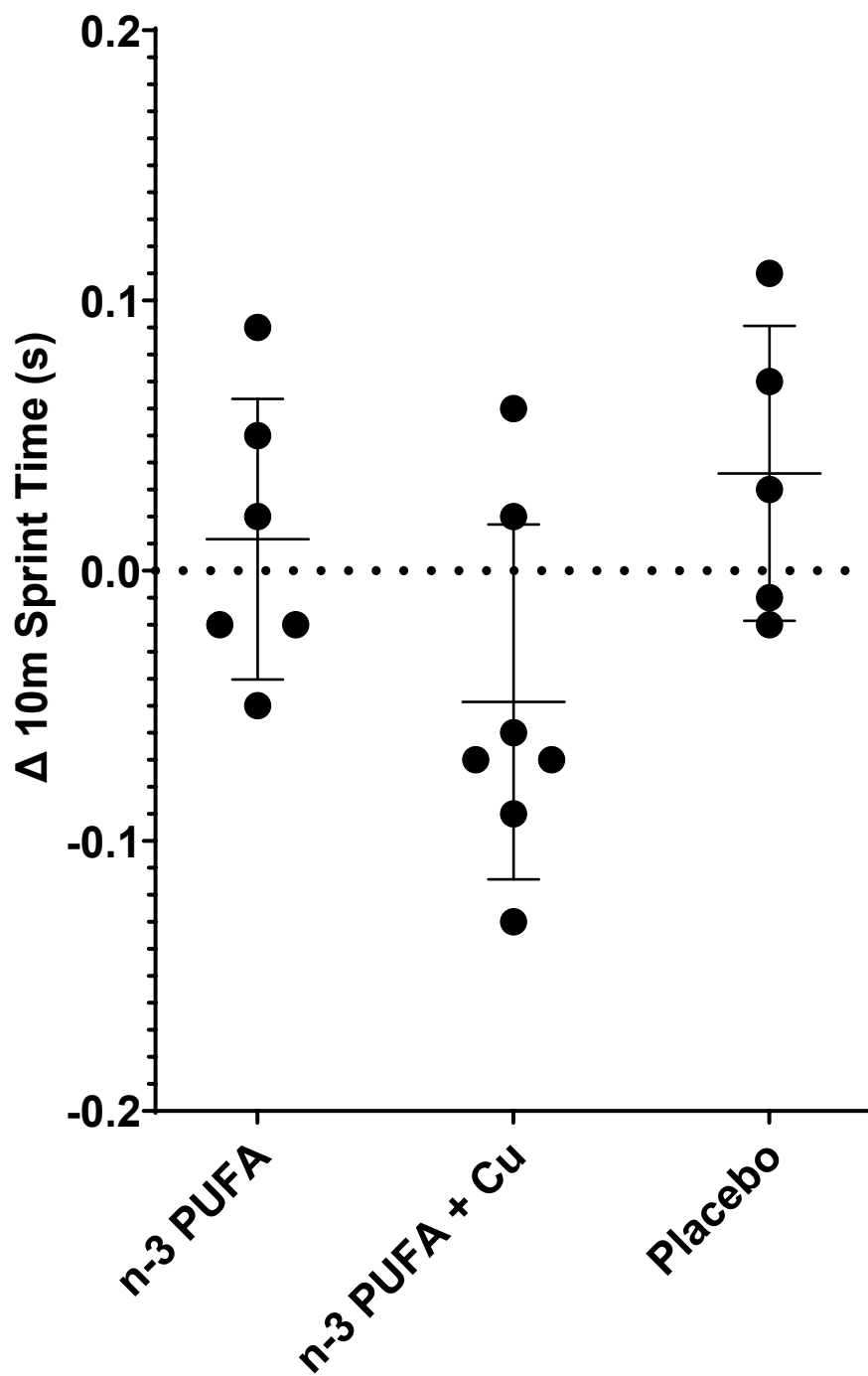
Figure 24 – Change in 10m sprint time (s) from pre- to post-supplementation (n = 18)

The muscle soreness was not different between groups pre- and post-supplementation and there was no significant rise in soreness resulting from the performance testing both immediately and 24h after testing (Table 16). In terms of muscle damage markers there were significant effects of time for CK ($p < 0.001$), LDH ($p = 0.019$) and Mb ($p < 0.001$), with all markers rising immediately post

performance test and then returning to baseline levels at 24 hours post performance test Table 17. There was a significant interaction effect of trial*time in CK with the levels of CK being consistently lower at all timepoints in the second performance test (p = 0.009). There were no supplement interaction effects across any of the systemic markers of muscle damage. There was a supplement x trial x timepoint effect on white blood cell count.

Table 16 – Effect of six weeks supplementation on perceived exertion and muscle soreness for the resistance-based exercise session

Variables / Group	Pre-supplementation			Post-Supplementation			P values (group; trial; time; interaction)
RPE (AU)							
n-3 PUFA	15 ± 1			14 ± 2			0.791;
n-3 PUFA + Cu	14 ± 2			15 ± 1			0.532;
Placebo	14 ± 2			14 ± 1			0.168
Soreness (AU)	Baseline	Post	24h	Baseline	Post	24h	0.783;
n-3 PUFA	5 ± 2	5 ± 1	5 ± 1	5 ± 3	5 ± 2	4 ± 2	0.870;
n-3 PUFA + Cu	5 ± 2	5 ± 2	6 ± 2	5 ± 2	5 ± 2	5 ± 1	0.873;
Placebo	4 ± 1	5 ± 1	6 ± 1	5 ± 2	5 ± 1	5 ± 1	0.801

Values are mean ± SD (n = 20: n-3 PUFA n = 6, n-3 PUFA + Cu n = 8, Placebo n = 6). RPE, rating of perceived exertion.

Table 17 – Effect of six weeks supplementation on muscle damage markers and white blood cell count for the resistance-based exercise session

Variables / Group	Pre-supplementation			Post-Supplementation			P values (group; trial; time; interaction)
	Baseline	**Post**	**24h Post**	**Baseline**	**Post**	**24h Post**	
CK (U/L)							
n-3 PUFA	587 ± 360	654 ± 393	540 ± 335	407 ± 325	454 ± 355	459 ± 323	0.826;
n-3 PUFA+ Cu	618 ± 204	702 ± 255	525 ± 204	592 ± 429	670 ± 486	539 ± 427	0.293; <0.001;
Placebo	609 ± 209	679 ± 218	489 ± 155	504 ± 86	600 ± 95	590 ± 166	0.301
LDH (U/L)							
n-3 PUFA	381 ± 25	364 ± 95	342 ± 27	349 ± 42	346 ± 50	341 ± 35	0.923; 0.416;
n-3 PUFA+ Cu	381 ± 46	407 ± 113	328 ± 67	363 ± 79	365 ± 73	335 ± 56	0.019;
Placebo	398 ± 102	378 ± 47	323 ± 75	354 ± 61	367 ± 42	384 ± 24	0.608
Mb (ng/ml)							
n-3 PUFA	48.6 ± 17.7	71.6 ± 26.0	35.8 ± 8.8	34.1 ± 9.8	61.8 ± 30.4	39.5 ± 12.0	0.586; 0.611;
n-3 PUFA + Cu	52.2 ± 15.1	87.2 ± 30.8	35.8 ± 9.1	41.5 ± 11.2	78.7 ± 32.2	37.2 ± 9.2	<0.001;
Placebo	49.0 ± 9.3	74.6 ± 8.5	33.9 ± 4.5	40.1 ± 7.6	94.5 ± 59.9	42.8 ± 10.8	0.419
WBC (10^9/L)							
n-3 PUFA	6.41 ± 1.74	6.48 ± 1.77	4.88 ± 1.05	5.53 ± 1.05	5.93 ± 1.08	5.06 ± 0.55	0.910; 0.511;
n-3 PUFA + Cu	5.42 ± 0.73	5.95 ± 0.90	6.52 ± 2.23	5.97 ± 2.26	6.31 ± 2.35	5.72 ± 1.68	0.091;
Placebo	6.04 ± 0.78	6.53 ± 1.14	5.57 ± 1.14	5.51 ± 0.90	5.95 ± 1.08	5.81 ± 1.63	0.011

Values are mean ± SD (n = 20: n-3 PUFA n = 6, n-3 PUFA + Cu n = 8, Placebo n = 6). CK, creatine kinase; LDH, lactate dehydrogenase; Mb, myoglobin; WBC, white blood cell.

