The modulation of metabolic stress responses by ω-3 fatty acids: molecular to whole-body perspectives

Niels Jeroen Bootsma

A thesis submitted to the University of Stirling in partial fulfilment
for the degree of Doctor of Philosophy

Physiology, Exercise and Nutrition Research Group
Faculty of Health Sciences and Sport
University of Stirling

May 2022

Declaration

I declare that this thesis was composed by myself, and all the data contained herein was collected by myself, under the supervision of Dr Oliver Witard, Dr Lee Hamilton, Dr Iain Gallagher and Professor Stuart Galloway, with the following exceptions:

i. Data collection for the study detailed in Chapter 2 was completed alongside Dr Jordan Philpott.
ii. Mr Mario van der Westhuizen and Mr Samuel Harrison assisted in making up the diets provided to participants for Chapters 2 & 3.
iii. Muscle biopsy and blood samples for the study detailed in Chapter 3 were collected by myself and Dr Jordan Philpott. Skinfold measurements were taken by Dr Nidia Rodriguez-Sanchez.
iv. Professor Kevin Tipton, Dr Oliver Witard and Dr Iain Gallagher undertook the muscle biopsies for Chapter 3.
v. Mr Stephen Walker, Mr Brannon Garnes, Ms Maša Srdić and Ms Ivana Ovcina assisted in data collection for the study detailed in Chapter 4.

Some DEXA data from the study detailed in Chapter 2 was previously submitted as part of Dr Jordan Philpott's thesis, however for this thesis different fractions were analysed and presented in original form. Neither this thesis, nor the original work contained herein have been submitted to this or any other institution for a higher degree. This thesis does not infringe upon anyone's copyright and any material from the work of others included in this thesis is fully acknowledged in accordance with common reference practices.

01/05/2022

Abstract

Although there are indications that n-3 PUFA may be beneficial for different aspects of physiological function, particularly in situations of metabolic stress, little is known about their potential modulatory effects in healthy, young adults. In **Chapter 2**, we investigated whether 4g/d n-3 PUFA (FO) for 4 weeks prior to and during 2-week, 40% energy restriction (ER), could preserve lean mass. ER facilitated a 3.2kg loss of body mass, of which 1.1kg lean mass, which was unaffected by n-3 PUFA supplementation. In addition, n-3 PUFA did not influence whole-body changes in energy metabolism or muscle function. In **Chapter 3**, we measured the phosphorylation status of mTOR and rpS6 in muscle from healthy males, as a proxy for muscle protein synthesis, to determine the effect of n-3 PUFA supplementation for 4 weeks on muscle anabolic signalling during 2-week, 40% ER. We demonstrated that muscle anabolic signalling during ER, as the change in phosphorylation status of mTOR and rpS6, was unaffected by n-3 PUFA in the basal state. RpS6 status was significantly potentiated by n-3 PUFA following resistance exercise and consumption of 10g protein, during ER, while mTOR status was not. N-3 PUFA did not significantly change the expression of genes related to muscle development and autophagy. In **Chapter 4**, we demonstrated that n-3 PUFA supplementation ingested 1 hour before exercise does not elicit worthwhile improvements in exercise function and time trial performance in trained cyclists. Using a Bayesian statistical approach, we determined there was a low-to-very low probability of improvements in exercise function. Collectively, the findings of this thesis suggest n-3 PUFA supplementation in healthy males does not elicit wortwhile improvements in whole-body physiology and muscle function.

Acknowledgements

A big thank you to Olly, Lee and Kevin for giving me the opportunity to undertake this journey, and for their guidance and expertise throughout the first part of my PhD studies. Stuart and Angus, and everyone else in PENRG, thank you for fostering an environment of collaboration and excellence. Gill and Chris, thank you for your support with everything technical (and non technical).

Kev, your passion for communicating science clearly has formed a key part of my character. I cherish the memories of our discussions, research away days and hill walks.

Iain, your patience and understanding allowed me to transform my way of thinking about myself and about science, and for that I will be eternally grateful. You revitalised my love for learning through your inspiring tutelage and perspectives on science.

Hgyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyyy (that would be Nugget standing on my keyboard). Couldn’t have done it without you buddy.

Thank you also to my friends, for jokingly calling me Dr from day one, your belief in me was instrumental.

De grootste dank gaat naar mijn ouders, Kees en Arentien, die altijd in me geloven en achter me staan. Waar ik maar voor koos te streven, jullie steun is altijd een bron van motivatie geweest.

Table of contents

List of tables

List of figures

List of abbreviations

Fatty acids
ALA – alpha linolenic acid (18:3n-3)
ARA – Arachidonic acid (20:4n-6)
DHA – Docosahexaenoic acid (22:6n-3)
EPA – Eicosapentaenoic acid (20:5n-3)
FA – Fatty acid
HUFA – highly unsaturated fatty acid (i.e. n-3 + n-6), equivalent to PUFA
LDL – low density lipoprotein
n-3 PUFA – omega-3 polyunsaturated fatty acid
n-6 PUFA – omega-6 polyunsaturated fatty acid
n-9 PUFA – omega-9 polyunsaturated fatty acid
PUFA – polyunsaturated fatty acid
TG – triglyceride
VLDL – very low density lipoprotein

Muscle metabolism
3MH – 3-methylhistidine
4EBP-1 – eukaryotic translation initiation factor 4E-binding protein 1
Akt – protein kinase B
eEF – eukaryotic translation elongation factor
eIF – eukaryotic translation initiation factor
IRS1 – Insulin Receptor Substrate-1
MPS – muscle protein synthesis
MPB – muscle protein breakdown
mTOR – mammalian Target Of Rapamycin / mechanistic Target Of Rapamycin
p70s6k – Ribosomal Protein S6 Kinase beta-1
PI3K – Phosphoinositide 3-Kinase
rpS6 – Ribosomal Protein S6
TSC2 – Tuberous Sclerosis Complex 2, also known as Tuberin
WB – Western blot

General

1-RM – 1 repetition maximum
AA – amino acid
BF% - body fat percentage
COX – lipoxygenase
CV – coefficient of variation
CYP450 – cytochrome P450
DEXA – dual energy x-ray absorptiometry
ER – energy restriction
GPCR – G-protein coupled receptor
GPR120 – G-protein coupled receptor 120, also known as Free Fatty Acid Receptor 4 (FFAR4)
ISAK – International Society for the Advancement of Kinanthropometry
LOX – lipoxygenase
MAP – mean arterial pressure
mRNA – messenger RNA
PPAR – peroxisome proliferator-activated receptor
PPO – peak power output
RPP – rate pressure product
RER – respiratory exchange ratio
REx – resistance exercise
RMR – resting metabolic rate
ROS – reactive oxygen species
RNS – reactive nitrogen species
SD – standard deviation
SUM8 – sum of 8 skinfold sites (triceps, biceps, iliac crest, supraspinale, thigh, medial calf, subscapular, abdominal)
SWC – smallest worthwhile change
TE – typical error
TT – time trial

Chapter 1 – General introduction

1.1 – n-3 PUFA

The family of omega-3 polyunsaturated fatty acids (n-3 PUFA) consists of 11 different variations of long-chain fatty acids between 16 and 24 carbon atoms in length and containing between 3 and 6 double bonds. These fatty acids (FA) are widely believed to improve aspects of cardiovascular and neural function, amongst improvements in other physiological systems, although in most cases evidence for these effects in humans is still equivocal. Since the 1970s, when interest into the health modulatory effects of n-3 PUFA arose, most research has gone into eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) as these n-3 PUFA are directly incorporated into tissue membranes and are precursors for production of bioactive eicosanoids. Principally present in fatty fish, such as salmon, mackerel and tuna, they are often colloquially called fish oils. Contrastingly, α-linolenic acid (ALA, 18:3n-3), another of the principal n-3 PUFA, is present primarily in plant sources.

In various organisms, elongase and desaturase enzymes aid the structural alteration of FA (figure 1.1). Elongase enzymes facilitate the addition of a carbon molecule to the chain, thereby elongating it. Desaturase enzymes act on the bonds between two carbon molecules and facilitate the addition of two extra electrons, thereby creating a so-called double bond. The numbering of FA is based on the carbon atoms in the chain, and starts at the methyl end (CH_3). Desaturase enzymes act on specific carbon molecules in the chain, but these are numbered in reverse order: starting from the carboxyl (COOH) end. As such, in an 18-carbon chain, the Δ-12 desaturase acts on the 6th carbon-carbon bond to create an omega-6 FA. Similarly, the Δ-15 desaturase acts on the 3rd carbon-carbon bond to create an omega-3 FA. However, the Δ-12 and Δ-15 desaturase enzymes

are not available in humans, meaning saturated FA cannot be converted into unsaturated FA. However, humans do have Δ-5, Δ-6 and Δ-9 desaturases which act further along the carbon chain [1]. As such, ALA can be converted into more highly unsaturated n-3 PUFA such as EPA and DHA. Similarly, linoleic acid (LA; 18:2n-6) can be converted into more highly unsaturated n-6 PUFA, such as γ-linolenic acid (γ-LA; 18:3n-6) and arachidonic acid (ARA; 20:4n-6). These processes are thought to be slightly more efficient in females compared to males, possibly due to the influence of oestrogen on the activity of desaturase and elongase enzymes, and the lower proportion of ALA partitioned towards β-oxidation [2]. However, the conversion rate of ALA into EPA and DHA in both females and males is extremely low at <1% for EPA and <0.1% for DHA [2]. The conversion of PUFA is a complex process, and requires their transport across different cellular compartments, and association with enzymes therein. In addition, the low rate of ALA conversion to EPA and DHA has been suggested to be due to competition between ALA and LA for Δ6-desaturase, the rate-limiting enzyme in this process [3]. Thus, while only alpha-linolenic acid and linoleic acid are classed as essential fatty acids, to achieve the levels of EPA and DHA suggested to be adequate for optimal functioning, direct consumption is required.

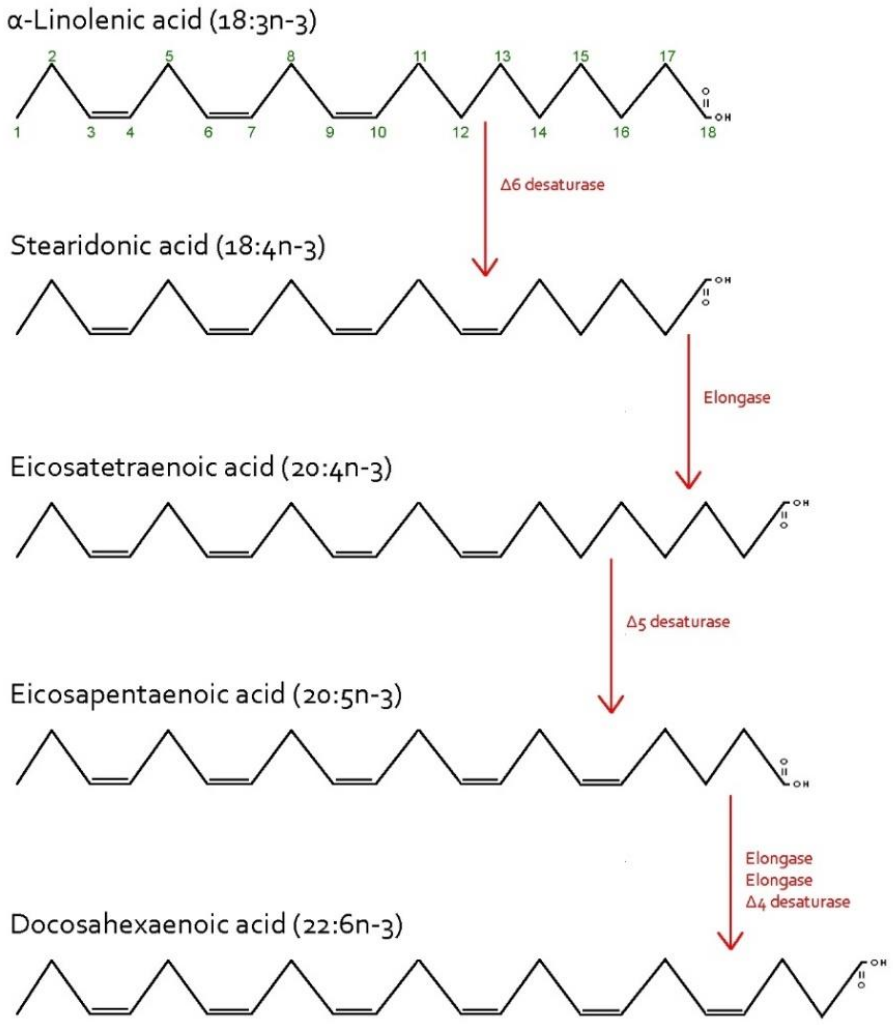

Figure 1.1 – Elongation and desaturation steps in the metabolism of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from α-linolenic acid (ALA).

1.2 – Bioavailability and metabolic fates

Following their ingestion, dietary lipids in the form of fats (solid at room temperature) or oils (liquid at room temperature) have several metabolic fates. As triacylglycerols (or triglycerides; TG), consisting of three FA linked to a glycerol backbone, they enter the digestive system before being hydrolysed by lingual, gastric and pancreatic lipases into free FA, monoglycerides and free glycerol. This allows micelle-mediated absorption across the intestinal wall. Inside epithelial cells, free FA and glycerol are reconstituted into TG before being transported into the bloodstream as chylomicrons via the lymphatic system. Due to their hydrophobic nature, lipids require lipoprotein structures to carry them around the bloodstream. As low density lipoprotein (LDL) and very low

density lipoprotein (VLDL) carry cholesterol and TG from adipose tissue and the liver towards peripheral tissues, high blood concentrations are associated with increased risk for cardiovascular disease [4]. Some studies suggest a dose of 4g/d n-3 PUFA can lower TG in those with high levels by ~20-30% [5], although the American Heart Association only recommends supplementation after diet and lifestyle changes are implemented. The effects of n-3 PUFA on TG metabolism are speculated to be (in part) due to their ability to influence metabolic fuel partitioning and FA synthesis in liver and skeletal muscle [6]–[8], and to reduce VLDL secretion and increase chylomicron clearance [9]. However, despite the widely believed notion that n-3 PUFA reduce cardiovascular disease risk, the evidence from human studies does not support this [5], [10], [11].

Regardless, n-3 PUFA, like other fats are carried through the circulation and may be delivered to any cell, where they can be incorporated into phospholipid bilayers (also known as plasma membranes). Within these bilayers exists a complex environment of molecules and interactions, protected by the hydrophillic heads of phospholipids, which form the outside of the membrane. Towards the middle of the bilayer are the hydrophobic ends of phospholipids. N-3 PUFA contain unsaturated bonds starting from the 3rd position of the carbon chain, from the hydrophobic (CH_3) end. Therefore, when n-3 PUFA is placed in a phospholipid bilayer, the unsaturated bonds are positioned further towards the middle of the membrane compared to other FA. The positioning of these unsaturated bonds has been suggested to influence membrane function by altering the physical properties of the membrane. That is, FA interactions with cholesterol and other FA determine the fluidity, permeability and thermodynamics of the membrane, and these are thought to be improved when n-3 PUFA are present [1]. In addition, the positioning of unsaturated bonds also has been suggested to alter the nature of interactions with other molecules inside the membrane [1]. Furthermore, n-3 PUFA may become constituents of lipid rafts, floating microdomains within the plasma membrane, which regulate cellular signalling and trafficking. The

presence of n-3 PUFA in lipid rafts therefore further influences membrane function and signalling [12], [13].