Game-based Testing

There was a main effect of trial (p = 0.021) in the minutes played between pre-supplementation (80 ± 18 mins) versus minutes played post-supplementation (90 ± 5 mins) (Figure 25). The overall RPE for the game play was not different between groups or games despite the different playing times.

☐ Pre-supplementation

■ Post-supplementation
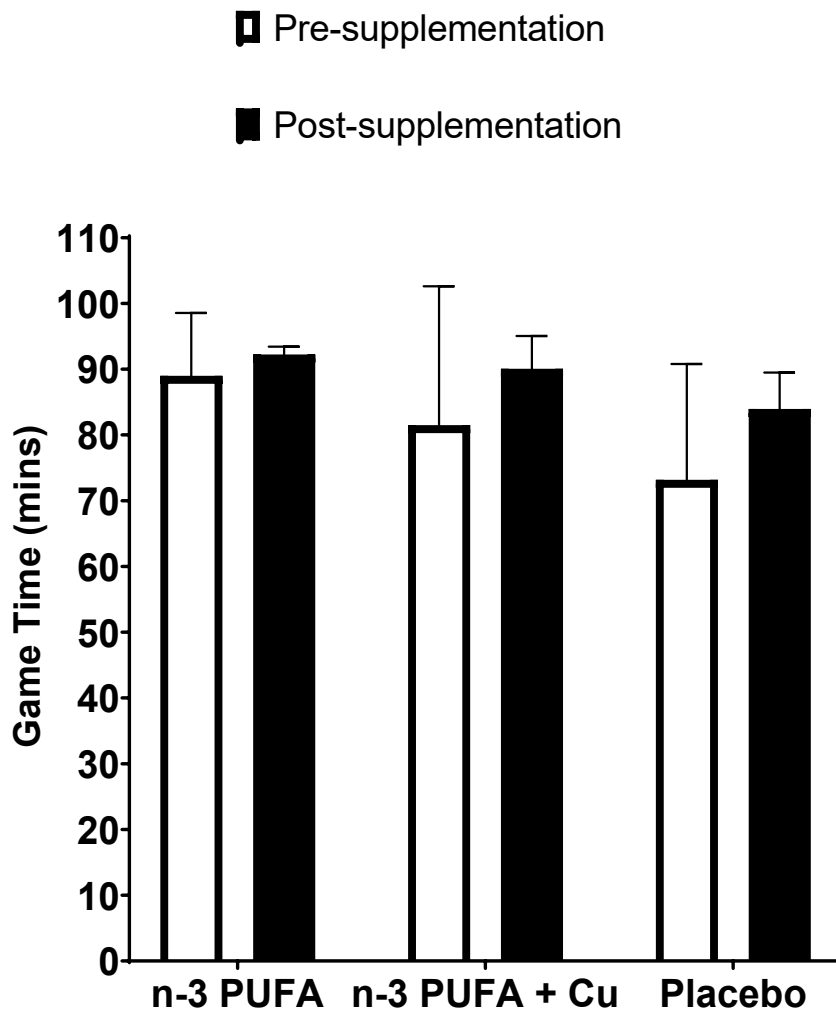
Figure 25 – Game time (mins) for pre- and post-supplementation of n-3 PUFA, n-3 PUFA + Cu and Placebo (n = 16: n-3 PUFA n = 6, n-3 PUFA + Cu n = 4, Placebo n = 6).

There was a significant main effect of time on muscle soreness (p = 0.003) with post-hoc analysis revealing soreness immediately post game was greater than 40h post

game. There were no differences in soreness between groups or games. To account for the longer game time in the post-supplementation testing the soreness score was divided by the game time for each player and this was analysed. Again, a main effect of time was revealed (p = 0.006) with the greater soreness reported immediately post game, this was not different across groups or games (Table 18).

Table 18 – Effect of six weeks supplementation on perceived exertion and muscle soreness for the rugby game

Variables / Group	Pre-supplementation		Post-Supplementation		P values (group; trial; time; interaction)
RPE (AU)					
n-3 PUFA	16 ± 3		16 ± 1		0.618;
n-3 PUFA + Cu	17 ± 2		18 ± 2		0.194;
Placebo	15 ± 5		17 ± 4		0.480
RPE/ game time					
n-3 PUFA	0.18 ± 0.03		0.17 ± 0.01		0.280;
n-3 PUFA + Cu	0.23 ± 0.07		0.20 ± 0.03		0.096;
Placebo	0.24 ± 0.08		0.20 ± 0.05		0.723
Soreness (AU)	Post-game	40h post-game	Post-game	40h post-game	0.055;
n-3 PUFA	7 ± 2	6 ± 2	6 ± 1	6 ± 0	0.901;
n-3 PUFA + Cu	7 ± 1	5 ± 2	7 ± 1	5 ± 1	0.003;
Placebo	5 ± 2	4 ± 1	5 ± 2	4 ± 3	0.747
Soreness/ game time	Post-game	40h post-game	Post-game	40h post-game	0.543;
n-3 PUFA	0.08 ± 0.03	0.06 ± 0.02	0.07 ± 0.01	0.07 ± 0.00	0.066;
n-3 PUFA + Cu	0.09 ± 0.03	0.06 ± 0.03	0.07 ± 0.01	0.06 ± 0.01	0.006;
Placebo	0.08 ± 0.03	0.06 ± 0.03	0.06 ± 0.02	0.04 ± 0.03	0.543

Values are mean ± SD (n = 16: n-3 PUFA n = 6, n-3 PUFA + Cu n = 4, Placebo n = 6). No p value for main effect of time for RPE due to only one time pont. RPE, rating of perceived exertion.

There was a significant main effect of time on CK, with CK increasing immediately post game, increasing further at 40h post game in all supplemented groups, in both games (p = 0.050). When CK was divided by minutes played in the game, the difference in time was removed and there were no differences detected within or between groups or trials.

There was a significant main effect of time on LDH with LDH peaking immediately post game and then decreasing back to baseline by 40h post game (p = 0.001). When concentrations of LDH were divided by minutes played in the game, there was

a significant main effect of trial with LDH levels higher in the game pre-supplementation than post-supplementation (p = 0.009), this was not different between groups. There was also a significant main effect of time with LDH peaking immediately post game and decreasing 40h after the game (p = 0.001), this was not different between groups or games.

There was a significant main effect of time on Mb (p = 0.004) with a peak immediately post game that was significantly higher than pre-game (p = 0.013) and 40h post-game (p = 0.012), this was not different between groups or games. When concentrations of Mb were divided by minutes played in the game, there was a significant main effect of trial with Mb levels higher in the game pre-supplementation than post-supplementation (p = 0.031), this was not different between groups. There was also a significant main effect of time with Mb peaking immediately post game and decreasing 40h after the game (p = 0.011), this was not different between groups or games.

A significant main effect of time was observed in the white blood cell count with a peak immediately post game that was significantly higher than pre-game (p < 0.001) and 40h post-game (p < 0.001), this was not different between groups or games. When white blood cell count was divided by game playing time, there was a significant main effect of trial with the white blood cell count higher in the pre-supplementation trial than the post-supplementation trial (p = 0.056), with no difference between groups. In addition, there was a significant main effect of time with a higher white blood cell count detected immediately post-game compared with 40h post-game (p < 0.001), with no difference between groups.