Across the natural world, organisms with various degrees of unsaturation in their phospholipid membranes exist. Typically, organisms with high degrees of unsaturation (e.g. fish) have a high metabolic rate, while organisms with low degrees of unsaturation (e.g. desert lizards) have a low metabolic rate [1]. For humans, it might seem desirable to increase the overall degree of unsaturation in the phospholipid membranes of tissues such as skeletal muscle. That is, an increased capacity for metabolism may improve fuel utilisation and therefore energy production during physical exercise. However, in humans it appears that attempts to increase the occurrence of specific PUFA (e.g. EPA & DHA) comes at the cost of other unsaturated FA (i.e. monounsaturated FA or n-6 PUFA). As such, changes in the overall degree of unsaturation are minimal [1], [14], [15]. Thus, manipulating the ratio of n-3 PUFA to other unsaturated FA, and specifically n-6 PUFA, is often the target of research studies attempting to investigate the physiological benefits n-3 PUFA consumption.

PUFA are also highly susceptible to oxidation via enzymatic and non-enzymatic pathways, resulting in the production of oxylipins, or eicosanoids. Eicosanoids are named after their main precursors, *eicosa*tetraenoic and *eicosa*pentaenoic acids, which contain 20 (*eicosa*) carbon chains. Eicosanoids are a subclass of oxylipins, oxidised products of FA. Eicosanoids are lipid peroxidation products which may take many different forms, depending on the initial substrate and oxidative pathway. Cyclooxygenase (COX), lipooxygenase (LOX) and cytochrome p450 (CYP450) enzymes catalyse the oxidation of PUFA to specific subsets of eicosanoids [16]. Additionally, PUFA may be subject to free radical oxidation [17]. As such, PUFA, including those released from phoshpolipid membranes by phoshpolipase A2 (PLA2), may be oxidised to produce prostanoids (prostaglandins, leukotrienes,

prostacyclin), thromboxanes, hydroxyeicosatetraenoic acids (HETE), resolvins, and many other subclasses of eicosanoids [2], [16]. These eicosanoids, which tend to be short-lived due to their radical nature, act as local signalling mediators, thereby influencing intracellular signalling and gene expression [18]. As a result of direct and indirect actions, PUFA and their oxidation products may therefore influence physiology on a wide-ranging scale, although the strength of these effects is unclear.

Finally, N-3 PUFA, like other fats, also may be catabolised for energy production in mitochondrial beta-oxidation. Although n-3 PUFA may influence the overall rate of beta-oxidation, it is unclear if this is a selective process [6], [19]. That is, does n-3 PUFA influence which types of fats are oxidised? Regardless, n-3 PUFA do appear to increase mitochondrial biogenesis, and induce beta-oxidation, in various tissues [6], [20]. Thus, n-3 PUFA may increase the capacity for oxidation of FA, which may further explain their suggested anti-triglyceridaemic and anti-diabetic effects [7], [21]. The relative proportion of n-3 PUFA partitioned to each of the discussed metabolic fates (i.e. incorporation, eicosanoid metabolism, beta-oxidation) is unclear, and may depend on many factors, including metabolic state (e.g. oxidative stress and antioxidant status), cell type and location, hormone and cytokine regulation, and expression of genes involved in these metabolic pathways.

1.3 – Eicosanoid actions

Of the oxylipins, eicosanoids derived from ARA and EPA, have been studied mostly in the context of inflammation. Of particular interest are changes in their production as a result of changes in the availability of n-3 versus n-6 PUFA, as this can be influenced by changes in diet, and may influence the state of inflammation within the body. n-6 PUFA are often suggested to be pro-inflammatory, while n-3 PUFA are suggested to be anti-inflammatory. However, this may be an oversimplification. The presence of n-3 PUFA inhibits ARA metabolism, and decreases the production of eicosanoids from ARA [22]. Due to structural differences, n-3 PUFA binding to the lipid peroxidation enzymes is thought to be strained compared to n-6 PUFA, resulting in reduced production of total eicosanoids [16]. Additionally, n-3 PUFA-derived eicosanoids produced with COX or LOX are suggested to have lower bioactivity compared to those derived from n-6 PUFA. Furthermore, the availability of n-3 PUFA also is thought to influence the catabolism of eicosanoids [16], [23]. Thus, differences in the overall inflammatory state may not be due to 'anti-inflammatory' actions of n-3 PUFA *per se*, but may (in part) revolve around their influence on the profile and quantities of eicosanoids synthesised and catabolised. In addition, specific subsets of eicosanoids, such as lipoxins, resolvins, maresins and protectins, may act as mediators of resolution. Resolution happens at the end of the inflammatory process, as it terminates the recruitment of (pro-inflammatory) leukocytes and return to a normal homeostatic state [24]–[26]. So, increases in the ratio of n-3 PUFA:n-6 PUFA may allow better self-regulation of the inflammatory climate.

Eicosanoids can bind to G-protein coupled receptors (GPCR) on cell membranes, thereby influencing production of second messengers, which subsequently activates intracellular signalling mechanisms [16], [18]. It is believed there are specific fatty acid receptors for each different type of eicosanoid, although cross-reactivity may also occur [18]. GPR120 (or FFAR4) was identified as a n-3 PUFA-specific receptor in mouse macrophages and adipocytes [27]–[29]. As such, activation of

this receptor by n-3 PUFA inhibited inflammation and improved insulin sensitivity, which was not the case in GPR120 knockout mice. N-3 PUFA or eicosanoids may also interact with nuclear receptors, thereby influencing expression of genes. Specifically, interactions between n-3 PUFA and peroxisome proliferator-activated receptors (PPAR-δ/γ/α), nuclear factor kappa B (NFkB), and other types of transcription factors have been observed [9]. In addition, n-3 PUFA may affect expression of the transcription factors themselves [30], [31]. In healthy individuals, n-3 PUFA supplementation has been observed to result in changes in the expression of hundreds of genes, mainly related to regulation of oxidative stress and fatty acid metabolism [32]. The specific actions of n-3 PUFA on expression of genes (e.g. up- or down-regulation) may depend on various contexts, including cell type [18]. Nevertheless, PUFA-mediated changes in gene expression may have beneficial effects on various cellular functions yet to be fully explored.

1.4 – Key to health throughout the lifetime?

Diet plays an important role in our physical health, and manipulating dietary components can significantly influence our body's ability to respond to physiologically stressful situations such as exercise, changes in energy availability, or disease. However, changes in the quantity and quality of foods consumed over the last two centuries have fueled the emergence of non-communicable, degenerative 'civilisation diseases' such as cancer, type-II diabetes mellitus (T2DM), obesity, and cardiovascular disease. A key factor in this is an increase in sucrose consumption from <10 kg to >50 kg per capita per year, across Western developed countries, over the last 200 years [33]. Over-reliance on carbohydrate (CHO) in the diet is associated with increased risk for all-cause mortality, especially at CHO intakes >50% of the total diet [34]. In addition, over the last 100 years, the addition of refined vegetable oils changed the types and amounts of fats consumed, which included new types of fats altogether in *trans* fats [33]. Consumption of saturated fat >10% of total energy consumption, of monounsaturated fat <20%, and of polyunsaturated fat >8% are all

associated with increased risk of all-cause mortality [34]. Besides changes in the make-up of diets, the overall calorie content also has risen in modern times. That is, among participants in the National Health and Nutrition Examination Survey (NHANES, USA data), energy intake increased from 9832 to 11652 kJ in men, and 6418 to 8142 kJ in women, between 1971 and 2004 [35]. It has been argued that the human genome has been unable to keep up with this rapid shift in the overall content and composition of our diets [36]. Thus, while the risk of death from infection has decreased, the leading causes of death are now diet and lifestyle-related, non-communicable degenerative diseases such as obesity, diabetes, cardiovascular disease, and cancer [33].

Strategies are required to mitigate the negative health effects of these 'Western' diets. Besides their effect on our health, diets containing high amounts of meat and dairy are also not environmentally sustainable. An increasingly larger proportion of the population in the Western world are becoming aware that a move towards more plant-based sources of protein is required, albeit this has long been the norm in large parts of the world such as Africa and Asia [37]. Although more environmentally sustainable, vegetarian and vegan protein sources are often less anabolically stimulating, due to deficiencies in specific essential amino acids and anti-nutritional factors [38]. Therefore, if dietary composition is not carefully considered, these types of diets may increase the risk of impaired muscle health. Aging muscle undergoes the additional challenge of anabolic resistance, in which its responses to anabolic stimulation start to become impaired as people approach the age of 50 [39]–[41]. As a result of these dietary challenges, there lies great value in dietary strategies which can improve the body's natural responses to anabolic stimuli and metabolic stress, protect against the risk of disease, and maintain our physical functioning and metabolic health throughout the lifetime. N-3 PUFA have long been suggested as a potential protective agent against cardiovascular disease [42]. However, more recently they have also been suggested to act as modulators of muscle health through their observed effects on muscle

anabolism and catabolism [43], [44]. In older adults, n-3 PUFA supplementation has been observed to improve muscle accretion with exercise programmes, and increase muscle mass and function, even in the absence of training [45]–[47]. As increased n-3 PUFA status is suggested to improve the sensitivity of the protein synthetic machinery to stimulation, it may be a useful tool in the fight against sarcopenia [48], [49]. In addition to its observed effects on muscle mass and function in older adults, n-3 PUFA also have been suggested to improve glucose control through improvements in insulin sensitivity, although meta-analysis does not support such an effect [50]. Additionally, in athletic populations, n-3 PUFA have been promoted as modulators of physical performance, as it is suggested they may improve exercise function and recovery, although evidence for this is also still limited [51]–[55]. The widely held belief that n-3 PUFA may be a key component of nutritional strategies to prevent or combat impairments in physical function and health throughout our lifetime, still requires further scrutiny.

1.5 – Potential ability to modulate stress responses

Within the wide field of n-3 PUFA research, investigators have attempted to elucidate the role of n-3 PUFA and n-3 PUFA-derived eicosanoids on processes ranging from the molecular to whole-body level. As mentioned, many pathways exist through which n-3 PUFA may mediate effects on gene expression, cell signalling, protein expression and activity. However, the understanding of the exact mechanisms of n-3 PUFA-mediated changes in physiological function is still fairly limited. An important aspect of the potential for n-3 PUFA to influence physiological processes are its n-6 PUFA counterparts. The 'Western diet' does not typically contain much in the way of n-3 PUFA sources, which has resulted in high n-6:n-3 PUFA ratios. It is suggested that historically, hunter-gatherers had n-6:n-3 ratios near 2:1, whereas in modern Western populations this ratio may be nearer to 20:1 [36], [56]. N-3 and n-6 PUFA compete for spaces in the phospholipid membranes of cells, for the enzymes that catalyze eicosanoid formation, and may both act as scavengers of free radicals

during periods of oxidative stress. The resulting changes in cellular function, and products from these reactions, influence cell signalling, gene expression, cytokine production (by associating with immune cells), and (regulation of) inflammation and resolution.

The interaction with the immune system is a critical one: immune cells are adept producers of cytokines, which act as signals to other cells, and thereby also influence the activity of other immune cells. In addition, some immune cells, such as macrophages, produce eicosanoids [57]. The interplay between eicosanoids and cytokines is a complex, but potentially crucial aspect of inflammatory regulation that may be influenced by changes in PUFA availability. Therefore, changes in eicosanoid metabolism may influence the ability for the immune system to self-regulate. Many disease states are marked by dysregulation of inflammatory cytokines such as interferons, interleukins and tumor necrosis factors [58], [59]. However, n-3 PUFA may inhibit production of some cytokines, while enhancing others, and thereby improve the overall inflammatory climate [28], [60], [61]. All inflammation is not by definition bad. Under normal (healthy) conditions, inflammatory mediators are important signalling molecules, which direct tissue responses to areas under metabolic stress through altered energy state or oxidative stress [62], [63]. Immune cells and their associated inflammatory cytokines also play a crucial role in the recovery from damage created by these metabolic stress states, and to this end may modulate satellite cell proliferation [63], [64]. This is an important consideration for those who wish to achieve maintenance or improvements in physical function. Does interfering with the body's natural responses to this metabolic stress lead to long-term impairments? In the context of athletic functioning, it has been suggested that reducing the inflammatory response (e.g. with ice baths, NSAIDs or n-3 PUFA) during and after exercise may impair training adaptation as it limits the natural inflammatory response required for tissue remodeling [65]–[67]. This may lead to a potential dilemma: faster recovery may allow improved performance in periods where

competitions come thick and fast, but it may concomitantly impair long-term outcomes.

A key aspect of n-3 PUFA-regulated changes in function is their interaction with oxidative stress. During exercise, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the natural result of the transfer of energy between molecules [68]. The production of these reactive species during muscle contraction contributes to force production and muscle endurance [63], [69]. However, excessive production of reactive species, which exceeds antioxidant buffer systems, may impair muscle function [69]. N-3 PUFA are believed to improve antioxidant capacity, or act as antioxidants themselves, thereby protecting against oxidative stress-induced damage [70], [71]. The state of oxidative stress induced by exercise promotes acute local inflammation that induces the release of inflammatory cytokines [72]. This inflammatory state further promotes the release of PUFA, evident through an increase in arachidonic acid (ARA), making these PUFA available for the production of eicosanoids [72], [73]. The initial stage of inflammation is followed by the production of anti-inflammatory and pro-resolving mediators, which may act as 'braking-signals' [24]. As n-3 PUFA are suggested to limit the formation of pro-inflammatory, and promote the formation of pro-resolving mediators, increasing their availability during and following metabolically stressful situations, such as exercise, may allow better self-regulation of the inflammatory state [22], [28], [74]. The measurement of specific eicosanoids is challenging however, due to their structural complexity, and short-lived and localised nature. Nevertheless, systemic levels of their stable metabolic endproducts may be detected in blood or urine [75]. Increased concentrations of eicosanoids such as thromboxane B_2 (TxB_2), prostaglandin E_2 (PGE_2), and 6-keto-$PGF_{1\alpha}$ have been observed during and after endurance exercise [72], [73]. Additionally, a recent report demonstrated the lipidomic profile of resistance exercise, with changes in dozens of eicosanoids during and in the 24 hours following exercise [66]. These eicosanoids may mediate vascular tone and reactivity, and trafficking of immune cells, among a plethora of other local

functions [24], [74], [76]. However, production of eicosanoids during exercise may be inhibited by β-blockers and NSAIDs [66], [73]. Therefore, athletes may wish to consider carefully their use during training and competitive season, as they may hinder the natural resolution of metabolic stress [77], [78].

In a wider context, n-3 PUFA may have positive effects on pathophysiologies associated with diseases such as cancer, atherosclerosis, asthma and diabetes, as these are associated with excessive inflammation [58], [79]. However, investigations into the effects of n-3 PUFA on inflammatory markers in healthy (typically athletic) populations report mixed results. Some have observed decreases in factors such as IL-6, TNF-α or C-reactive protein [53], [55], [80], [81], while others have not [82]–[87]. This may be due to limited consistency in the measurement of inflammatory markers in terms of timing in relation to exercise and type of specific cytokines [53]. Although cytokines may be active on a more systemic scale than eicosanoids, their measurement is still challenging. For example, studies that have investigated changes in inflammatory markers with exercise suggest increases in IL-6 may only be observed following strenuous or long-duration exercise [88], [89]. There may still be potential minor and acute changes in cytokines, and it is unclear if their detection would be improved with more reliable measurement in the future. Still, strategies that influence their production, activity and clearance may provide collectively worthwhile benefits even if we are unable to detect them reliably.

1.6 – Effects of n-3 PUFA on muscle protein turnover

Muscle plays a critical role in metabolic and functional health [90], and there are indications that n-3 PUFA may positively influence muscle mass and muscle health [43], [44]. At a molecular level, improvements in the signalling activity of key proteins regulating muscle protein synthesis (MPS) and muscle protein breakdown (MPB) have been observed with n-3 PUFA [49], [86], [87], [91]–

[95]. Although it is unclear how n-3 PUFA influence these processes exactly, several pathways have been proposed as being involved (figure 1.2). Generally, the main pathway of muscle protein turnover regulation is understood to revolve around mammalian/mechanistic target of rapamycin (mTOR, historically known as FRAP) [96]. The mTOR complex regulates many aspects of cellular functioning, which includes processes promoting cell survival/growth (development) and cell death (apoptosis). Through (dis)association with several factors, mTOR may be up- or down-regulated in response to alterations in the metabolic state of the cell. Growth factors, such as IGF-1 or insulin, stimulate Akt (PKB) through PI3K/IRS1, which results in inactivation of the TSC1/2 complex. TSC1/2 in turn is a regulator of Rheb, which is suggested to be required for the activation of mTOR through their combined association with the endomembrane system [97]. To facilitate this activation, mTOR requires translocation from the cytoplasm, an action which is disabled by AMPK-mediated phosphorylation of Raptor in response to energy stress [96]. On the other hand, availability of AA within the cell promotes association of mTOR with Rag GTPases, which facilitate its translocation. Thus, when the cell is not in a state of energy stress, and AA are available, mTOR may be translocated to the endomembrane system, associate with Rheb, and thereby become active [97], [98]. When active, mTOR promotes MPS through phosphorylation of p70 s6 kinase (p70s6k) and eukaryotic translation initiation factor 4E-binding protein (4E-BP1). The activation of p70s6k stimulates mRNA translation, and biogenesis of ribosomes through ribosomal protein s6 (rpS6) [99]. The phosphorylation of 4E-BP1 by mTOR stimulates its disassociation from eIF4E, which allows formation of the eIF4F complex and consequently enables cap-dependent RNA translation [96]. mTOR is also an inhibitor of autophagy, through phosphorylation of ULK1 [100]. Autophagy is an essential component of the protein breakdown machinery, which is responsible for the recycling of damaged or old proteins [101]. Thus, autophagy may be induced to maintain adequate AA for MPS, when nutritional AA are unavailable (and mTOR is inactive). It has been suggested that alternate pathways exist for regulation of MPS in the postabsorptive state, as

inhibition of mTOR does not affect protein metabolism in this state [102]. However, these pathways have not been elucidated.

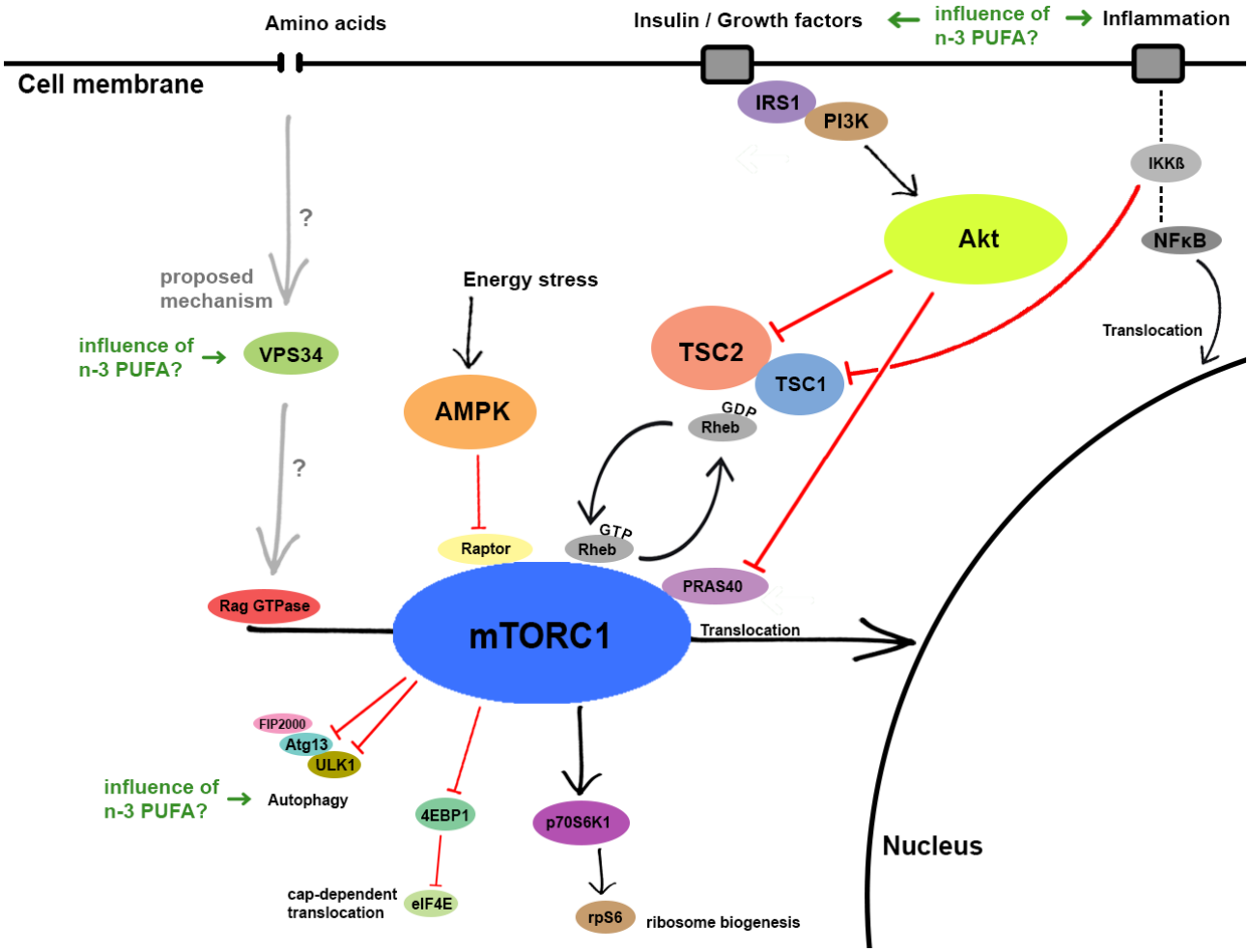

Figure 1.2 – Simplified diagram of the mTOR pathway regulation muscle protein turnover. Adapted from Laplante and Sabatini (2009) [96].

N-3 PUFA can positively influence the (in)activation of various proteins along the pathways regulating MPS and MPB (Table 1.1). As such, n-3 PUFA infusion for 5 weeks in steers was observed to positively influence insulin-stimulated amino acid disposal and significantly increased Akt and p70s6k phosphorylation, and non-significantly increased mTOR and 4E-BP1 phosphorylation [103]. Interestingly, although the rate of weight gain was not different, these animals required less feed intake to gain the same amount of weight. In growing chickens, an n-3 PUFA-rich diet significantly increased muscle insulin sensitivity and p70s6k activation, and non-significantly increased Akt

activation, following the injection of insulin [104]. Similar effects are observed in humans, with n-3 PUFA inducing a greater increase in activation of the mTOR/p70s6k pathway following amino acid and insulin infusion, resulting in increased mixed muscle MPS [86], [87]. McGlory et al. (2016) investigated the influence of n-3 PUFA on the myofibrillar MPS response to protein feeding and exercise, as a more direct measurement of muscle hypertrophy in a real-world setting [49]. Interestingly, they observed a suppression of Akt activity at rest, compared to placebo. In addition, they also observed a suppression of the 30g protein and resistance exercise-induced increase in Akt and p70s6k signalling in the fish oil group. As the exercise was unilateral, they were able to investigate the effects of 30g protein feeding independently. Following protein feeding, n-3 PUFA appeared to induce a greater increase in MPS in both the rested and exercised legs, although this did not reach statistical significance. As a result of the observed suppression of Akt and p70s6k, and no significant change in MPS, the authors suggested n-3 PUFA may have improved the efficiency of anabolic protein signalling to the protein synthetic machinery [49]. However, they also speculate n-3 PUFA may have induced a greater increase in MPS if muscle had not been 'maximally' stimulated with 30g protein. Another study in older adults demonstrated that n-3 PUFA may also influence postabsorptive MPS, with significantly greater increases in mitochondrial and sarcoplasmic MPS, and non-significant increases in mixed muscle and myofibrillar MPS with n-3 PUFA [48]. In addition, the post-exercise increase in mitochondrial MPS was significantly greater with n-3 PUFA, and was greater but did not reach statistical significance for myofibrillar, sarcoplasmic and mixed muscle MPS. As it became clear that n-3 PUFA may be beneficial in catabolic situations associated with muscle atrophy, their effects in conditions of muscle disuse have also been investigated. In rats, n-3 PUFA reduced the loss of Akt and p70s6k phosphorylation during hindlimb immobilisation, which was associated with reduced muscle atrophy [105]. A similar effect was observed in young female humans, with n-3 PUFA preserving myofibrillar MPS and reducing atrophy during leg immobilisation for 2 weeks [106].

Others have attempted to investigate the role of n-3 PUFA in other conditions associated with muscle atrophy, such as cancer cachexia and energy restriction/starvation, although specific measurements of anabolic signalling proteins or MPS are limited in these contexts. In cachectic mice, which experienced a 80% reduction in MPS, n-3 PUFA alone was unable to exert a preserving effect [93]. However, when n-3 PUFA supplementation was combined with essential amino acids (EAA), MPS was preserved to a greater degree. In addition, these mice experienced extremely high rates of MPB, which was significantly reduced by n-3 PUFA, regardless of EAA co-ingestion. In another catabolic context, mice undergoing starvation for 24 hour experienced reduced ubiquitin-dependent proteolysis when fed EPA for 24 hour prior to starvation [92]. Together, the studies on the effects of n-3 PUFA suggest attenuation of MPB-related processes, irrespective of anabolic state; and potentiation of MPS-related processes, which may be more potent when the protein synthetic machinery is anabolically stimulated. In addition, it was speculated that n-3 PUFA may increase the efficiency of muscle anabolic signalling and protein synthesis, such that less stimulation is required to reach the same effect [49]. Therefore, n-3 PUFA may provide especially useful benefits on muscle protein turnover in catabolic states where MPS is impaired.

1.7 – Effects of n-3 PUFA on gene expression

Besides improvements in signalling activity of proteins regulating protein turnover, several studies have also attempted to investigate the effects of n-3 PUFA on transcriptomic changes in various tissues. In peripheral blood mononuclear cells (PBMC) of healthy people, 3g/d n-3 PUFA supplementation for 6 weeks was shown to induce detectable changes in expression of 610 genes in men, and 250 genes in women [32]. The gene pathways that were most affected were related to nuclear receptors, transcription factors and oxidative stress. In whole blood of obese women, 1.8g/d n-3 PUFA induced changes in the expression of hundreds of genes, notably including those

related to the NFkB pathway, fatty acid oxidation and synthesis, eicosanoid synthesis and receptors, antioxidant enzymes, and the mitochondrial electron transport chain [31]. In normo- and dyslipidemic men, supplementation with 2.6g/d n-3 PUFA for 12 weeks also induced changes in expression of genes, assessed in whole blood [107]. As such, this study observed global changes in regulation of oxidative stress pathways, oxidative and anti-oxidative enzymes, and the NFkB and MAPK pathways. Following up this microarray analysis with targeted measurement of relative gene expression using quantitative polymerase chain reaction (qPCR), they also found n-3 PUFA-induced changes in expression of catalase (CAT), heme oxygenase 2 (HMXO2), and cytochrome P450 enzyme 1 A2 (CYP1A2). Thus, n-3 PUFA supplementation at typical doses seen in intervention studies induces potential positive changes in the regulation of oxidative and other types of metabolic stress. Other studies that have investigated expression of individual genes further demonstrate potential beneficial effects on muscle metabolism. In the study by You, et al. (2010), the preservation of muscle mass with n-3 PUFA, in immobilised rats, was associated with an abolition of the increase in MAFbx, MuRF1 and COX2 expression [105]. As MAFbx and MuRF1 are key components of the ubiquitin-proteasome pathway, responsible for targeted degradation of proteins, this suggests a potential mechanism for the preservation of muscle mass by n-3 PUFA. Further, they also observed preservation of iNOS expression with n-3 PUFA, during immobilisation. Together with the suppressed expression of COX2, this observation supports a potential role for n-3 PUFA in the regulation of oxidative stress and inflammation during a catabolic condition. In arthritic rats, 1g/kg/d EPA also attenuated the increase in expression of MAFbx, MuRF1 and TNF-α, which was associated with reduced muscle atrophy [94]. A similar observation was made in pigs, where a 5% fish oil diet for 21 days reduced expression of MAFbx, MuRF1, TNF-α and COX2 [95]. Together, these studies suggest changes in gene expression contribute to the observed effects of n-3 PUFA on oxidative stress, inflammation and muscle protein turnover.

Table 1.1 - The influence of n-3 PUFA supplementation on muscle protein synthesis and activity of anabolic signalling protein kinases. Arrows denote difference compared to control or placebo, with * denoting significant differences. Cells left blank were not measured or not reported. Abbreviations: male (M), female (F), amino acids (AA), infusion (inf.), muscle protein synthesis (MPS), muscle protein breakdown (MPB), Resistance exercise (REx).

Study	Population	Intervention	Anabolic stimulus	Akt	mTOR	p70s6k	4EBP1	MPS	MPB
Gingras et al. (2007)[103]	Growing steers	4% menhaden oil inf., 5 wks	Insulin inf.	↑*	↑	↑*	↑		
You et al. (2010)[105]	Immobilised rats	5% cod liver oil diet, 2 wks	Last feed unclear	↑*		↑*			
Smith et al. (2011a)[86]	Young M & F	4g/d n-3 PUFA, 8 wks	Insulin + AA inf.	→	↑*	↑*		↑*	
Smith et al. (2011b)[87]	Older M & F	4g/d n-3 PUFA, 8 wks	Insulin + AA inf.		↑*	↑*		↑*	
Kamolrat et al. (2013)[108]	Aging mice	2.5g/d EPA + DHA	Dextrose + AA inf.	→	→	↑*	↑		
Kamolrat & Gray, (2013)[91]	Mouse myotubes	50 µM EPA or DHA		→	↑	↑*	↑	↑*	↓*
Tesseraud et al. (2014)[104]	New-born chickens	ALA-rich diet, 3 wks	Insulin injection	↑		↑*			
		n-3 PUFA-rich diet, 3 wks		→		→			
McGlory et al. (2016)[49]	Young M	5g/d n-3 PUFA, 8 wks	Basal	↓*		→		→	
			Protein feeding	→		→		↑	
			Protein feeding + REx	↓*		↓*		↑	
Lalia et al. (2017)[48]	Older M & F	4g/d n-3 PUFA, 16 wks	Basal					↑*	
			REx					↑*	
McGlory et al. (2019)[106]	Immobilised young F	5g/d n-3 PUFA, 6 wks	Free-living condition					↑*	

1.8 – Exercise function and metabolism

N-3 PUFA supplementation has been suggested to have several beneficial effects on aspects of exercise function, including oxygen kinetics, vascular function, neuromuscular function, fatigue resistance and psychological strain. Generally, these studies provide supplementation for several (3-10) weeks and examine changes in exercise function and performance. As such, some studies have observed improvements in specific aspects of exercise function, although generally these effects have not been replicated, nor have they translated into improvements in performance (summarised in table 1.2). Specifically, n-3 PUFA is suggested to improve fatigue resistance and oxygen kinetics in rats, and trained human cyclists [109]–[112]. The suggested mechanism for this effect was an improvement in muscle cell Ca^{2+} cycling, associated with the increase in DHA incorporation. Although n-3 PUFA were demonstrated to improve oxygen efficiency during submaximal cycling in active males, and trained cyclists, this effect has not been shown to translate to improvements in exercise performance [113], [114]. Similarly, it has been suggested n-3 PUFA supplementation improves aerobic and anaerobic capacity [115], [116]. Others have also suggested n-3 PUFA lowers heart rate during submaximal exercise, and heart rate recovery following exercise [117]–[119]. Furthermore, one study observed improvements in neuromuscular function and fatigue resistance with 3-week n-3 PUFA supplementation in male athletes [120]. Finally, some have also suggested n-3 PUFA may reduce ratings of perceived exertion during submaximal exericse [113]. Nevertheless, the changes in aspects of exercise function with chronic n-3 PUFA supplementation have not been shown to induce worthwhile improvements in performance settings.