6.4 Discussion

In the first study to investigate the impact of n-3 PUFA supplementation on rugby union performance and recovery, it was hypothesised that 6 weeks of n-3 PUFA supplementation would reduce the EIMD following a rugby game, or a resistance-based training session compared with a placebo. The findings of this study oppose this hypothesis.

Exercise Performance

The rugby players perceived the performance testing combined with the resistance-based exercise session to be of similar exertion pre- and post-supplementation, however, the squat jump in all supplemented groups was significantly higher post-supplementation and there was a tendency for the maximum weight lifted in the hip bridge to be greater post-supplementation in all groups. Whilst these measurements were taken mid-season and a training effect was not expected, it is possible that a small training effect was detected due to the limited match-play prior to the pre-supplementation trial as several fixtures were cancelled due to poor weather conditions. During the six weeks of supplementation the weather conditions improved, and more match-play occurred, which may have contributed to the improved performance.

No difference was observed in markers of performance as a result of n-3 PUFA supplementation. In accordance with the findings, four weeks of n-3 PUFA supplementation during football training in competitive male and female football players did not affect 1RM leg extension, CMJ performance or 20m sprint time (Gravina *et al.*, 2017). Vertical jump height was not different between n-3 PUFA supplemented groups and a placebo group following 7.5 weeks of supplementation in resistance trained males (VanDusseldorp *et al.*, 2020) Furthermore, there was no effect of n-3 PUFA supplementation on quadriceps MIVC in recreationally active young males (Gray *et al.*, 2014), healthy male athletes (Lewis *et al.*, 2015) or trained male cyclists and runners (Hingley *et al.*, 2017). In contrast to the present findings, 5 weeks of a protein based supplement containing n-3 PUFAs improved CMJ performance by 4.6% compared with a decrease of 3.4% after 5 weeks of a protein based placebo (Black *et al.*, 2018). However, it is important to interpret these results with caution as the 5-week period was during pre-season training and therefore it is normal for improvements in CMJ in this training period (Grainger *et al.*, 2020). Black *et al.*, (2018) do not provide an explanation for the drop in performance in the placebo group and it was probably the decrease in CMJ performance in the placebo group that enabled the likely beneficial effect to be detected between groups. Philpott *et al.*, (2019) found an increase in 1RM leg extension in the non-dominant leg of resistance trained men supplemented with n-3 PUFA following two weeks of

an energy-restricted diet that was not seen in those supplemented with a placebo, however there were no differences between groups in other performance indicators including leg press 1RM, maximum voluntary contraction and muscular endurance. Although speculative, it may be that a higher dietary protein intake, as provided in the study by Black *et al.*, (2018), is required to optimise the interactive effect of protein and n-3 PUFA in the regulation of muscle protein metabolism, which can improve muscle performance.

Interestingly, there was an interaction effect in 10m sprint time with the n-3 PUFA combined with curcumin group improving mean sprint time by 0.05 s after supplementation Figure 24. In practical terms this could mean the difference in a rugby game between getting to the ball first when it has been kicked or getting to a player to make a crucial tackle, making this a key finding of this study. However, this was the only beneficial effect of this supplement observed and intriguingly the same pattern was not seen in those supplemented with just n-3 PUFA. Participant numbers for this test was low due to dropouts and injuries, making it difficult to ascertain whether this was a true finding or if other factors came into it such as participant motivation and fatigue.

Recovery from resistance-based exercise

Muscle soreness rated on the 0-10 scale did not increase post-exercise in any group, suggesting that the exercise session did not induce soreness, or the scale used was insufficient to pick up small changes in soreness. Consequently, there was no effect of supplementation. However, this scale was used because it was part of a daily questionnaire that the players completed to help monitor training and, to make the study more ecologically valid, only limited changes were made to their normal routines. For a more detailed assessment of muscle soreness, future studies may wish to use a soreness scale for individual muscle groups (Roberts *et al.*, 2011). Contrary to the soreness data, the resistance-based exercise did cause the markers of MD; CK, LDH and Mb, to rise immediately post exercise, returning to baseline 24h post exercise. There was a training effect of CK, with CK levels being lower post-supplementation in all groups compared with pre-supplementation, however, this was not observed in the other markers of muscle damage. As only one marker of

muscle damage demonstrated a training effect, it should be interpreted with caution, especially as CK can be highly variable.

Recovery from match play

Rugby match play induced increases in CK, LDH, Mb and WBC count, suggesting the match play induced muscle damage and inflammation. The time course of serum muscle damage markers followed the same trend as Roberts *et al.*, (2011), with CK peaking 40h post exercise and Mb peaking immediately post exercise. Increases in Mb and resting and 40h post-match CK levels were much higher in the present study than in the simulated rugby union match play study (Roberts *et al.*, 2011), suggesting simulating rugby union match play does not induce the same level of muscle damage as actual match play and this is likely due to the lack of physical contacts. In support of this CK levels 24h following a competitive rugby union game were comparable to the present study (Minett, Duffield and Bird, 2010). Soreness scores immediately post game also indicated that muscle damage had occurred using a subjective scale. Due to the ecological nature of the study, muscle function was not assessed pre- or post-match play, so it is impossible to ascertain whether any muscle damage that occurred would cause subsequent training and match performance to be impaired. However, as the study was performed on elite athletes, it was not appropriate to alter their training regimes in the middle of the season. Supplementation of either n-3 PUFA or n-3 PUFA and curcumin did not attenuate the increase in systemic markers compared with pre-supplementation or placebo.

Due to the nature of the study, it was impossible to control for muscle damage and inflammation caused by the game as both games that players were tested in were competitive National League division 1 games. The main concern with this is that there was a significant difference between the minutes played in the two games that players were tested in with the post supplementation game being 9.7 minutes longer than the pre supplementation game. Interestingly, players did not differentiate between the games in terms of the RPE score given to the game. To overcome this issue, further analysis was performed dividing muscle damage, inflammation, and soreness scores by the number of minutes played per individual in each game. The only variables affected by this change in analysis were Mb and WBC count, where

the pre-supplementation game induced higher levels of Mb and higher WBC count than the post-supplementation game. Together these findings may suggest that the pre-supplementation game caused greater levels of damage and inflammation than the post-supplementation game possibly due to the difference in playing style and ability of the opposing team, which may have masked any effect of n-3 PUFA supplementation. This was unavoidable due to the nature of this real-world sporting scenario study.

Relative to placebo, supplementation with n-3 PUFA or n-3 PUFA with curcumin did not reduce markers of muscle damage immediately or 40h after exercise. This finding concurs with a number of studies involving exercise with a large eccentric component to induce muscle damage (Gray *et al.*, 2014; Lembke *et al.*, 2014; Tsuchiya *et al.*, 2016; Jakeman *et al.*, 2017). Others have reported that n-3 PUFA supplementation attenuates the rise in CK and LDH (Rajabi *et al.*, 2013; Ramos-Campo *et al.*, 2020; VanDusseldorp *et al.*, 2020) post exercise. Although these studies have all employed a muscle damaging eccentric exercise protocol, rather than a sports specific, ecologically valid protocol. One reason why the current study did not find an effect of n-3 PUFA supplementation may be because the participants were highly trained and accustomed to the damage caused by playing rugby union, meaning they may have been responding optimally already.

6.5 Conclusion

In conclusion, rugby union resistance training induced small increases in markers of muscle damage but not muscle soreness. Rugby union match-play induced large increases in markers of muscle damage and smaller increases in soreness in elite rugby union players. Six weeks of n-3 PUFA supplementation or n-3 PUFA supplementation with curcumin did not influence exercise performance or markers of muscle damage and soreness after resistance-based exercise or rugby union match play. Individuals accustomed to a particular mode of exercise do not gain any benefit from n-3 PUFA or n-3 PUFA and curcumin supplementation in terms of reducing muscle damage.