Table 1.2 – Influence of n-3 PUFA on exercise function and performance in healthy adults. Abbreviations: male (M), female (F), Australian (Aus.), recreationally active (rec.), endurance (end.), fish oil (FO), krill oil (KO), seal oil (SO), time to exhaustion (TTE), time trial (TT), oxygen consumption (VO_2), peak power output (PPO), heart rate (HR), rate pressure product (RPP), maximum voluntary contraction (MVC), counter-movement jump (CMJ), respiratory exchange ratio (RER), rate of perceived exertion (RPE).

Study	Population	Daily dose	Duration	Key findings
Raastad et al. (1997)[115]	M soccer	5.2g FO: 1.6g EPA + 1.04g DHA	10 weeks	No improvement in maximal aerobic power, anaerobic power or running TTE
Oostenbrug et al. (1997)[85]	M cyclists	6g FO: 1.05g EPA + 0.75g DHA	3 weeks	No improvement in 1-hour cycling TT
Toft et al. (2000)[83]	M runners	6g FO: 1.9g EPA + 1.1g DHA	6 weeks	No improvement in marathon time
Peoples et al. (2008)[111]	M cyclists	8g FO: 0.8g EPA + 2.4g DHA	8 weeks	Ramp test: no difference in VO_{2peak} or PPO, but lower HR; Submaximal cycle: lower HR, VO_2 and RPP
Buckley et al. (2009)[117]	M Aus. football	6g FO: 0.36g EPA + 1.56g DHA	5 weeks	Reduced HR during submaximal exercise, no improvement in TTE
Nieman et al. (2009)[82]	M & F cyclists	2g EPA + 0.4g DHA	6 weeks	No improvement in 10-km TT
Kawabata et al. (2014)[113]	M rec.	3.6g FO: 0.9g EPA + 0.4g DHA	8 weeks	Reduced VO_2 during submaximal cycle, no change in HR or RPE
Zebrowska et al. (2015)[81]	M cyclists	0.66g EPA + 0.44g DHA	3 weeks	Improved endothelial function and maximal oxygen uptake
Da Boit et al. (2015)[84]	M & F	2g KO: 0.24g EPA + 0.12g DHA	6 weeks	No improvement in work-target TT (~80min)
Lewis et al. (2015)[120]	M athletes	5g SO: 0.375g EPA + 0.23g DHA	3 weeks	Increased VL EMG, reduced Wingate power drop, no improvement in MVC or 250 kJ TT
Hingley et al. (2017)[114]	M end. athletes	0.14g EPA + 0.56g DHA	8 weeks	Reduced oxygen consumption during TT, but no improvement in power output during 6s sprints, 5-min TT or MVC
Black et al. (2018)[121]	Rugby players	1.1g EPA + 1.1g DHA	5 weeks	Improvement in CMJ force
Gravina et al. (2017)[116]	M & F soccer	0.1g/kg FO: 70% EPA + 20% DHA	4 weeks	No improvements in strength, power or speed, but improvement in Yo-Yo test performance
James et al. (2020)[122]	M cyclists	3.2g EPA + 1.5g DHA	4 weeks	No change in VO2, HR, RER or RPE during submaximal cycle, no improvement in 15-min TT

1.9 – Incorporation and measurement

Regular consumption of n-3 PUFA is known to result in their incorporation into phospholipid membranes. There are different ways of reporting n-3 PUFA status, which is typically done in relation to other fatty acids. One such method of reporting is the omega-3 index, which indicates the relative amount of EPA plus DHA in red blood cells, as a percentage of total fatty acids [123]. It has been suggested that an omega-3 index ≥ 8% is optimal for general health outcomes, as values of 8.5-9.5% are observed in Japanese people with low sudden cardiac death [123]. However, n-3 PUFA are incorporated into a wide range of tissues, and the optimal range for various physiological outcomes is not known. In addition, optimal values for different tissues are also unknown. For example, it has been suggested that DHA is preferentially incorporated into neurons, as relatively high concentrations are observed in the nervous system [124]. There are several studies which have investigated the incorporation of n-3 PUFA into various tissues. On an acute timeframe, these studies suggest incorporation into leukocytes and platelets may occur as quickly as 1 hour following their injection into the circulation [125], [126]. An investigation of regular oral n-3 PUFA supplementation observed increases in EPA, DHA and total n-3 PUFA into lipid profiles of blood and muscle over a duration of 4 weeks [15]. In this study, 3.5g/d EPA plus 0.9g/d DHA increased EPA from 0.9 to 4.8% (of total FA), DHA from 2.7 to 3.6% and total n-3 PUFA from 5.9 to 11.2%. Similarly, in muscle, EPA increased from 0.6 to 2.4%, DHA from 1.5 to 2.1% and total n-3 PUFA from 3.8 to 6.8%. At the same time, n-3 PUFA supplementation led to decreases in monounsaturated FA and n-6 PUFA, in both blood and muscle lipid pools, with no change in saturated FA. Therefore, in a healthy young population, n-3 PUFA supplementation at palatable oral doses results in clear increases in blood and muscle n-3 PUFA status.

1.10 – Studies in the field of n-3 PUFA

Despite observed positive effects of n-3 PUFA on various aspects of oxidative stress, inflammation, energy metabolism, and muscle protein turnover, meta-analyses on whole-body effects in various contexts often fail to demonstrate significant benefits. For example, a Cochrane review on the ability for n-3 PUFA to relieve symptoms associated with cancer cachexia concluded there was insufficient evidence to support an effect [127]. Similarly, there is not enough high-quality evidence to support an effect of n-3 PUFA on mortality or cardiovascular health [11]. Although some studies observe improvements in these parameters, the authors suggest support for an effect of n-3 PUFA mainly stems from studies with a high risk for bias. Meta-analyses assessing the influence of n-3 PUFA on body composition, as total, fat and lean mass, mainly in overweight individuals, also fail to find a worthwhile effect [128]–[130]. Further, meta-analysis suggests n-3 PUFA consumption is not significantly associated with development of type 2 diabetes mellitus [131]. Meta-analyses on the effect of n-3 PUFA on insulin sensitivity in the general population found no benefit, although they did find a significant increase in insulin sensitivity in people with metabolic disorders [50], [132]. A recent meta-analysis on the ability for n-3 PUFA to reduce inflammation and muscle soreness after exercise concluded there was insufficient evidence to support an effect [53]. A meta-analysis on n-3 PUFA and muscle mass, strength and function in older adults found minor benefits for muscle mass gain and function, especially with supplementation periods lasting >6 months [133]. Further, another meta-analysis demonstrates that n-3 PUFA reduces heart rate in different populations at rest [134]. N-3 PUFA supplementation was also demonstrated to improve endothelial function in various populations [135]. Furthermore, n-3 PUFA appears to increase adiponectin, reduce leptin and improve the adiponectin-leptin ratio [136], [137]. Finally, one meta-analysis on the effects of n-3 PUFA on depression suggested they may have beneficial effects in this context [138]. However, another meta-analysis on the same topic suggests the benefits of n-3 PUFA were removed after adjusting for publication bias [139].

That is, the efficacy of n-3 PUFA was suggested to be increased in trials of lower quality, of shorter duration, and with greater baseline symptoms. Taken together, many meta-analyses on the effects of n-3 PUFA on different whole-body outcomes indicate methodological issues and high risk of bias in the analysed studies. It has been argued that generally, most published research findings are false, due to factors including publication bias, financial interests, study design and statistical techniques [140]. Thus in a general sense, there is a lack of reproducibility of effects, which appears to hold true for studies on n-3 PUFA. Therefore, more high-quality randomised controlled trials are required to corroborate the suggested effects of n-3 PUFA on various physiological parameters.

1.11 – Aims of thesis

Although researchers have studied the influence of n-3 PUFA on various physiological processes in detail, many questions still remain. Largely, the focus has been on the potential for n-3 PUFA to protect against, or reverse, negative health outcomes associated with various disease states. However, n-3 PUFA and eicosanoids may fundamentally affect gene expression and cellular signalling, which may also result in physiological benefits on a whole-body level in healthy individuals. This thesis will attempt to build on existing knowledge by studying the effects of dietary n-3 PUFA on physiological responses, ranging from molecular to whole-body level, to acute and chronic stressors in healthy, active adults. It will achieve this through the following aims:

1. To investigate the influence of n-3 PUFA supplementation on lean mass loss during the catabolic situation of energy restriction.
2. To investigate the influence of n-3 PUFA supplementation on whole-body metabolic changes during energy restriction.
3. To investigate the influence of n-3 PUFA on muscle anabolic signalling and gene expression during energy restriction.
4. To investigate the influence of *ex vivo* n-3 PUFA rich serum on *in vitro* muscle cell development.
5. To investigate the acute influence of n-3 PUFA on exercise performance in trained cyclists.
6. To investigate the acute influence of n-3 PUFA on whole-body exercise function in trained cyclists.
7. To investigate differences in bioavailability of n-3 PUFA emulsion versus standard oil, during exercise.

Chapter 2 – randomised controlled trial of n-3 PUFA supplementation shows no effect on lean mass changes during energy restriction in healthy active young males

2.1 - Introduction

During diet-induced energy restriction (ER), individuals typically lose both fat and lean mass. Loss of muscle, as the primary constituent of lean mass, has negative effects on metabolic health and physical functioning, which includes activities of daily living and exercise performance [90]. Although some small studies show high-protein diets and exercise can moderate lean mass loss during ER, additional strategies may have substantial beneficial effects on its preservation [141]. Omega-3 polyunsaturated fatty acids (n-3 PUFA) are one nutritional candidate that has been suggested may preserve muscle mass through its actions on muscle anabolism and catabolism [43], [86], [87], [91]. Given the n-3 PUFA status of the UK population is low [142], supplementation can be used to increase their incorporation into phospholipid pools of various tissues. The positive effects of n-3 PUFA on physiological functioning (as reviewed in the general introduction – Ch1) show these molecules may be useful in situations that induce metabolic stress, such as ER, muscle disuse, inflammation and disease. As such, their ability to influence inflammation, cell signalling and gene expression may result in positive whole-body changes in body composition, energy metabolism and physical function in healthy adults [86], [87], [143]–[145]. To date, no studies have investigated the influence of n-3 PUFA on changes in lean mass and parameters of metabolic and functional health during ER in healthy, normal weight adults.

Optimisation of health and functional outcomes during and after voluntary or involuntary weight loss is desirable for many, be it general population, athletes, soldiers or astronauts. Without manipulating the protein content of the diet, weight loss results in loss of lean mass, including

skeletal muscle. The negative energy balance, paired with reduced intake of amino acids, results in an impaired balance in muscle anabolic and catabolic processes. By breaking down muscle proteins, energy and materials can be freed up to maintain novel protein synthesis for core processes. In the early phase of weight loss, lean mass losses include water, glycogen and protein [146]. Typically, the proportion of weight lost as lean mass ranges from 20-50%, with greater proportions of lean mass loss observed in lean individuals [146]–[149]. Studies show that exercising during ER may preserve muscle mass and function [149]. High protein diets, which under current recommendations are considered to be diets above 0.8 g of protein/kg body weight [150], may successfully preserve muscle mass during weight loss in healthy active young individuals [147], [151]. However, this effect is small, and the increased protein intake does not affect muscle function [149]. Therefore, alternative or additional strategies to preserve muscle mass and function during ER are required.

Most studies that have attempted to investigate the influence of n-3 PUFA on changes in body composition do not report lean mass, as they have focused on changes in total and fat mass and associated metabolic changes. There are some reports that n-3 PUFA improves maintenance or accretion of lean mass during habitual living or exercise training programmes, especially in older adults [46], [143], [152], [153]. However, other studies have observed no effect of n-3 PUFA on lean mass during energy balance (EB) conditions [128], [152], [154]–[156]. In the context of ER, in overweight adults (BMI 30-40 kg/m^2), supplementation with 1.62g EPA and 0.42g/d DHA during a 4-week very low energy diet (3000kJ/d) did not influence lean mass loss [157]. In a similar study, but with a ‘healthy eating weight loss diet’ (5000kJ/d for females; 6000kJ/d for males), n-3 PUFA also did not affect lean mass loss. However, metabolic control of muscle protein turnover is known to be impaired in obese individuals [158]. The effects of n-3 PUFA on lean mass during energy restriction in normal weight adults have not been studied.

In older adults, small studies suggest n-3 PUFA can increase muscle strength improvements during strength training [45], improve muscle function [46], [47], [144], [159] and mass [46], [144]. However, other studies that have investigated changes in muscle strength [144], [160], function [45], [47], [116], [159]–[162] and mass [116], [161], [162] have not observed an effect of n-3 PUFA. Meta-analysis shows n-3 PUFA may have beneficial effects on muscle strength and function, but not muscle mass, in older adults [163]. As aging muscle is known to differ from that in young adults, in terms of muscle protein turnover, and the observed anabolic effects of n-3 PUFA in young and middle aged adults [86], [87], their effects in young people warrant further investigation.

The effects of n-3 PUFA on muscle physiology have been suggested to stem from improvements on muscle protein turnover, which in turn are made up of improvements in both muscle protein synthesis (MPS) and muscle protein breakdown (MPB) [48], [49], [86], [87], [91], [106]. Therefore, n-3 PUFA may preserve lean mass during ER, which is characterised by reductions in MPS and increases in MPB [164]–[166]. The effects of n-3 PUFA on muscle protein turnover are apparent in muscle disuse, a context in which anabolic stimulation through mechanical load is sparse. Here, n-3 PUFA have been shown to reduce muscle atrophy, through improvements in MPS and MPB [105], [106]. During diet-induced ER, anabolic stimulation is also sparse due to the limited availability of amino acids. In rodents, n-3 PUFA was able to reduce MPB during short-term starvation [92], although lean mass changes were not reported.