Chapter 7: General Discussion

7.1 Aims and Summary

The aim of this thesis was to investigate whether n-3 PUFA supplementation could improve exercise performance and recovery from strenuous exercise. The initial chapter demonstrated an increase in the levels of n-3 PUFAs in whole blood and PBMCs following 4 weeks of n-3 PUFA supplementation. Despite this incorporation of n-3 PUFAs into the tissues, there was no translation into significant changes in cycling performance or markers of MD and inflammation resulting from the exercise. Previous studies investigating the effects of n-3 PUFA supplementation on cycling performance and inflammation have produced mixed results, however, there have been multiple issues in terms of research design which may have caused bias in the results. In order to address this lack of consensus, chapter 4 employed a robust repeated-measures, crossover, double-blind, placebo-controlled design with a pre-supplementation trial. There was a wide inter-individual variability in the levels of n-3 PUFA in the whole blood and PBMCs following supplementation, however, the repeated measures design allowed for control of this variable, despite this no effect of supplementation on exercise performance or recovery was observed. It was suspected that the non-significant results were due to the trained status of the cyclists and the minimal MD or inflammation caused by the cycling protocol. To overcome the lack of MD and inflammation caused by cycling, chapter 5 employed a muscle damage protocol with eccentric contractions to ensure biomarkers for MD were present and at a 'high' level. Eccentric contractions produce direct muscle injury with leukocyte infiltration and the cytokine response from eccentric exercise is more damaging for the muscle compared to concentric exercise, such as cycling (Toft *et al.*, 2002). This finding was confirmed in this thesis with much greater responses in CK and Mb to exercise involving a high eccentric component compared to cycling which is mainly concentric in nature.

The well-trained cyclists in chapter 4 are likely to have increased their natural defence system through training, thus attenuating the requirement of the anti-inflammatory effects of n-3 PUFAs. Indeed, Baum, Telford and Cunningham (2013) reported a more pronounced effect of n-3 PUFA on lesser trained runners than well-

trained runners on CK and DOMS after a 30 km run. Hence, the participants in chapter 5 were untrained to see whether there was a greater effect of supplementation. The recruitment of untrained individuals allowed participants to abstain from exercise 48 hours post exercise to enable blood sampling when the peak of soreness and MD was expected. It was not feasible to ask the cyclists, that train daily, to refrain from exercise for 24 hours prior to the trial and then for 48 hours after the trial.

Following 3 weeks of n-3 PUFA supplementation the reduction in isometric peak torque following MD was attenuated in the untrained males, however, there were no significant differences in other markers of damage or inflammation between the n-3 PUFA group and the placebo group, nor were there any improvements in strength and power performance. Furthermore, following 8 weeks of eccentric training combined with supplementation, there were no differences between groups. Importantly supplementation did not blunt the strength and power adaptations to eccentric training. This was an important finding since pharmaceutical anti-inflammatories such as NSAIDs have been shown to blunt adaptations to training (Trappe *et al.*, 2002; Lilja *et al.*, 2018). Therefore, if individuals are taking n-3 PUFA supplements for the known health benefits (Lemaitre *et al.*, 2003; Mickleborough, Ionescu and Rundell, 2004; Goldberg and Katz, 2007; Calder, 2012b) then they should not worry about interference with adaptations to training.

Chapter 5 introduced the encouraging finding of not blunting adaptation to training in untrained individuals. However, athletes are unlikely to train in the same manner as the method employed in chapter 5 making it difficult to apply the findings to athletes. Chapter 6 addressed this issue by investigating elite rugby union players during 6 weeks of a playing season and found no difference in strength or power between an n-3 PUFA supplemented group and a placebo group. However, there were also no differences between groups for the recovery parameters measured following a training session and match play, indicating no effect of n-3 PUFA supplementation in these trained individuals.

The mechanisms responsible for the potential beneficial effects of n-3 PUFA supplementation on exercise performance and recovery have not been fully elucidated but are likely complex and multifactorial. It was beyond the scope of this

exercise performance-based thesis to examine the mechanisms by which n-3 PUFA may optimise recovery and enhance exercise performance. In terms of strength and power improvements it has been suggested that n-3 PUFA augments the anabolic response via the mTOR pathway (Smith *et al.*, 2011b, 2011a; Strandberg *et al.*, 2019). In terms of recovery from muscle damaging exercise, n-3 PUFA is thought to dampen exercise-induced inflammation (Mickleborough *et al.*, 2015; Ramos-Campo *et al.*, 2020) through its incorporation into leukocytes and skeletal muscle altering the cell membrane permeability.

7.2 Limitations

It is important to acknowledge that several limitations were identified in the interpretation of the findings of this thesis.

7.2.1 Sample size

A common theme with intervention studies in sport and exercise science is a small sample size. A power calculation was used to determine the sample size in chapter 4; however, it was based upon one of the secondary outcome measures and not the primary one, performance. Therefore, it is possible that the study was underpowered to establish significant performance results. Whilst the cross-over design used may have mitigated the small sample size, it is possible that a type II error may have occurred. In chapter 5 recruitment issues (due to the training element of the study) and lack of investigator time (due to the nature of the part time PhD) resulted in a smaller sample size than initially anticipated particularly for part 2 of the study. In chapter 6, despite recruitment going well, it was constrained by the fact that all players were from one team so there was only a certain number of players to recruit from. This was then further limited by the request of the company providing the supplements to make the study a 3-arm study, meaning the participants had to be split into 3 groups rather than the 2 groups planned in the initial study design. There were further reductions in sample size as the study progressed due to injuries and lack of selection for the games when testing took place. However, this was an

ecologically valid study where injuries and non-selection are unavoidable when investigating elite athletes in a performance setting.

7.2.2 Female Participants

Females were not investigated in this thesis, meaning the findings cannot be applied to females. There is a clear sex data gap in sport and exercise science research (Cowley *et al.*, 2021) and this thesis has added to this issue. The main reason for focusing on males in this thesis was due to wanting to give participants the same duration of n-3 PUFA supplementation. If testing had been arranged to allow pre- and post- testing to be conducted at the same phase in the menstrual cycle, then some participants would have had longer or shorter durations of supplementation due to longer or short cycle lengths. This would have potentially affected the amount of n-3 PUFA incorporated.

7.2.3 n-3 PUFA Incorporation

Measuring n-3 PUFA levels in the whole blood and PBMCs for all investigations in this thesis was the initial plan, however, it was due to have been conducted by another PhD student within the Chemistry department, which unfortunately did not happen. GCMS analysis is a technique beyond my capabilities and time constraints did not allow me to develop these skills, therefore, only samples from chapter 4 were presented. Data presented in chapter 4 does show an increase in n-3 PUFA in the blood and PBMCs and as similar doses were used in chapter 5 and 6 it could be inferred that similar increases in n-3 PUFA in the blood and PBMCs occurred. Furthermore, previous research showed that a similar dose was sufficient to induce changes to the fatty acid profile of both blood and skeletal muscle (McGlory *et al.*, 2014).

7.3 Future Directions

This thesis found no effect of n-3 PUFA supplementation in trained individuals, whilst a small effect was observed in untrained individuals with regards to attenuating the strength decrements following EIMD. These findings suggest that n-3 PUFA

supplementation may be most beneficial in a population that has lower physiological function as opposed to trained males who already have an efficient anabolic response to exercise through their training. The anabolic response to exercise may be attenuated in the elderly, which is demonstrated by a lack of muscle fibre hypertrophy in response to resistance training in older adults (Kosek *et al.*, 2006; Strandberg *et al.*, 2019). This anabolic resistance may be the reason for the greater effects of n-3 PUFA supplementation on strength and hypertrophy in older people demonstrated in the literature (Rodacki *et al.*, 2012; Smith *et al.*, 2015; Gray and Mittendorfer, 2017; Strandberg *et al.*, 2019). The lack of similar findings in younger adults, such as the studies detailed in this thesis, is possibly due to the anabolic response to resistance exercise in younger adults being sufficient. n-3 PUFA rich diets have caused a down-regulation in the gene expression of IL-1β in skeletal muscle (Strandberg *et al.*, 2019) and inhibition of IL-1β synthesis in PBMCs (Caughey *et al.*, 1996). Muscle hypertrophy during aging may be limited by elevated skeletal muscle IL-1β (Przybyla *et al.*, 2006), therefore downregulation of IL-1β may contribute to the hypertrophic response seen in older females. Whether this same mechanism exists in older males remains to be found.

In a non-supplemented, 18-week resistance training study the increase in maximal torque was 15.8 ± 10.6% in females, whereas there was a much greater increase of 41.7 ± 25.5% in males (p < 0.05). Muscle quality increased by 8.8 ± 17.5% in females, compared to a greater increase of 33.7 ± 25.6% in males (Da Boit, Sibson, Meakin, *et al.*, 2016). These findings suggest that older women require a greater resistance exercise stimulus to achieve the same improvements as in men. Therefore, there may be a greater capacity for older women to maximise strength and hypertrophy by combining resistance training with n-3 PUFA supplementation. However, there is a clear lack of female participants in the current n-3 PUFA literature. In terms of resistance training, one study in older adults has shown superior strength gains following 18 weeks of resistance exercise combined with n-3 PUFA supplementation in older women compared with older men (Da Boit, Sibson, Sivasubramaniam, *et al.*, 2016). The resistance training-induced increases in muscle quality and maximal isometric torque were enhanced by supplementation in older females but not older males, suggesting that the response to resistance exercise was not saturated in the females like it was in males. It is possible that older females

have greater anabolic resistance compared with older males and n-3 PUFAs can reduce this, resulting in greater improvements from resistance training. Whether this is also true in young females remains to be seen.

Another area that warrants further investigation is the use of n-3 PUFA supplementation during the recovery from injury in athletes. Recent evidence suggests that supplementation may alleviate muscle loss caused by limb immobilisation in young females and any losses detected were recovered after 2 weeks of normal activity (McGlory, Gorissen, *et al.*, 2019). This protective effect on skeletal muscle was linked to reduced losses in mitochondrial protein content and respiration kinetics (Miotto *et al.*, 2019). These findings suggest that n-3 PUFA supplementation could attenuate the reductions in muscle mass/size during injury and help young females return to play quicker. It is important to note that these studies were conducted in healthy young females that were not in a pro-inflammatory state, which would not be the case if they had been injured. Therefore, it remains unknown whether the effects would be the same in a pro-inflammatory state (this is generally the case when injured) or indeed in males.

Aside from further investigation into the populations mentioned above, it is crucial that any future work should include analysis of n-3 PUFA levels, as performed in chapter 4. This will enable confirmation of n-3 PUFA incorporation into tissue, in addition to a further check of compliance to ensure participants consumption of supplements. This analysis can also check whether dietary controls have been adhered to and whether the increase in n-3 PUFAs is associated with a decrease in other fatty acids. If possible, muscle tissue samples should be obtained to directly assess the effects of n-3 PUFA in the muscle and identify the associated cell signalling pathway to deduce the potential mechanisms involved.

7.4 Practical Recommendations

This thesis did not find significant evidence within the constraints of the experimental conditions employed to support the use of n-3 PUFA supplementation to improve exercise performance or recovery from exercise. However, contrary to current opinion within elite sports medicine, the anti-inflammatory properties of n-3 PUFAs did not appear to negatively impact adaptations to training in untrained or trained

individuals. Consequently, athletes supplementing with n-3 PUFAs for health reasons need not be concerned that they are dampening adaptations to training. It is also important to appreciate that whilst there was no positive evidence for the use of n-3 PUFA supplements to improve performance or recovery, the findings were limited by many variables including dosage and duration, type of exercise, biochemical markers, number, and demographic of the populations studied. A wealth of evidence is available in the literature to suggest exercise performance and recovery can be enhanced by n-3 PUFA supplementation when different populations (such as older females) and experimental designs have been used to the ones presented in this thesis. Moreover, although not directly assessed in this thesis, the literature suggests athletes could minimise reductions in strength and muscle mass caused by disuse by supplementing with n-3 PUFAs. Therefore, it may be beneficial for athletes to consume at least 5g/day of n-3 PUFAs following an injury if immobilization is unavoidable.

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Appendices

Appendix 1:

Example Recruitment Poster from Chapter 4

Do fish oils make you fit?

It is well known that fish oil has a protective effect in relation to cardiovascular inflammatory diseases and cancer. However, there is a lack of studies relating to physical performance. It is possible that fish oils enhance physical performance through similar mechanisms as the protective effect found in health, which could provide a rationale for endurance athletes to use omega-3 supplements.

We need participants who are:

- Male
- 18 – 45
- Not currently taking any fish oil or antioxidant supplementation
- Regularly taking part in cycling training
- Available for 5 sessions between October - January

What is involved:

5 x laboratory visits:

1 x VO$_2$ max test followed by either fish oil or placebo supplementation for 4 weeks with a performance test (70% Watt max for 45 minutes, followed by 15 minute distance trial) before and after supplementation. You will then do the same on the other supplementation.

N.B. A cannula (small plastic tube) will be used to take blood during the performance tests.

If you are interested in being a participant in the study please contact -

Lynsey Wilson
L.Wilson2@lboro.ac.uk

01509 228779

Fish Oil Study	Fish Oil Study	Fish Oil Study	Fish Oil Study	Fish Oil Study
Contact Lynsey @	Contact Lynsey @	Contact Lynsey @	Contact Lynsey @	Contact Lynsey @
L.Wilson2@lboro.ac.uk	L.Wilson2@lboro.ac.uk	L.Wilson2@lboro.ac.uk	L.Wilson2@lboro.ac.uk	L.Wilson2@lboro.ac.uk
01509 228779	01509 228779	01509 228779	01509 228779	01509 228779

Example Recruitment Poster from Chapter 5

Loughborough University

Resistance Exercise Study

We are conducting a resistance exercise study in addition to the supplementation of fatty acids to investigate whether certain fatty acids reduce the muscle damage and reductions in strength following a bout of resistance leg exercise.

We need participants who are:

- Male
- 18 – 55 years of age
- Not currently taking supplements
- Not resistance trained
- Available for 3 testing sessions between October – December 2016

What is involved?

- **4 x laboratory visits (1 x 1 hour, 3 x 6 hours):**

Approximate Testing Session Timings								
30 min	30 min	30 min	30 min	3 hr	30 min		30 min
Anthropometrics / Measurements	Samples / Warm up	Performance test / Eccentric Exercise	Performance test / Samples / Measurements	3 hr Rest in Lab	Samples / Measurements / Performance test	45 hour Rest at home	Samples / Measurements / Performance test

- **Eccentric exercise of the quadriceps**
- **Blood samples**

If you are interested in being a participant in the study, please contact -

Lynsey Wilson (Postgraduate research student)

L.Wilson2@lboro.ac.uk **or 01509 228779**

Resistance Training Study
Contact Lynsey @
L.Wilson2@lboro.ac.uk
01509 228779

Resistance Training Study
Contact Lynsey @
L.Wilson2@lboro.ac.uk
01509 228779

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01509 228779

Example Recruitment Poster from Chapter 6

Appendix 2: Example Health Screen Questionnaire

Health Screen Questionnaire for Study Volunteers

As a volunteer participating in a research study, it is important that you are currently in good health and have had no significant medical problems in the past. This is (i) to ensure your own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

If you have a blood-borne virus, or think that you may have one, please do not take part in this research.