There are indications that n-3 PUFA also have distinct effects on fat metabolism, which may affect changes in fat mass during ER. At a molecular scale, n-3 PUFA has been shown to promote fat oxidation and reduce lipogenesis [167], [168]. As such, less lipids are available in the circulation, as exemplified by a reduction in triglycerides with n-3 PUFA supplementation [7]. In

overweight/obese adults, n-3 PUFA improve abdominal parameters of adiposity, such as waist-to-hip ratio and waist circumference, despite a lack of change in overall fat mass [129], [130]. In normal weight adults, some have observed increased fat loss with n-3 PUFA during EB [143], [169], although others have not observed such an effect [152]. Nevertheless, n-3 PUFA may alter underlying drivers of changes in body composition, such as substrate metabolism and metabolic rate [170]. Like other modulatory aspects of n-3 PUFA, previous results regarding their effects on energy metabolism are ambiguous, and may further depend on age and sex [144], [145], [171], [172]. In males, some investigators have observed no change in resting metabolic rate (RMR), while others observed an increase with n-3 PUFA [171], [172], while in females both increases and decreases have been observed [144], [145]. Therefore the effects of n-3 PUFA on energy metabolism, if any, remain unclear.

n-3 PUFA may also improve muscle function, independent from changes in muscle mass. Previous studies in older adults have shown that muscle function following exercise training is improved to a greater degree with n-3 PUFA supplementation [45]–[47]. Additionally, n-3 PUFA may alter specific parameters of muscle function such as neuromuscular function, oxygen consumption, heart rate and anaerobic capacity [111], [114], [116], [120]. It is possible to maintain or even increase on measures of exercise performance during ER combined with exercise, despite loss of lean mass [173], [174]. Nevertheless, potential preservation of muscle mass by n-3 PUFA supplementation may improve performance outcomes even further.

Previous studies have established that significant increases in n-3 PUFA of blood and muscle lipid profiles can be observed after 4 weeks of supplementation at 4g/d, and that this duration and dose results in increased muscle anabolism in healthy adults [15], [86], [87]. As there have been no studies to investigate the influence of n-3 PUFA supplementation on lean mass during ER in healthy

adults, our primary aim was to establish whether 4g/d n-3 PUFA for 6 weeks could preserve lean mass during ER in healthy active young males. In this study, we utilised a relatively severe 2-week, 40% energy restriction, with a standard macronutrient composition of the diet (i.e. not high-protein). As such, we aimed to determine whether n-3 PUFA supplementation could form a viable additive strategy to maintenance of exercise, for the preservation of lean mass during ER. To this end, we recruited resistance trained healthy males to study the potential ameliorating effects of n-3 PUFA supplementation on body composition. Our secondary aims were to determine the influence of n-3 PUFA supplementation on changes in whole-body energy metabolism and muscle function during ER with maintenance of exercise.

2.2 – Methods

2.2.1 – Participants

20 male participants volunteered to take part in the study. Participants were actively resistance training (≥ 2 times/week for at least 6 months prior to commencement of the study). Participants were excluded if they were already taking n-3 PUFA supplementation, which was confirmed verbally. They were healthy as determined by our pre-participation health questionnaire. The study conformed to the principles set out by the Declaration of Helsinki and was approved by the NHS West of Scotland Research Ethics Committee 4 (16/WS/0248) and the University of Stirling Research Ethics Committee. During an initial pre-screening visit, participants were informed of the study procedures and provided their written consent. Following this, a blood sample was taken from an antecubital vein for the determination of baseline whole blood n-3 PUFA status.

2.2.2 – Supplements

Participants were randomised into a n-3 PUFA (FO) or a placebo (PLA) supplementation group. The FO and PLA supplements were in the form of a 200 mL juice box (Smartfish, Oslo, Norway), to be consumed twice daily for 6 weeks. Due to the make-up of the FO and PLA supplements, they were not matched for caloric content. The FO supplement (180 kcal) contained 18 g carbohydrate, 5 g protein and 9.9 g fat of which 2g EPA plus DHA (800-1000 mg EPA and 1000-1200 mg DHA). Thus, the FO group consumed 4g/d EPA plus DHA. The placebo supplement (96 kcal) consisted of 1.5 g fat, 5 g protein and 18 g carbohydrate. As we provided participants with their entire diet during weeks 4-6, and the FO and PLA supplements were not matched for caloric content, only the participants were blinded. The supplements were provided to participants in unmarked whitepackaging and matched for taste using orange flavouring. Adherence to supplementation was assessed by analysis of whole blood PUFA content, taken from blood samples obtained at the start and end of the study. Participants were instructed not to consume oily fish, fish oil supplements

and to maintain habitual exercise volume and intensity throughout the 6-wk period.

2.2.3 – Diet control

During the first 3 weeks, participants consumed their habitual uncontrolled diet. To determine baseline energy balance, habitual energy intake and expenditure were recorded over three days during the first week, using a protocol adapted from Mettler et al. (2010) [147]. First, participants completed a food diary over two weekdays and one weekend day. Each food diary was analysed for total energy intake (EI) in calories/day as well as the macronutrient composition using dietary software (Nutritics, Dublin, UK). In addition, participants wore an Actiheart heart rate and activity monitor (CamNtech, Papworth Everard, UK) continuously on the same days the food diary was completed. Actiheart data was analysed using Actiheart 4.0 software to establish active energy expenditure (AEE) and total energy expenditure (TEE). Furthermore, participants completed a Bouchard physical activity record (BAR) [175] during the same three-day period. The EI, TEE and BAR data were averaged to establish a baseline energy balance (EB) in kcal/day. During week 4, participants were fed a control diet matching EB (100%). During weeks 5 and 6 participants were provided with an energy restricted (ER) diet comprising of 60% calories relative to EB. The macronutrient composition of all diets was fixed at 50% carbohydrate, 15% protein and 35% fat. The FO and PLA supplements were accounted for by incorporating their energy and macronutrient content into the diets, provided during weeks 4-6.

2.2.4 – Experimental trials

Participants attended trials in the morning of five different days spread over the 6-week period. There was no familiarisation session. Visits 1, 2 and 4 (days 7, 27, 42) were identical to each other and consisted of body composition, metabolic and performance assessments. Unfortunately, some participants did not attend either visit 1 or 2, resulting in missing data. This was accounted for in

the statistical analysis. Participants were requested not to undertake strenuous exercise in the 2 days before each trial. Before attending the laboratory for the morning trials, participants were instructed to fast overnight (no food and drink other than water from 10 pm) and to consume at least 500 mL water in the morning, to ensure euhydration [176]. Upon arrival, participants were asked to empty their bladder before stature was recorded on a Harpenden stadiometer (Holtain Ltd., Crosswell, UK), and body mass was recorded on digital scales (Seca, Birmingham, UK). They then underwent a whole-body dual energy x-ray absorptiometry scan (Lunar iDXA; GE Healthcare, UK) to determine total mass, fat mass and lean mass of total body, arms, legs, trunk, android and gynoid regions. All scans were performed by the same researcher. The position of participants on the scan bed (196 x 66 cm) was standardised with the top of their head 3 cm away from the upper limit of the scan region. Their hands were positioned alongside their body in an upright position. Laying supine and wearing only underwear, their feet were rested against a foam positioning aid. As some of the participants did not fully fit on the scan bed, in some cases part of their arms and/or feet were outside of the scan field. In the cases where one arm was not fully scanned, the iDXA software (enCORE v18, GE Healthcare) was used to apply corrections by estimating one half from the other. That is, if for example part of the left arm was missing, data from the right side was used to estimate the left. In the cases where feet were missing, no corrections were applied. However, given the participants heads were always placed 3cm away from the upper limit of the scan region, the part of the feet that was not scanned was consistent for each individual. Some fat and lean mass data for this cohort was previously reported in Philpott, et al. (2019) [177]. In the present study, corrected fat and lean mass data are presented for total body, trunk, arms, legs, android and gynoid regions only.

Following the DEXA scan there was a short walk, up some stairs, to the metabolic laboratory, where resting metabolic rate, respiratory exchange ratio and oxygen consumption were determined via indirect calorimetry using an Oxycon Pro (Becton Dickinson, Wokingham, UK).

Participants rested for 5 minutes prior to commencement of indirect calorimetry. The participant lay supine, and a canopy connected to the Oxycon Pro was placed over their head. Participants were instructed to remain as still as possible for 15 minutes. Minutes 5:00-15:00 were averaged for the determination of respiratory exchange ratio (RER) and resting metabolic rate (RMR). RMR was determined automatically by the Oxycon software via the following formula (de V. Weir, 1949) [178]:

$$RMR\ (kcal/min) = (1.59 * VCO_2 + 5.68 * VO_2 – 2.17 * 15) / 1440$$

Where VCO_2 is CO_2 produced and VO_2 is oxygen consumed. As we did not measure urinary nitrogen, the default setting of 15g/day was used by the Oxycon software.

To determine body fat percentage (BF%), measures of 8 skinfold sites (biceps, triceps, subscapular, iliac crest, supraspinale, abdominal, front thigh, medial calf) were taken and the sum of these skinfolds (SUM8) calculated. Measures were completed by the same level 3 trained anthropometrist following the International Standards for Anthropometric Assessment [179] using a Harpenden skinfold caliper. Withers' formula was applied to determine percentage body fat (WIT-BF%) [180]:

$$WIT\text{-}BF\% = 495 / (1.0988 – 0.0004 * \Sigma\ 7) – 450$$

Where Σ 7 is the sum of all 8 skinfold sites minus the iliac crest. Many formulas exist to estimate BF% from skinfolds, and Withers' formula was selected due to its applicability to our cohort of healthy active young males [181].

A Philips ATL HDI-5000 ultrasound scanner (ATL Ultrasound, Bothell, WA, United States) was used to determine changes in muscle size of the vastus lateralis (VL), semitendinosus (ST), biceps brachii (BB) and triceps brachii (TB). Ultrasound gel was applied to the scanner head and the skin at the target area. The distances from the upper and lower side of the scan head to reference points on the body were recorded for repeat measurement on subsequent visits. The scan location was set as 2/3rds of the length between the lower and upper reference points. For example, the bicep scan

was performed at 2/3rds of the length between the antecubital crease and the acromion [182]. A still image was captured and the distance across the widest part of the muscle was recorded to determine changes in muscle size.

To measure exercise performance, participants performed counter-movement jumps (CMJ). Vertical jump height was recorded using a linear transducer (Celesco, Toronto, Canada), connected to a BioPac MP100 data acquisition system (BIOPAC, Norfolk, UK). The transducer was fixed to a FT700 power cage (Fitness-technology, Melbourne, Australia), directly above the participant's head. The end of the transducer cable was attached to the centre of a dowel rod by a Velcro strap. Participants stood directly under the transducer with the dowel rod resting on their shoulders and maintaining its position with their hands on either side. The vertical displacement was recorded using Acqknowledge software (version 3.9.1, BioPac Systems, Inc.) and saved for offline analysis. Jump height was calculated as the displacement from the starting position to the highest point recorded at the top of each jump by the linear transducer. The maximum height from 3 attempted jumps was recorded as maximum CMJ height. Simultaneously, jump force was determined using a force platform, also connected to the BioPac MP100 system. The force platform was calibrated before each use with standard weights (0 and 1852 Newton). The force of each jump was calculated as the maximum force following take-off from the platform minus the force on the platform during the starting position. As such, maximum CMJ force was recorded as the highest force produced during the 3 jumps. During this visit, participants also completed leg press and leg extension 1RM, muscular endurance and maximum voluntary contraction (MVC) tests which are reported elsewhere [177]. Set up and testing for the jump tests, oxycon, and ultrasound were completed by NB, DEXA and MVC were completed by the other student, while other muscle function tests were supervised by both investigators.

2.2.5 – Lipid extraction of whole blood samples

Whatman 903 blood spot cards were analysed by the Institute of Aquaculture at the University of Stirling, as previously described [183]. In short, the blood spots were placed into vials containing 1.25M methanol-HCl and placed in a hot block at 70°C for 1h. They were allowed to cool to room temperature, after which 2mL distilled water and 2mL of saturated HCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 2 x 2 mL of isohexane. FAME were quantified by gas-liquid chromatography (GLC).

2.2.6 – Data presentation and statistical analysis

Data were analysed in Rstudio (version 1.4) using R (version 4.1.1) and the *lme4* package. Where appropriate, for each analysis a linear mixed effects model was created with the participant ID as a random factor. A type III analysis of variance (ANOVA) was then performed using the Kenward & Roger approach for approximation of degrees of freedom, as is recommended for small sample sizes [184], [185]. Where significant effects were found, post-hoc analysis was performed using Tukey's method [186]. For some analyses, the random factor did not significantly improve the model compared to the simpler model (when Chi^2 for the random factor was $p > 0.05$). When this was the case, the usual linear model was used. Correlations were estimated as Pearson's R [187]. Effect sizes were expressed as Hedges' *g*, which applies a correction factor to Cohen's *d* for small sample size [188], [189]. Data are presented as mean ± standard deviation unless otherwise stated.

2.3 - Results

2.3.1 – Participant characteristics

Participant baseline characteristics are displayed in table 2.1. Participants in FO and PLA did not significantly differ in baseline age ($p = 0.60$), stature ($p = 0.28$), body mass ($p = 0.22$), WIT-BF% ($p = 0.09$) or SUM8 ($p = 0.07$). The variability in stature (range: 1.63 – 1.91 m) and body mass (range: 58.3 – 137.0 kg) of participants at baseline was high.

Table 2.1 - Baseline characteristics of resistance-trained male participants. Participants were randomly assigned to supplemental groups. Data are displayed as mean ± standard deviation.

	Fish oil (n = 10)	Placebo (n = 10)	Mean (n = 20)
Age (yr)	23.8 ± 4.5	22.1 ± 4.8	22.95 ± 4.5
Stature (cm)	179.8 ± 18.7	177.8 ± 7.3	178.8 ± 16.2
Mass (kg)	89.1 ± 19.4	77.3 ± 9.6	83.8 ± 16.6
Body fat (%)	11.9 ± 3.3	9.2 ± 2.1	10.7 ± 3.1
Sum of 8 skinfolds (cm)	118.0 ± 42.8	81.2 ± 28.2	101.1 ± 40.4

Total n-3 PUFA, EPA and DHA values are displayed in table 2.2. After 6 weeks of supplementation, whole blood total n-3 PUFA (as % of total PUFA) increased by 16.2 ± 6.0 in FO ($p < 0.0001$), with no change (-1.0 ± 2.6) in PLA ($p = 0.48$). The effect size of total n-3 PUFA change was $g = 3.6$ (95% CI: 1.9 – 5.3). DHA increased by 1.83 ± 0.60 ($p < 0.0001$) in FO, with no change (-0.13 ± 0.88) in PLA ($p = 0.67$). The effect size of DHA change was $g = 2.4$ (95% CI: 1.0 – 3.8). EPA increased by 1.75 ± 1.09 ($p < 0.0001$) in FO, with no change (-0.15 ± 0.13) in PLA ($p = 0.54$). The effect size of EPA change was $g = 2.4$ (95% CI: 1.0 – 3.8).

Table 2.2 – total n-3 PUFA, EPA and DHA values at the start and end of the study. Total omega-3 values are as a percentage of total polyunsaturated fatty acids (i.e. n-3 plus n-6 PUFA). EPA and DHA values are as a percentage of total fatty acids. Irregularities in sample size are due to missing samples.