Please complete this brief questionnaire to confirm your fitness to participate:

1. **At present,** do you have any health problem for which you are:

   (a) on medication, prescribed or otherwise .... Yes ☐ No ☐

   (b) attending your general practitioner .... Yes ☐ No ☐

   (c) on a hospital waiting list .... Yes ☐ No ☐

2. **In the past two years,** have you had any illness or injury which required you to:

   (a) consult your GP .... Yes ☐ No ☐

   (b) attend a hospital outpatient department .... Yes ☐ No ☐

   (c) be admitted to hospital .... Yes ☐ No ☐

3. **Have you ever** had any of the following:

   (a) Convulsions/epilepsy .... Yes ☐ No ☐

   (b) Asthma .... Yes ☐ No ☐

   (c) Eczema .... Yes ☐ No ☐

   (d) Diabetes .... Yes ☐ No ☐

   (e) A blood disorder .... Yes ☐ No ☐

   (f) Head injury .... Yes ☐ No ☐

   (g) Digestive problems .... Yes ☐ No ☐

   (h) Heart problems/chest pains .... Yes ☐ No ☐

   (i) Problems with muscles, bones or joints .... Yes ☐ No ☐

   (j) Disturbance of balance/coordination .... Yes ☐ No ☐

   (k) Numbness in hands or feet .... Yes ☐ No ☐

   (l) Disturbance of vision .... Yes ☐ No ☐

   (m) Ear/hearing problems .... Yes ☐ No ☐

   (n) Thyroid problems .... Yes ☐ No ☐

   (o) Kidney or liver problems .... Yes ☐ No ☐

   (p) Problems with blood pressure .... Yes ☐ No ☐

If YES to any question, please describe briefly if you wish (eg to confirm problem was/is short-lived, insignificant or well controlled.)

....

4. Smoking, physical activity and family history

    (a) Are you a current or recent (within the last six months) smoker?   Yes ☐   No ☐

    (b) Are you physically active (30 minutes of moderate intensity, physical activity on at least 3 days each week for at least 3 months)?   Yes ☐   No ☐

    (c) Has any, otherwise healthy, member of your family under the age of 35 died suddenly during or soon after exercise?   Yes ☐   No ☐

5. Allergy Information

    (a) Are you allergic to any food products?   Yes ☐   No ☐

    (b) Are you allergic to any medicines?   Yes ☐   No ☐

    (c) Are you allergic to plasters?   Yes ☐   No ☐

    (d) Are you allergic to latex?   Yes ☐   No ☐

If YES to any of the above, please provide additional information on the allergy

....

6. Additional questions for female participants

    (a) Are your periods normal/regular? .... Yes ☐   No ☐

    (b) Are you on "the pill"? .... Yes ☐   No ☐

    (c) Could you be pregnant? .... Yes ☐   No ☐

    (d) Are you taking hormone replacement therapy (HRT)?   Yes ☐   No ☐

7. Are you currently involved in any other research studies at the University or elsewhere?

         Yes ☐   No ☐

If yes, please provide details.

....

8. Have you recently given blood or been involved with research involving blood samples?

         Yes ☐   No ☐

If yes, please provide details.

....

9. Please provide contact details of a suitable person for us to contact in the event of any incident or emergency.

Name

……………………………………………………………………………………………………….

Telephone Number

………………………………………………………………………………………….

Work ☐ Home ☐ Mobile ☐

Relationship to Participant

……………………………………………………………....................

Appendix 3: Example Consent Forms

Example Informed Consent Form from Chapter 4

The Effect of Fish Oils on Performance, Inflammation, Oxidative Stress and Epigenetics

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.

I understand that any blood taken from me will only be used for the research purposes detailed in the participant information sheet.

I have read and understood the information sheet and this consent form.

I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in the study.

I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.

I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.

I agree to participate in this study.

Your name   _____

Your signature   _____

Signature of investigator   _____

Date   _____

Example Informed Consent Form from Chapter 5

Effect of Combined Fatty Acid Supplementation and Eccentric Exercise Training on Exercise Performance, Inflammation, Gene Expression and Epigenetic signatures.

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.	Yes ☐	No ☐
I have read and understood the information sheet and this consent form.	Yes ☐	No ☐
I have had an opportunity to ask questions about my participation.	Yes ☐	No ☐
I understand that I am under no obligation to take part in the study.	Yes ☐	No ☐
I understand that I have the right to withdraw from this study at any stage for any reason, and that I will not be required to explain my reasons for withdrawing.	Yes ☐	No ☐
I understand that all the information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others.	Yes ☐	No ☐
I agree to participate in this study.	Yes ☐	No ☐
I agree that the bodily samples taken during this study can be stored for future research.	Yes ☐	No ☐
If No to above, I confirm that the bodily samples taken during this study can **only be** used for this study and should be disposed of after 5 years.	Yes ☐	No ☐

Your name   _____

Your signature   _____

Signature of investigator   _____

Date   _____

Example Informed Consent Form from Chapter 6

The effects of fatty acid supplementation on markers of performance and inflammation on elite rugby union players

INFORMED CONSENT FORM

(to be completed after Participant Information Sheet has been read)

Taking Part	**Please initial box**

The purpose and details of this study have been explained to me. I understand that this study is designed to further scientific knowledge and that all procedures have been approved by the Loughborough University Ethics Approvals (Human Participants) Sub-Committee.

☐

I have read and understood the information sheet and this consent form.

☐

I have had an opportunity to ask questions about my participation.

☐

I understand that I am under no obligation to take part in the study, have the right to withdraw from this study at any stage for any reason, and will not be required to explain my reasons for withdrawing.

☐

I agree to take part in this study.

☐

Use of Information

I understand that all the personal information I provide will be treated in strict confidence and will be kept anonymous and confidential to the researchers unless (under the statutory obligations of the agencies which the researchers are working with), it is judged that confidentiality will have to be breached for the safety of the participant or others or for audit by regulatory authorities.

☐

Bodily Samples

I agree that the bodily samples taken during this study can be stored until 01/09/2027 for future research in the same research theme as this project.

☐

[Or] I agree that the bodily samples taken during this study can **only be** used for this study and will be disposed of within 5 years.

☐

_____   _____   _____
Name of participant   [printed]   Signature   Date

_____   _____   _____
Researcher   [printed]   Signature   Date

Appendix 4: Published Peer Reviewed Paper

Four Weeks of Omega-3 Supplementation does not Improve Cycling Time Trial Performance in Trained Cyclists

Lynsey S James[1]*, Alex J Wadley[2], Boakye Gyimah[3], David J Hunter[1], Tomos G Rosser[1], James C Reynolds[3], Sarabjit S Mastana[1] and Martin R Lindley[1]

[1]*School of Sport, Health and Exercise Science, Loughborough University, Epinal Way, Loughborough, England*
[2]*School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, Birmingham, England*
[3]*Department of Chemistry, Loughborough University, Epinal Way, Loughborough, England*

Abstract

Objectives: This study examined whether omega-3 polyunsaturated fatty acid (n-3 PUFA) supplementation lowered the heart rate (HR), rating of perceived exertion (RPE) and oxygen uptake ($\dot{V}O_2$) and accordingly improved cycling performance in a time trial.

Design: In a randomised, crossover, double-blind study, trained male cyclists (n = 10) were supplemented for 4 weeks with n-3 PUFA (5.7 g/day of eicosapentaenoic acid (EPA) and docosahexaenoic (DHA)) and 4 weeks with placebo (6g olive oil), with a 4-week washout period.

Methods: Cycling performance trials (45 min preload at 70% maximal work rate (Wmax) followed by 15 min time trial) were carried out prior to and following both supplementation periods. Fatty acid composition of blood total lipids was analysed prior to and in response to supplementation.