	Fish oil		Placebo		
	week 0	**week 6**	**week 0**	**Week 6**	**p (change by group)**
EPA (% FA)	1.15 ± 1.05	3.17 ± 1.11	0.66 ± 0.16	0.49 ± 0.16	0.0035
DHA (% FA)	2.79 ± 0.82	4.90 ± 0.86	2.60 ± 0.70	2.49 ± 0.49	0.0002
Total n-3 PUFA (% PUFA)	26.7 ± 6.8	44.8 ± 7.5	25.3 ± 2.8	23.9 ± 3.1	0.0001
sample size (n)	8	8	8	10	15

2.3.2 – Energy restriction

During initial habitual energy balance monitoring, participants self-reported energy intake (EI) of 12.0 ± 2.4 MJ (2857 ± 569 kcal) per day, with no significant difference between groups (p = 0.62; Table 2.3). Their self-reported energy expenditure, via Bouchard activity record (BAR), was 17.2 ± 5.0 MJ (4101 ± 1192 kcal), with no significant difference between groups (p = 0.10). Energy expenditure assessed via Actiheart (TEE) was 14.9 ± 3.9 MJ (3558 ± 939 kcal), with no significant difference between groups (p = 0.30). We attempted to further measure energy expenditure in participants during EB and ER, however this was only successful in a limited subset of participants (FO: n = 4; PLA: n = 4) due to methodological issues. In these participants, active EE (AEE) was maintained from EB (3.13 ± 1.13 MJ) to ER (3.31 ± 1.09 MJ), with no effect of FO (p = 0.55). In addition, total EE (TEE) was also maintained from EB (12.0 ± 1.54) to ER (12.2 ± 1.68), with no effect of FO (p = 0.55).

The energy intake and macronutrient composition of the diets provided to participants is displayed in table 2.3. There was no significant difference in protein intake between groups at EB (p = 0.92) or ER (p = 0.80) on an absolute basis. Relative to total body mass at baseline (g/kg), protein intake appeared higher in PLA compared to FO, although this was not statistically significant at EB (p = 0.21) or ER (p = 0.17).

Table 2.3 – Overview of dietary macronutrients and energy provided to participants during energy balance (EB; week 4) and energy restriction (ER; week 5-6). Participants were provided with all their food and drink during this period and the FO or PLA supplements were integrated into the diet. The g/kg values are based on baseline total body mass.

	Fish oil		Placebo		Total	
	EB	**ER**	**EB**	**ER**	**EB**	**ER**
Protein (g)	129 ± 16	83 ± 12	128 ± 26	82 ± 12	128 ± 21	83 ± 12
Protein (g/kg)	1.48 ± 0.25	0.95 ± 0.15	1.59 ± 0.15	1.03 ± 0.07	1.54 ± 0.21	0.99 ± 0.12
Carbohydrate (g)	432 ± 56	251 ± 37	443 ± 91	277 ± 49	438 ± 74	264 ± 44
Carbohydrate (g/kg)	4.90 ± 0.55	2.85 ± 0.37	5.57 ± 0.91	3.48 ± 0.41	5.24 ± 0.81	3.16 ± 0.50
Fat (g)	121 ± 31	75 ± 17	99 ± 22	59 ± 14	110 ± 29	67 ± 17
Fat (g/kg)	1.39 ± 0.42	0.86 ± 0.23	1.23 ± 0.15	0.74 **±** 0.09	1.31 ± 0.32	0.80 ± 0.18
Energy (Kilocalories)	3333 ± 497	2000 ± 298	3200 ± 609	1920 ± 365	3266 ± 545	1960 ± 327
Energy (MJ)	13.95 ± 2.08	8.37 ± 1.25	13.39 ± 2.55	8.04 ± 1.53	13.67 ± 2.28	8.20 ± 1.37

2.3.3 - Body composition

Total body mass

There was a significant time by group interaction effect ($F = 3.90$, $p = 0.029$) for the change in total body mass, and a significant main effect of time ($F = 123.9$, $p < 0.0001$), but no significance for the effect of group ($F = 1.827$, $p = 0.19$; Figure 2.1B). Total body mass was not significantly different between groups at any time point ($p > 0.05$; figure 2.1A). During EB, total body mass did not change in FO (0.28 ± 0.61; $p = 0.64$) but decreased significantly in PLA (-0.91 ± 0.59 kg; $p = 0.026$). During ER, total body mass decreased in both FO (-3.3 ± 1.0 kg; $p < 0.0001$) and PLA (-2.4 ± 1.2 kg; $p < 0.0001$). Over the entire 6-week period, there was no difference ($p > 0.99$) between groups in total body mass change (-3.2 ± 1.1 kg).

Despite the lack of a statistically significant difference between groups in baseline weight, there was considerable disparity. As such, we also examined total weight as percentage change (from baseline – week 4, and week 4 – week 6). With total body mass expressed as percentage change, there also was a significant time by group interaction effect ($F = 6.49$, $p = 0.026$), and a significant

main effect of time (F = 36.73, p < 0.0001), but no significant main effect of group (F = 0.73, p = 0.41). During EB, percentage change in total body mass was 0.36 ± 0.67% in FO and -1.16 ± 0.73% in PLA. During ER, percentage change in total body mass was -3.85 ± 1.35% in FO and -3.05 ± 1.35% in PLA.

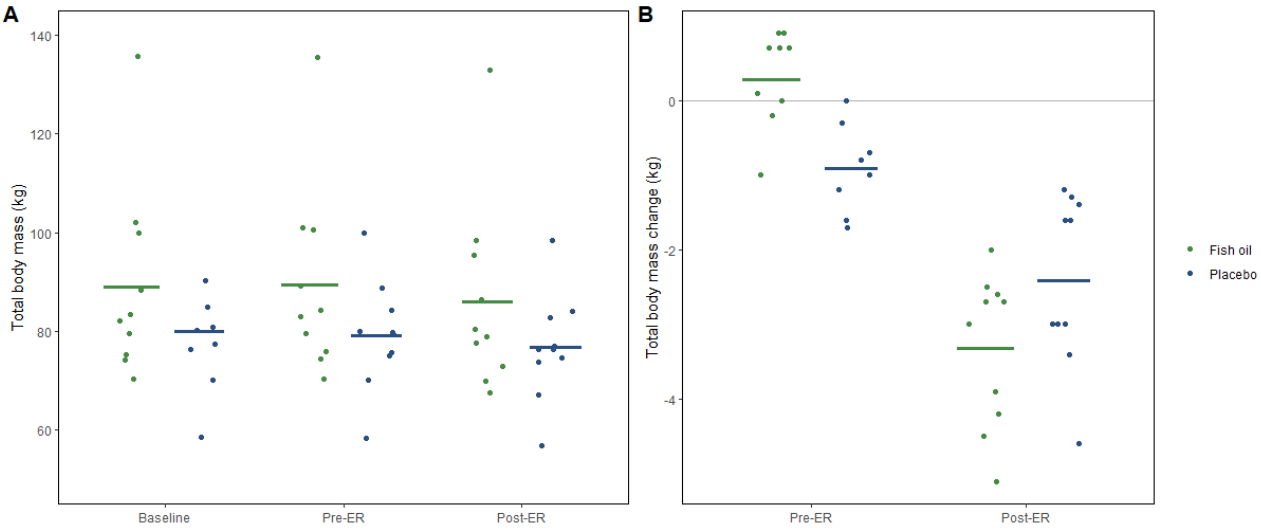

Figure 2.1 – **A)** Scatterplot of total body mass at baseline (day 7), pre-ER (day 27) and post-ER (day 42). **B)** Scatterplot of total body mass change during EB (Baseline to Pre-ER) and ER (Pre-ER to Post-ER). The horizontal bars represent model estimated means.

Table 2.4 – Model estimated values of lean and fat mass determined via DEXA. Means that do not share a letter are significantly different from each other ($p < 0.05$). Lower and upper limits of 95% confidence limits are displayed.

Lean mass (g)

	total		arms		legs		trunk		android		gynoid	
	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo
Baseline	**67558** [a]	**62242** [a]	**9580** [a]	**8544** [a]	**23654** [a]	**21497** [a]	**30661** [a]	**28558** [a]	**4520** [a]	**3963** [a]	**11208** [a]	**9985** [a]
lower CL	60505	55184	8220	7182	20854	18694	27763	25653	4090	3532	10064	8839
upper CL	74610	69299	10940	9906	26455	24299	33560	31463	4950	4394	12351	11131
Pre-ER	**67573** [a]	**61342** [a]	**9485** [a,b]	**8399** [a,b]	**23628** [a]	**21228** [a]	**30754** [a]	**28117** [a]	**4456** [a]	**3950** [a]	**11138** [a]	**9807** [a]
lower CL	60521	54290	8125	7039	20827	18427	27856	25219	4026	3520	9994	8664
upper CL	74626	68395	10845	9759	26428	24028	33652	31015	4886	4380	12281	10951
Post-ER	**66149** [b]	**60461** [b]	**9338** [b]	**8279** [b]	**23163** [b]	**20716** [b]	**30027** [b]	**27892** [b]	**4368** [b]	**3842** [b]	**10864** [b]	**9478** [b]
lower CL	59096	53409	7978	6919	20363	17915	27128	24993	3939	3412	9720	8335
upper CL	73201	67514	10698	9639	25964	23516	32925	30790	4798	4272	12007	10622

Fat mass (g)

	total		arms		legs		trunk		android		gynoid	
	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo	Fish oil	Placebo
Baseline	**17472** [a]	**14072** [a]	**1668** [a]	**1410** [a]	**5892** [a]	**5010** [a]	**8966** [a]	**6718** [a]	**1418** [a]	**952** [a]	**2842** [a]	**2330** [a]
lower CL	12762	9357	1263	1004	4487	3603	6028	3775	847	380	2038	1525
upper CL	22181	18786	2073	1816	7298	6418	11904	9660	1989	1523	3647	3136
Pre-ER	**17675** [a]	**14136** [a]	**1673** [a]	**1435** [a]	**6014** [a]	**5090** [a]	**9032** [a]	**6688** [a]	**1424** [a]	**943** [a]	**2841** [a]	**2400** [a]
lower CL	12965	9426	1268	1030	4609	3685	6095	3750	854	372	2036	1596
upper CL	22385	18845	2079	1840	7419	6495	11970	9626	1995	1513	3646	3205
Post-ER	**16179** [b]	**12793** [b]	**1580** [b]	**1309** [b]	**5725** [b]	**4704** [b]	**7942** [b]	**5866** [b]	**1227** [b]	**805** [b]	**2613** [b]	**2146** [b]
lower CL	11470	8084	1175	903	4320	3299	5004	2928	657	234	1808	1341
upper CL	20889	17503	1985	1714	7131	6109	10879	8804	1798	1375	3418	2951

Lean mass

Lean mass changes, assessed by DEXA, are displayed in table 2.4. There was no significant interaction effect ($F = 2.34$, $p = 0.11$), a significant effect of time on total body lean mass ($F = 22.5$, $p < 0.0001$), with no significant effect of group ($F = 1.47$, $p = 0.24$). During EB, total body lean mass decreased non-significantly ($p = 0.19$) by 416.8 ± 940.7 g. During ER, total body lean mass decreased significantly ($p = 0.0001$) by 1132.4 ± 1103.6 g. There was also a significant of time on lean mass in the android ($F = 9.29$, $p = 0.0006$), gynoid ($F = 19.5$, $p < 0.0001$), trunk ($F = 40.46$, $p < 0.0001$), arms ($F = 6.98$, $p < 0.0001$), and legs ($F = 20.4$, $p < 0.0001$) regions. There was no significant effect of group on lean mass in any individual region, although there was a trend ($p = 0.082$) for the android region. However, the changes in android lean mass during EB or ER were not significantly different between groups ($p > 0.99$).

Fat mass

Fat mass changes, assessed by DEXA, are displayed in table 2.4. Total fat mass significantly changed over time ($F = 29.7$, $p < 0.0001$), with no effect of FO ($F = 1.18$, $p = 0.29$). During EB, fat mass did not change ($p = 0.80$). During ER, fat mass decreased significantly ($p < 0.0001$) by 1494.4 ± 735.0 g. There was also a significant effect of time on fat mass in the android ($F = 27.6$, $p < 0.0001$), gynoid ($F = 27.6$, $p < 0.0001$), trunk ($F = 26.6$, $p < 0.0001$), arms ($F = 19.3$, $p < 0.0001$), and legs ($F = 14.1$, $p < 0.0001$) regions. There was no significant effect of group on fat mass in any individual region.

Body fat

Body fat percentages determined via Withers' formula (WIT-BF%) [180] and DEXA (DEXA-BF%) are displayed in table 2.5. There was a significant effect of time ($F = 21.9$, $p < 0.0001$) on WIT-BF%, but there was no significant effect of group ($F = 1.09$, $p = 0.31$). During EB, WIT-BF% did not change ($p = 0.96$). During ER, WIT-BF% decreased significantly (-1.35 ± 1.14%; $p < 0.0001$).

Table 2.5 – Body fat percentage (BF%) values determined by Withers' formula (1987) and DEXA scans. Upper and lower confidence levels of 95% confidence interval are displayed. Values are model estimates.

Body fat percentage				
	Skinfolds		DEXA	
	Fish oil	Placebo	Fish oil	Placebo
Baseline	**15.9**	**13.5**	**20.1**	**18.3**
lower CL	12.6	9.98	16.0	14.3
upper CL	19.2	17.0	24.1	22.4
Pre-ER	**15.8**	**13.7**	**20.2**	**18.5**
lower CL	12.5	10.2	16.2	14.5
upper CL	19.2	17.2	24.3	22.6
Post-ER	**14.7**	**12.1**	**19.1**	**17.2**
lower CL	11.4	8.57	15.1	13.2
upper CL	18.1	15.6	23.2	21.3

Muscle size

Muscle size of biceps brachii (BB), triceps brachii (TB), vastus lateralis (VL) and semitendinosus (ST), determined by ultrasound, are displayed in figure 2.2. Repeat measurements at EB (4 weeks apart) in a limited subset of participants (n=9) indicated there was a high degree of intra-individual variability for each of the examined muscles. During subsequent measurements under EB, at baseline and pre-ER, the coefficient of variation for each muscle was: biceps brachii (BB; 6.5%), triceps brachii (TB; 11.1%), vastus lateralis (VL; 3.7%) and semitendinosus (ST; 5.3%).

There was a significant effect of time on BB ($F = 9.04$, $p = 0.0012$) and ST ($F = 3.41$, $p = 0.050$), but not TB ($F = 1.26$, $p = 0.30$) or VL ($F = 0.061$, $p = 0.94$). There was no significant effect of group on any muscle ($p > 0.05$). Due to missing values, the following changes are reported as model estimated mean ± standard error (SE). BB significantly ($p = 0.0087$) increased by 0.13 ± 0.04 cm during EB, and significantly ($p = 0.0011$) decreased by 0.16 ± 0.04 cm during ER. ST appeared to decrease by 0.16 ± 0.07 cm during ER, but this did not reach statistical significance ($p = 0.087$). There were no other significant changes in muscle size, assessed via ultrasonography.

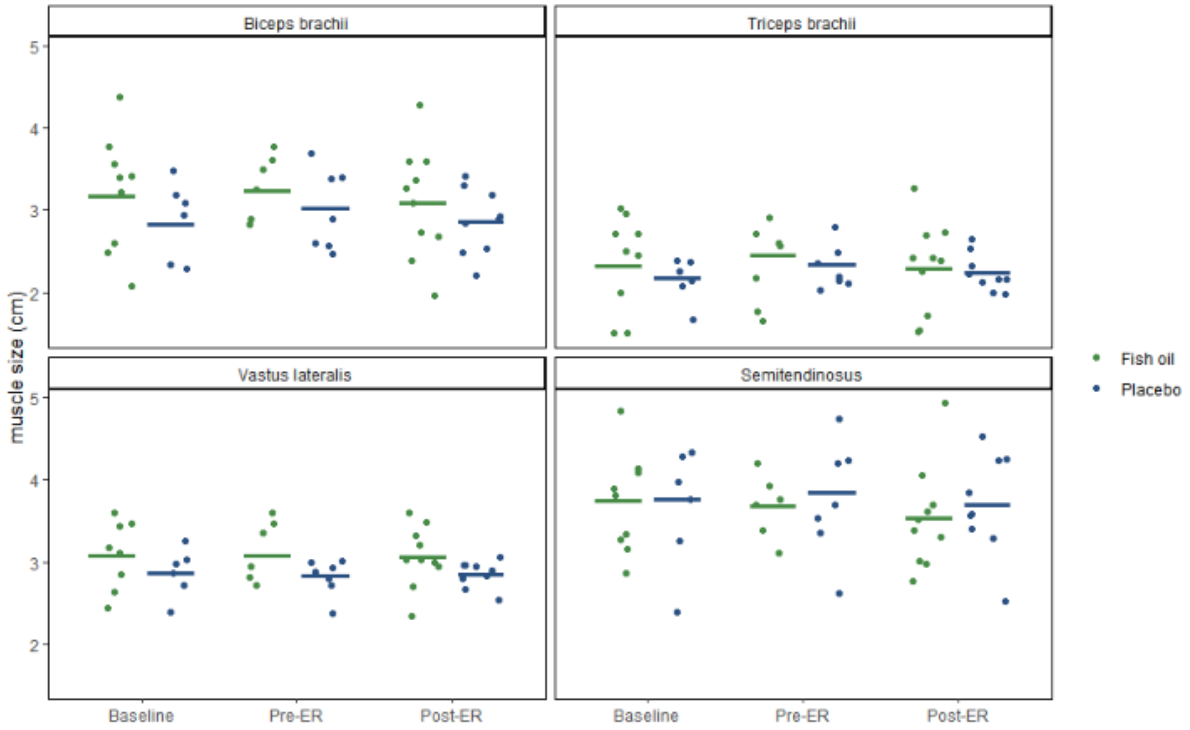

Figure 2.2 – Scatterplot of muscle sizes for biceps brachii (BB), triceps brachii (TB), vastus lateralis (VL) and semitendinosus (ST) assessed via ultrasonography at baseline (week 1), pre-energy restriction (week 4) and post-energy restriction (week 6). Dots represent observations from individual participants, while bars represent model estimated mean values.

2.3.4 – Whole-body energy metabolism

Resting whole body energy expenditure and substrate oxidation

RMR is known to be directly related to total body mass, which was confirmed by a significant correlation in our study ($R = 0.64$; $p < 0.0001$; figure 2.4). As such, RMR was normalised to total body mass to elucidate the independent effects of n-3 PUFA on ER (figure 2.3). Although RMR appeared lower in FO than PLA, there was no significant difference between groups ($F = 2.19$, $p = 0.16$). There was a trend for an effect of time on RMR ($F = 3.21$, $p = 0.055$), which was driven by a trend ($p = 0.057$) for a decrease (-0.0013 ± 0.0013 ml/min/kg) during EB, with no significant change ($p = 0.75$) during ER (0.00051 ± 0.0019 ml/min/kg).

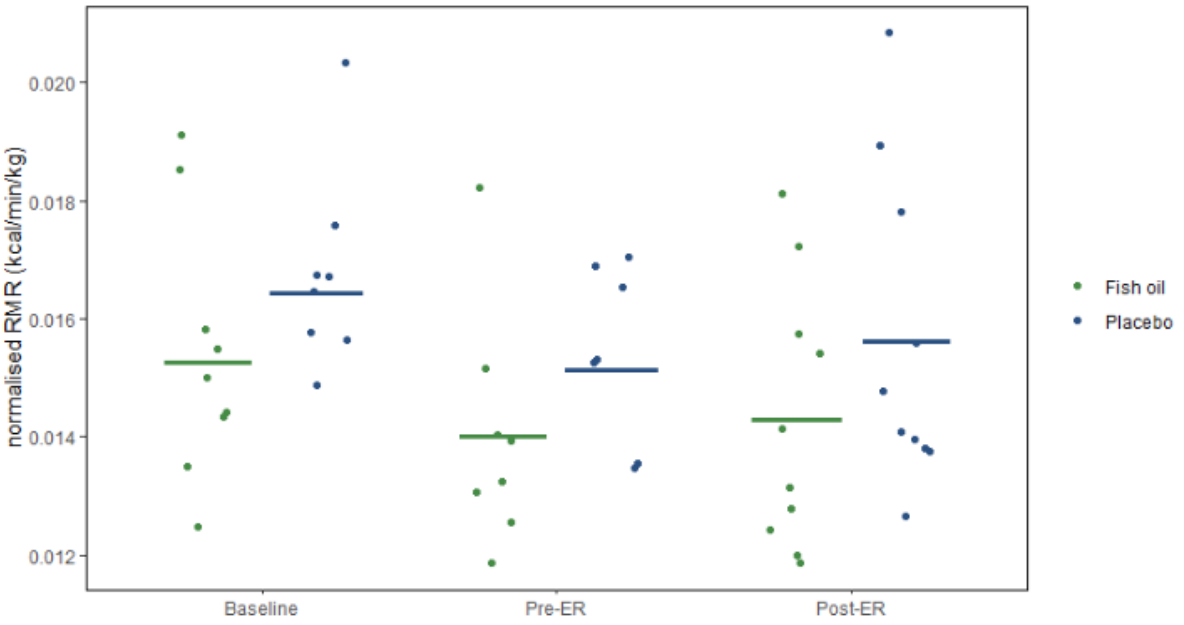

Figure 2.3 – Resting metabolic rate (RMR) normalised to total body mass, at baseline (n = 17), pre-ER (n = 15) and post-ER (n = 20).

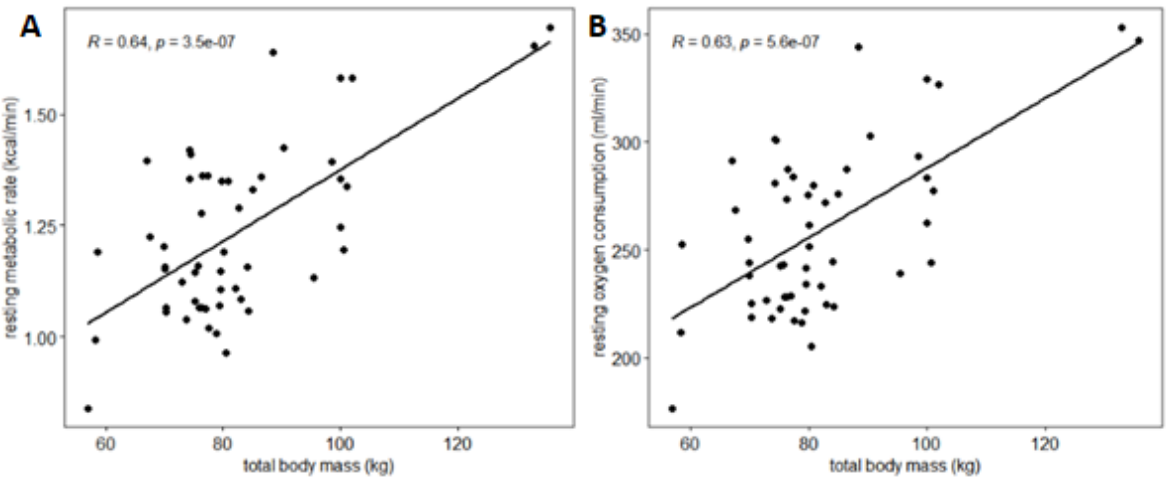

Figure 2.4 – Pearson's correlation between (**A**) resting metabolic rate and total body mass; (**B**) resting oxygen consumption and total body mass. Data from all time points (baseline, pre-ER and post-ER).

Respiratory exchange ratio

Resting respiratory exchange ratio (RER) values are displayed in figure 2.5. There was a trend for a time by group interaction effect (F = 3.13, p = 0.053) for the change in RER, with a main effect of time (F = 5.71, p = 0.006), but no main effect of group (F = 0.40, p = 0.53). At baseline, RER was significantly (p = 0.042) higher in FO compared to PLA. During EB, RER did not change in FO (0.00 ± 0.09; p = 0.87) or PLA (0.04 ± 0.04; p = 0.62). During ER, RER decreased significantly (p = 0.012) in FO (-0.07 ± 0.05) but not (p = 0.49) in PLA (0.01 ± 0.07).

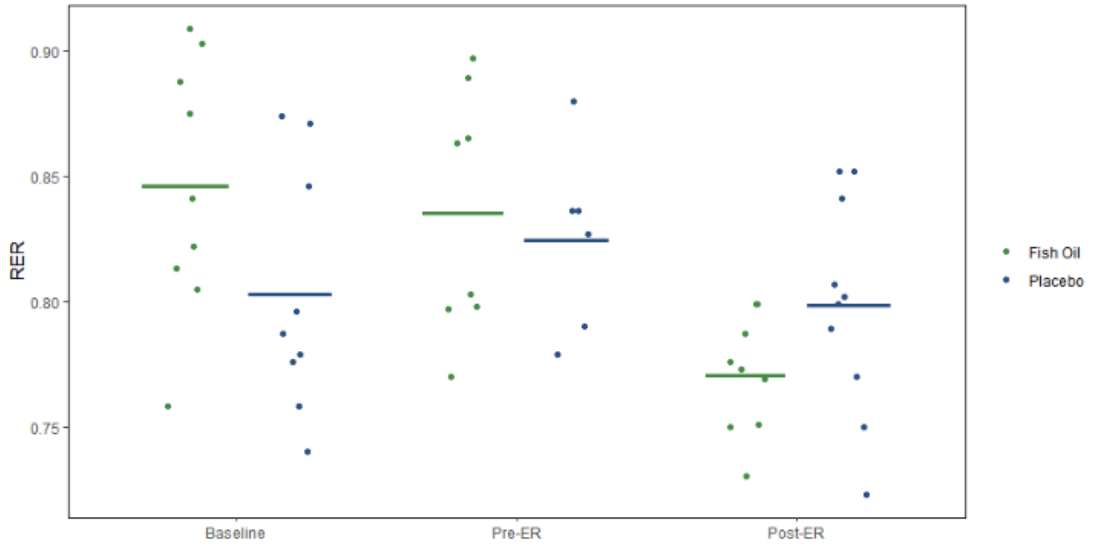

Figure 2.5 – Respiratory exchange ratio (RER), calculated as VCO_2/VO_2, at baseline (n = 18), pre-ER (n = 14) and post-ER (n = 19).

2.3.5 – Counter-movement jumps

CMJ maximum height and force are presented in figure 2.6. There was no significant effect of time (F = 1.03; p = 0.38) or group (F = 0.02; p = 0.88) on CMJ height. As such, CMJ height did not change during EB or ER. There also was no significant effect of time (F = 1.83, p = 0.19) or group (F = 0.0013, p = 0.97) on CMJ force. As such, CMJ force also did not change during EB or ER.

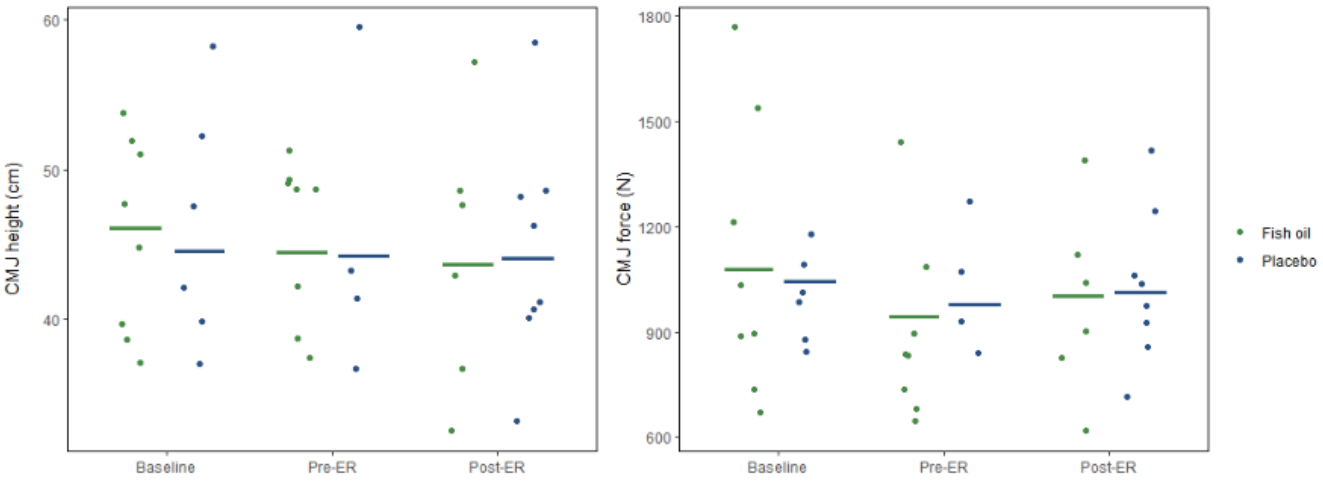

Figure 2.6 – Counter movement jump (CMJ) maximum height and force recorded from 3 attempts at baseline (n = 14), pre-ER (n = 12) and post-ER (n = 14). Dots represent individual values, while bars represent model estimated means.

2.4 - Discussion

The primary finding of this study was that 4g/d n-3 PUFA supplementation for 6 weeks did not preserve lean mass during 40% energy restriction in healthy, young, active males. This was the first study to investigate the influence of n-3 PUFA supplementation on changes in lean mass during energy restriction in normal weight young adults. Participants successfully lost the intended ~3kg of total body mass, of which approximately half was lean mass, over a 2-week period. Baseline n-3 PUFA status of participants in our study was low, and supplementation with 800-1000 mg/d EPA and 1000-1200 mg/d DHA for 6 weeks successfully increased the relative concentrations of EPA by 275%, DHA by 175% and total n-3 PUFA by 168%. However, we did not observe an effect of increased n-3 PUFA status on changes in body composition, be it lean mass, fat mass or total body mass. Additionally, we also did not observe an effect of n-3 PUFA on resting metabolic rate or respiratory exchange ratio. Furthermore, n-3 PUFA did not influence physical function, as we did not detect a change in either group despite significant loss of lean mass. Therefore, fish oil supplementation at 4g/d day for 4 weeks prior to, and during a 2-week energy restriction period, was not beneficial for optimisation of body composition, energy metabolism and muscle function in healthy active young males.

Despite a relatively severe period of energy restriction, we did not observe an effect of dietary n-3 PUFA on changes in total, lean or fat mass. As dietary n-3 PUFA have been suggested to positively influence muscle anabolism and catabolism, and to reduce muscle atrophy in situations of muscle disuse, we hypothesised this effect may also occur in the catabolic situation of energy restriction [43]–[45], [105], [106]. The weight loss model employed was designed to rapidly induce ~3kg weight loss through dietary ER and maintenance of EE [147]. The reduction in total body mass, and the proportion of lean and fat mass loss equalled that of the control group in the study by Mettler, et al. (2010), suggesting compliance with the weight loss diets was successful in our study.