Results: Whole blood n-3 PUFA (% total fatty acids) increased from 1.67% (SD = 0.99%) to 3.72% (SD = 1.22%) (p < 0.05) following 4 weeks n-3 PUFA supplementation. Submaximal measures of $\dot{V}O_2$, HR, respiratory exchange ratio (RER) and RPE were unaffected by supplementation. Time trial performance (mean power W) was unchanged by n-3 PUFA (pre 239 W, SD = 34 W vs post 243 W, SD = 33 W), as were measures of $\dot{V}O_2$, HR, RER and RPE during the time trial.

Conclusions: High dose n-3 PUFA supplementation for 4 weeks did not improve cycling performance or attenuate the physiological variables usually associated with improved cycling performance, i.e. $\dot{V}O_2$ and HR, in a repeated-measures, placebo-controlled, crossover design study. It is possible that the exercise protocol used in the study was of insufficient intensity for the n-3 PUFA to show beneficial affects due to the highly trained nature of the cyclists.

Keywords

Fish oil; n-3 PUFA, Exercise, Oxygen uptake, Heart rate, Time trial

Abbreviations

DHA: Docosahexaenoic Acid; EPA: Eicosapentaenoic Acid; FAME: Fatty Acid Methyl Esters; HR: Heart Rate, n-3 PUFA: Omega-3 Polyunsaturated Fatty Acid; PT: Performance Test; RER: Respiratory Exchange Ratio; RPE: Rating of Perceived Exertion; $\dot{V}CO_2$: Carbon dioxide Production; VE_{STPD}: Minute Ventilation; $\dot{V}O_2$: Oxygen Uptake; Wmax: Maximal Work Rate

Introduction

It is well documented that omega-3 long chain polyunsaturated fatty acids (n-3 PUFAs) have beneficial effects on human health; supplementing with n-3 PUFA has demonstrated positive effects on atherosclerosis [1] cardiovascular disease [2], rheumatoid arthritis [3], asthma [4], brain function [5], and the prevention of acute and chronic inflammation [6]. These affects are due to the anti-inflammatory, antithrombotic, antiarrhythmic, hypolipidemic and antiproliferative properties of n-3 PUFA [7] and it is for this reason that n-3 PUFA is one of the most popular dietary supplements used by elite athletes [8].

Nonetheless, evidence that n-3 PUFA supplementation can be advantageous for athletes is equivocal. Maximum oxygen uptake ($\dot{V}O_2$ max) appears to be unaffected by n-3 PUFA supplementation [9-12]. Conversely, some studies have reported a trend for increased time to fatigue [13] and a reduction in submaximal $\dot{V}O_2$ following periods of n-3 PUFA sup-

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Appendix 5: Published Peer Reviewed Abstracts

ACSM Annual Meeting 2015 Abstract

Fish oil supplementation and time trial performance

Fish oil supplementation is regularly taken by sports performers across a wide range of athletic activities and by recreational as well as elite level cyclists. However, due to the lack of well controlled research studies involving exercise performance and fish oil supplementation there is a lack of consensus within the current literature about whether fish oils can improve performance.

PURPOSE: To determine the effect of fish oil (rich in EPA and DHA) supplementation on cycling time trial performance and heart rate (HR) in trained cyclists. METHODS: Eleven trained male cyclists ($54ml \cdot kg^{-1} \cdot min^{-1}$ – 327 Wmax) performed a maximal oxygen consumption cycle test (LODE Excalibur Sport electro-magnetically braked ergometer) followed by four separate cycling performance tests (45 mins at 70% Wmax followed by 15 min Time Trial, TT) in a double blind, placebo controlled randomised order cross over research design. Performance Tests were conducted pre- and post- 4-week supplementation with FO - 5.7g per day (6 capsules) of fish oil (EPA - 4.08g, DHA - 1.63g) or placebo (PLA) - 6g per day (6 capsules) Extra Virgin Olive Oil (Holland and Barrett), separated by a 4 week washout period. Supplements were taken with meals evenly spread throughout the day (2 capsules per meal). Mean power, distance covered and heart rate during the 15 min TT were compared across the four trials using a one-way repeated measures ANOVA. RESULTS: There was no significant difference ($p>0.05$ ANOVA) in mean power during the 15 min TT between trials (pre-FO=248W, post-FO=245W: pre-PLA=252W: post PLA=241W). There was no significant difference ($p>0.05$) in distance covered over the 15 min TT between trials (pre-FO = 13.3km, post-FO= 13.5km: pre-PLA =13.6km, post PLA=13.2km). Concurrently there was no significant difference ($p>0.05$) in HR at the midpoint of the 15 min TT or max HR between trials (pre-FO=176 $b \cdot min-1$, post-FO=177 $b \cdot min-1$, pre-PLA=175 $b \cdot min-1$, post-PLA=176 $b \cdot min-1$). CONCLUSION: Neither cycling time trial performance nor heart rate during the TT is moderated by four weeks of fish oil supplementation in trained cyclists.

ACSM Annual Meeting 2019 Abstract

Eight weeks eccentric quadriceps training with omega-3 supplementation does not impair torque and power improvements

Lynsey S James, David J Hunter, Beth Hussey, Glenda Anderson, Sarabjit S Mastana, Martin R Lindley

Loughborough University, UK

Individuals who are unaccustomed to resistance exercise experience greater levels of exercise-induced muscle soreness, this can deter individuals from completing an exercise programme and improving strength and power. To alleviate the symptoms, they may consume Non-steroidal anti-inflammatory drugs (NSAIDS). Evidence suggests NSAIDS can blunt muscle protein synthesis (Trappe *et al.*, 2002) and attenuate strength and muscle hypertrophic adaptations from resistance training (Lilja *et al.*, 2018), negating the effects of the exercise. Omega-3 supplementation has been suggested as an alternative to NSAIDS but the impact of Omega-3 on resistance training is inconclusive.

PURPOSE

To determine the effects of omega-3 supplementation on eccentric training-induced increases in torque and power.

METHODS

Nine physically active but non-resistance trained males (29 ± 9 years) were pair matched for isometric and eccentric quadriceps strength and randomly assigned, in a double-blind manner, to either omega-3 (5.1g/d) or olive oil (6.0g/d) supplementation for 3 weeks prior to and for 8 weeks during eccentric training. Performance measures of peak torque (isometric, concentric, eccentric) and jump height were conducted before and after 8 weeks of training. Supervised training consisted of maximal eccentric quadriceps contractions on an isokinetic dynamometer at $60°s^{-1}$ through 80° range of motion. Two training sessions were conducted per week, with a minimum of 48 hours recovery between sessions. Number of repetitions and sets were increased over the 8 weeks.

RESULTS

Following 8 weeks of eccentric training, peak eccentric torque significantly increased by 40 ± 56 Nm in omega-3 group and 51 ± 52 Nm in olive oil group, with no differences between groups ($P > 0.05$). Both groups also significantly increased their maximal isometric torque ($P = 0.02$); omega-3 group increased by 21 ± 10 Nm and olive oil group increased by 23 ± 30 Nm, with no differences between groups ($P > 0.05$). There was no main effect of training on peak concentric torque ($P > 0.05$). Jump height increased by 1.0 ± 1.9 cm in the omega-3 group and decreased by 0.03 ± 1.33 cm in the olive oil group, with no difference between groups ($P > 0.05$).

CONCLUSION

Omega-3 supplementation does not impair or augment eccentric training-induced increases in torque or power in young males.

BASE Annual Conference 2019 Abstract

Eight weeks eccentric quadriceps training with Omega-3 supplementation does not impair torque and power improvements

Lynsey S James*, David J Hunter, Beth Hussey, Glenda Anderson, Sarabjit S Mastana, Martin R Lindley

Loughborough University, UK

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Individuals who are unaccustomed to resistance exercise experience greater levels of exercise-induced muscle soreness, this can deter individuals from committing to an exercise programme. To alleviate the symptoms, they may consume Non-steroidal anti-inflammatory drugs (NSAIDS). Evidence suggests NSAIDS can blunt muscle protein synthesis (Trappe et al., 2002, *American Journal of Physiology. Endocrinology and Metabolism*, *282*(3), E551–E556) and attenuate strength and muscle hypertrophic adaptations from resistance training (Lilja et al., 2018, *Acta Physiologica*, *222*(2), 1–16.), negating the effects of the exercise. Omega-3 supplementation has been suggested as an alternative to NSAIDS but the impact of Omega-3 on resistance training is inconclusive (Rossato, Schoenfeld, & de Oliveira, 2019, *Clinical Nutrition,* Article in Press). Therefore, the aim of the study was to determine the effects of omega-3 supplementation on eccentric training-induced increases in torque and power. With institutional ethical approval, 9 physically active but non-resistance trained males (29 ± 9 years) were match-paired for isometric and eccentric quadriceps strength and randomly assigned, in a double-blind manner, to either omega-3 (5.1g/d) or olive oil (6.0g/d) supplementation for 3 weeks prior to, and for 8 weeks during eccentric training. Performance measures of peak torque (isometric, concentric, eccentric) and jump height were conducted before and after 8 weeks of training. Supervised training consisted of maximal eccentric quadriceps contractions on an isokinetic dynamometer at $60°s^{-1}$. Two training sessions were conducted per week, with a minimum of 48 hours recovery between sessions. Number of repetitions and sets were increased over the 8 weeks. Following 8 weeks of eccentric training, peak eccentric torque significantly increased by 40 ± 56 Nm in

omega-3 group and 51 ± 52 Nm in olive oil group ($F_{1,7}$ = 6.35, P = 0.04, η^2_p = 0.48), with no differences between groups (P > 0.05). Both groups also significantly increased their maximal isometric torque (P = 0.03); omega-3 group increased by 21 ± 10 Nm and olive oil group increased by 23 ± 30 Nm, with no differences between groups (P > 0.05). There was no main effect of training on peak concentric torque (P > 0.05). There was a non-significant increase in jump height by 1.02 ± 1.91 cm in the omega-3 group and decrease by 0.03 ± 1.33 cm in the olive oil group, with no difference between groups (P > 0.05); however, a moderate to large effect was observed (η^2_p = 0.11). Omega-3 supplementation does not impair or augment eccentric training-induced increases in torque or power in young males.

Appendix 6: Absolute data for chapter 5 percentage change values

Table 19 – Effect of 3 weeks supplementation on systemic markers of muscle damage and inflammation following eccentric exercise

Variable/ Trial/ Group	Baseline	Post MD	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
LDH (U/L)						
Pre-supplementation						
Olive Oil	255.0 ± 130.5	255.9 ± 61.6	258.5 ± 72.9	306.9 ± 81.2	309.9 ± 165.2	0.748; 0.215;
n-3 PUFA	243.1 ± 62.4	262.1 ± 67.8	283.3 ± 64.5	302.4 ± 68.4	340.6 ± 102.2	0.016; 0.930
Post-supplementation						
Olive Oil	271.9 ± 169.2	231.4 ± 61.4	259.9 ± 58.7	271.9 ± 68.5	265.5 ± 64.8	
n-3 PUFA	251.6 ± 72.6	260.9 ± 74.8	269.1 ± 84.1	298.0 ± 78.9	299.0 ± 92.7	
CK (U/L)						
Pre-supplementation						
Olive Oil	150.6 ± 27.8	215.6 ± 63.6	273.4 ± 97.3	556.4 ± 352.9	601.6 ± 293.5	0.235; 0.008;
n-3 PUFA	174.2 ± 34.6	275.5 ± 58.7	332.5 ± 80.1	632.2 ± 321.3	1390.8 ± 1541.1	0.006; 0.284
Post-supplementation						
Olive Oil	163.6 ± 54.8	203.9 ± 65.4	238.3 ± 71.7	338.7 ± 110.4	346.3 ± 145.7	
n-3 PUFA	172.2 ± 72.1	236.2 ± 85.5	281.5 ± 114.6	415.0 ± 202.1	504.3 ± 194.2	
Mb (ng·mL^{-1})						
Pre-supplementation						
Olive Oil	33.0 ± 9.1	168.0 ± 78.8	294.5 ± 196.4	398.7 ± 324.3	63.9 ± 58.7	0.238; 0.006;
n-3 PUFA	43.6 ± 23.4	256.2 ± 151.2	406.4 ± 311.5	468.3 ± 367.2	480.4 ± 621.2	<0.001; 0.099
Post-supplementation						
Olive Oil	30.5 ± 8.2	91.6 ± 43.0	149.2 ± 74.2	156.2 ± 73.8	33.8 ± 10.3	
n-3 PUFA	35.0 ± 10.4	117.7 ± 59.0	196.7 ± 116.7	215.8 ± 133.5	38.6 ± 10.9	
Thigh girth (cm)						
Pre-supplementation						

Olive Oil	54.7 ± 6.0	-	54.5 ± 5.9	54.6 ± 5.9	54.8 ± 5.6	0.729; 0.020;
n-3 PUFA	55.5 ± 5.9	-	55.5 ± 6.0	55.4 ± 5.6	55.8 ± 5.8	0.002; 0.774
Post-supplementation						
Olive Oil	55.1 ± 5.7	-	55.2 ± 5.7	55.1 ± 5.5	55.3 ± 5.7	
n-3 PUFA	56.3 ± 6.5	-	56.5 ± 6.7	56.2 ± 6.5	56.6 ± 6.6	

Table 20 – Effect of 8 weeks training and supplementation on systemic markers of muscle damage and inflammation following eccentric exercise

Variables/ Trial / Groups	Baseline	Post MD	Post PT2	Post MD +3h	Post MD +48h	P values (group; trial; time; interaction)
LDH (U/L)						
Pre-training						
n-3 PUFA	236.3 ± 98.8	240.5 ± 99.1	253.0 ± 115.3	277.8 ± 105.1	267.0 ± 117.3	0.954; 0.633;
Olive Oil	242 ± 102.5	225.6 ± 67.2	252.0 ± 74.4	273.4 ± 80.8	269.4 ± 74.2	<0.001; 0.024
Post-training						
n-3 PUFA	211.3 ± 75.5	234.5 ± 67.3	237.8 ± 65.3	271.8 ± 75.2	252.0 ± 82.1	
Olive Oil	235.6 ± 48.9	238.4 ± 64.7	255.4 ± 62.7	266.2 ± 57.8	252.8 ± 57.5	
CK (U/L)						
Pre-training						
n-3 PUFA	205.3 ± 89.7	270 ± 115.4	335.7 ± 154.1	509.7 ± 274.6	505.0 ± 302.7	0.089; 0.710;
Olive Oil	180.8 ± 50.2	199.0 ± 72.1	218.8 ± 67.1	318.5 ± 133.2	304.8 ± 109.5	0.004; 0.808
Post-training						
n-3 PUFA	144.7 ± 30.3	252.7 ± 115.7	325.3 ± 176.7	480.0 ± 281.1	411.7 ± 221. 5	
Olive Oil	145.8 ± 71.2	180.5 ± 57.2	209.0 ± 68.1	282.8 ± 74.7	309.8 ± 94.1	
Mb (ng·mL^{-1})						
Pre-training						
n-3 PUFA	38.8 ± 12.8	135.4 ± 73.4	236.0 ± 146.6	241.0 ± 177.6	39.1 ± 14.6	0.855; 0.954;
Olive Oil	35.6 ± 12.2	103.2 ± 53.2	166.6 ± 86.0	195.3 ± 85.9	33.8 ± 11.6	<0.001; 0.815
Post-training						
n-3 PUFA	28.3 ± 4.3	121.1 ± 56.3	208.3 ± 118.2	189.2 ± 97.5	33.4 ± 12.1	
Olive Oil	78.2 ± 88.7	78.2 ± 88.7	190.3 ± 148.3	230.4 ± 134.2	32.5 ± 7.1	