Supplementation with fish oil, at a dose of 5g/d (of which 4.4g n-3 PUFA) for 4+ weeks, was previously shown to significantly increase incorporation of EPA and DHA into muscle tissue [15]. In our study, whole blood EPA, DHA and total n-3 PUFA increased significantly from week 0 to week 6. As whole blood lipid profiles correlate well with muscle lipid profiles in this population, it can reasonably be assumed that n-3 PUFA successfully incorporated into muscle in our study [15]. Nevertheless, the loss of lean mass was not attenuated by the supplementation.

Our observation, that n-3 PUFA does not influence lean mass loss during energy restriction in normal weight young adults, is the first in this context. However, our findings are in accordance with studies that have measured lean mass changes during ER in overweight/obese populations, which did not find a convincing influence of n-3 PUFA [128], [154]–[157]. We hypothesised n-3 PUFA would be able to preserve lean mass during ER in healthy males due to the higher proportion of lean mass loss in normal compared to overweight individuals, and the differences in anabolic control of muscle [146], [149], [190]. As no such effect was observed, it appears n-3 PUFA do not exert a protective effect on muscle mass during ER irrespective of weight status. Previous studies also show high protein diets and resistance exercise may successfully attenuate loss of lean mass during energy restriction [151], [165]. In our study, participants were instructed to maintain their exercise habits through ER but were provided protein at 15% of total calories. Therefore, the aim was to investigate whether n-3 PUFA may attenuate muscle loss during relatively severe ER, but with otherwise ‘standard’ conditions. However, despite ample anabolic stimulation, the suggested anabolic properties of n-3 PUFA did not result in a preservation of lean mass. Thus, at this time n-3 PUFA supplementation cannot be recommended for maintenance of lean mass during ER in healthy young adults.

Surprisingly, PLA lost a significant amount of total body mass during EB, while FO did not, although

the difference between groups did not reach significance. It is unclear why total body mass decreased in PLA during this time, as participants were instructed to maintain their habitual diet and exercise. The reduction in total mass in PLA appeared primarily driven by a (non-significant) reduction in lean mass during this time. Previous studies, that examined the effects of n-3 PUFA on body composition in healthy adults continuing with their habitual diet and exercise during EB, have also observed minor, although ambiguous changes [143], [152]. In healthy young men and women, supplemented with 4g/d fish oil for 6 weeks, lean mass increased significantly, while fat mass decreased significantly, with no changes in the control group [143]. In healthy young men, maintaining their habitual lifestyle for 12 weeks, lean mass was maintained with 3g/d n-3 PUFA plus 3g/d conjugated linoleic acid (an n-6 PUFA), but decreased slightly in PLA, although this difference was not significant [152]. Thus, preliminary evidence suggests that n-3 PUFA may promote a maintenance of, or an increase in lean mass under situations of EB which include (uncontrolled) exercise training. The increase in fat-free mass in the study of Noreen, et al (2010) is particularly striking. As FO supplementation in our study was of equal dose and similar duration (4 vs 6 weeks at EB), it is unclear why they observed an increase in lean mass while lean mass was unaffected by FO in our study. The discrepancy may (in part) be explained by the inclusion of females in their study. A study of changes in lean mass following strength training, which included both older men and women, showed greater increases in muscle mass with dietary n-3 PUFA [46]. However, the change in lean mass was not reported for women and men separately in the case of Noreen et al (2010), so it is unclear if the increase in lean mass was driven by females. Another study in older adults undergoing strength training observed improvements in muscle function and quality with older females but not males supplemented with FO [47]. As such, the authors suggested the effects of dietary n-3 PUFA on changes in muscle mass and function may be more pronounced in women compared to men. In young healthy women undergoing leg immobilisation for 10d, supplementation with n-3 PUFA attenuated the loss of lean mass [106]. The differences in

responses to dietary fish oil between females and males have been suggested to be related to differential influences of sex hormones on PUFA metabolism [2]. Thus, repetition of this study with a female cohort may yield different results.

Furthermore, it is unclear what influence age has on the potential for dietary n-3 PUFA to exert beneficial effects on lean mass. In the study of Noreen, et al. (2010), participants were aged 18-55, and therefore the potential effects of dietary n-3 PUFA on body composition may not have been the same in the older participants. Aging is believed to impair MPS through a concept known as 'anabolic resistance', which describes a reduced ability for our protein synthetic machinery to respond to anabolic stimulation [39], [191]. As n-3 PUFA are suggested to amplify MPS responses to anabolic stimulation, such an effect may be especially useful in older adults experiencing such anabolic resistance. Furthermore, exercise training was not controlled, as participants were merely instructed to maintain habitual activity levels, including exercise training intensity and duration. As such, heterogeneity in the type and intensity of training may have resulted in different interactions with dietary n-3 PUFA and body composition. Future studies may wish to investigate whether n-3 PUFA can maintain and/or increase lean mass during EB or ER in normal weight adults in a more controlled situation. Although, given the evidence so far, such an effect would likely be trivial.

Besides the lack of an effect of n-3 PUFA on lean mass retention in our study, the lack of a modulatory effect on total and fat mass loss adds to the already conflicting literature on this topic. These indicate that n-3 PUFA may have (minor) effects on changes in total and fat mass, although the overall evidence is ambiguous [128]–[130]. In terms of total body mass changes, one meta-analysis demonstrated that dietary n-3 PUFA increases total body mass loss during ER in overweight/obese individuals, with no effect on fat mass or lean mass [128]. However, since then, two other meta-analyses suggest there is insufficient evidence to support an effect of dietary n-3

PUFA on total body weight or fat mass in overweight/obese individuals [129], [130]. Yet, studies in overweight/obese populations do demonstrate an effect of n-3 PUFA on specific aspects of adiposity, such as waist-to-hip ratio and waist circumference. Using DEXA and skinfolds, we did not observe an effect of FO on regional changes in body composition of abdominal (skinfolds; individual site data not shown) or android, gynoid or trunk (DEXA) regions. The contrast in the lack of an effect of n-3 PUFA on fat mass in these regions may have been due to a relatively low level of adiposity in our cohort of young healthy active males. Heymsfield, et al., (2011) suggest, although based on limited evidence, that individuals with higher baseline body fat may be more likely to lose more fat mass during diet-induced ER. Fat mass loss approximated lean mass loss in our study, which is consistent with other studies of weight loss in similar populations and of similar magnitude [147], [151].

The ambiguity in results from studies on the effects of n-3 PUFA on body composition, including our own, suggests any effect of n-3 PUFA, if there is one, is likely small. Although we did not collect coefficient of variation (CV) data for the reliability of DEXA measurements, others suggest CVs ranging from 0.5-2.5% for lean mass and 0.8-5.0% for fat mass [192]. As our observed changes in lean and fat mass fall within the range of expected variation with repeated measurements, it is unclear if they represent 'real' change. Thus, although minor changes in body composition with n-3 PUFA supplementation may be clinically relevant, our ability to observe them may have been limited. The small (but mostly non-significant) differences between groups in body composition changes we observed may also have been due to randomness in factors other than the supplementation. That is, the study was advertised as a weight loss study, and as such participants may have changed their habits as they became more aware of their diet and energy expenditure following completion of food and energy diaries during the initial assessment period. Additionally, participants were recreationally active and/or involved in team sports, and as such may already

have been striving to improve their body composition. Therefore, slight variations in fat and lean mass were to be expected over a 6-week duration and may not necessarily have reflected an effect of n-3 PUFA.

One of the mechanisms through which dietary n-3 PUFA has been suggested to influence whole-body composition is a change in energy metabolism [1]. However, we did not observe an influence of dietary n-3 PUFA on changes in RMR, either during EB or ER. The observed maintenance of relative RMR (i.e. energy expenditure/kg body weight) during ER is consistent with other studies [193]. Although short-term ER typically reduces absolute RMR, this seems to be mainly driven by the reduction in body mass. Further, the addition of exercise to dietary ER is known to preserve metabolic rate [194]. To our knowledge, this was the first study to investigate the influence of dietary n-3 PUFA on changes in RMR during ER in normal weight adults. However, one previous study that investigated the change in RMR in overweight/obese males and females during ER also observed no effect of dietary n-3 PUFA [195]. Thus, it appears n-3 PUFA also do not influence RMR during ER irrespective of weight status.

However, the trend for a decrease in relative RMR during EB was surprising, because energy metabolism would not normally be expected to change over a duration of 3 weeks and without changes in lifestyle. It is possible the higher observed RMR at week 1 compared to week 4 were due to measurement error. Participants were not familiarised with the indirect calorimetry protocol before the first recording, and as such may not have been fully at ease during the first visit, resulting in altered breathing and restlessness, and therefore inflated RMR rates. In addition, participants may not have spent enough time at rest before recording took place. To measure RMR accurately, it has been suggested a resting period of 10-20 minutes is required before recording is initiated [196]. RMR measurement took place on a bed, in supine position, and participants rested

for 5 minutes before recording started. Therefore, they may not have been truly in a resting state during the recording. In addition, the testing took place in the early morning and sleep during the night prior was not controlled in this study, which may have influenced our ability to accurately measure RMR [196]. Furthermore, while participants were instructed not to perform strenuous exercise in the 24h before trials, compliance with this instruction was only checked verbally. Therefore, variations in exercise and sleep in the 24h before measurement may have caused additional disturbances .

In terms of changes in metabolic rate during EB with dietary n-3 PUFA, previous studies have observed conflicting results. In young males, like in our study, one previous study observed a significant (but minor) increase in RMR with 3g/d EPA plus DHA for 12 weeks [172]. Another study in young males, supplementing for the same dose and duration did not observe a change in RMR [171]. Another study, including both males and females, also did not observe an effect of 4g/d fish oil for 6 weeks on RMR [143]. Participants in these studies all continued their habitual lifestyle, like in our study. Potentially, the lack of a change in RMR with n-3 PUFA in our study was due to the relatively short length of supplementation. However, had we supplemented participants for longer, the ambiguity of previous studies with longer supplementation periods suggests an effect on RMR would still likely have been minor. Interestingly, it has been suggested the influence of dietary n-3 PUFA on metabolic rate may be different in females compared to males. Additionally, this may be confounded by age as opposing directions of change in RMR have been observed with dietary n-3 PUFA in young (decrease) and older (increase) females [144], [145]. In the current study, RMR appeared lower (although not significantly) in FO compared to PLA. Unfortunately, as the first measurement (“baseline”) took place 7 days following the start of supplementation, it is unclear if the observed lower metabolic rate was due to changes in n-3 PUFA status. As blood and muscle n-3 PUFA levels are known to increase rapidly [15], a true baseline with low n-3 PUFA status was

lacking. Additionally, as mentioned earlier, participants were not familiarised with the measurement and as such the decrease in RMR may simply reflect an improved resting state during later visits. Given previous studies have found no convincing evidence of changes in RMR with n-3 PUFA supplementation, it is unlikely they are able to affect energy metabolism in a meaningful way. Nevertheless, due to the limitations mentioned, we are unable to corroborate these previous reports and it remains unclear if n-3 PUFA affect RMR during ER in normal weight adults.

The suggested potential for dietary n-3 PUFA to alter metabolic rate stems from their observed effects on several underlying processes. Of overall oxygen consumption, approximately 65% is used for mitochondrial ATP production to fuel cellular activities [1], [197], [198]. In muscle cell and rodent models, changes in n-3 PUFA mitochondrial membrane content are associated with changes in mitochondrial respiration kinetics and expression of related genes [199]–[201]. However, in older men and women, no changes in mitochondrial respiration were observed following FO supplementation for 16 weeks [48]. On the other hand, n-3 PUFA did reduce mitochondrial reactive oxygen species (ROS) production in these older adults [48]. ROS react with other cellular components and influence cell signalling pathways, and excessive ROS production may cause damage to DNA and proteins [71]. Therefore, although no direct changes on energy production were observed, dietary n-3 PUFA may preserve energy by preventing the need for repairing ROS-induced damage. Further, about 20-25% of energy demand within muscle is believed to be required for protein turnover. As such, changes in protein synthesis and breakdown, and suggested improvements in the efficiency of protein synthetic machinery with dietary n-3 PUFA may lead to alterations in energy requirements within cells. Another large proportion of RMR is associated with maintenance of ion gradients across plasma membranes [198]. As PUFA are important components of these membranes (as phospholipids and in lipid rafts), increased presence of n-3 PUFA may

influence signalling within, and activity across the membrane [198]. That is, increased presence of n-3 PUFA in membranes of various tissues has been suggested to improve efficiency of cell signalling and processes, which may further aid in preservation of energy [112], [202]. Finally, metabolic rate also includes energy expended for calcium cycling within cells. In a study of trained male cyclists, oxygen consumption during submaximal exercise was reduced with dietary n-3 PUFA, which the authors suggested may have been due to improvements in calcium cycling in muscle [109], [111]. Nevertheless, although dietary n-3 PUFA appear to influence various molecular aspects of energy metabolism in cell and animal models, these were not reflected in whole-body measurements of RMR in our study.

N-3 PUFA supplementation also did not affect substrate metabolism (as RER) during ER in our cohort of healthy active males. To our knowledge, this was the first study to investigate changes in RER with n-3 PUFA during ER. During ER, fat oxidation increases [203], [204], which is consistent with our observation that RER seemed to decrease in both groups. The decrease did not seem as large in PLA as in FO, but there was no statistically significant difference between groups. Evidence from rodent models indicates n-3 PUFA may increase mitochondrial fat oxidation [167], [205] and lipogenic gene expression [168]. Further, dietary n-3 PUFA have been observed to lower the appearance of triglycerides in blood in humans, which is consistent with increased uptake and oxidation of fat [7], [206]. The lack of an effect of dietary n-3 PUFA on RER during EB is also consistent with studies in young males and females [143], [171], [172]. Our data add to this by showing n-3 PUFA do not influence RER during ER either. However, several limitations of the current observations should be considered. Although participants were instructed to fast from 10pm the night before testing, fasting status of participants was not confirmed on the morning of measurement. As such, participants may not all have been fasted, resulting in variations in substrate metabolism. Conforming with this are observations of RER exceeding 0.85 in several

individuals, possibly indicating non-fasting rates of carbohydrate oxidation in these individuals. Like RMR, RER also may have been affected by the resting time prior to recording, as it appears this may not have been enough to achieve a resting steady state. In addition, as the variation in RER appears to be considerably smaller during the final (post-ER) measurement, it is possible a 'learning effect' occurred in regard to the ability for participants to be at rest and lie motionless. Furthermore, measurements took place year-round, which may have added inter-individual variability. Seasonal differences in substrate utilisation have been observed, with increased fat oxidation and decreased carbohydrate oxidation during winter [171]. Thus, limitations in our measurements mean we are unable to form a conclusion on whether n-3 PUFA affect energy metabolism during ER in healthy males.

Despite a significant reduction in lean mass (including legs), we did not observe a decline in countermovement jump height and force. Maintenance of muscle function during energy restriction is crucial for performance in athletes and soldiers, and aspects of daily living in the general population. During ER, changes in specific aspects of muscle function (i.e. fatigue and patterns of muscle contraction) may precede changes in body composition [90], [207]. However, the lack of a decrement in muscle function in the current study is consistent with most previous studies of muscle function during ER combined with exercise training, with some even observing improvements [149]. As such, it appears that the habitual exercise during ER, despite losses of lean mass, was able to preserve muscle function in the current study. This was the first study to investigate the influence of dietary fish oil on changes in muscle function during ER. We used CMJ height and force as measures of muscle function, as CMJ force is correlated with lower limb muscle strength in healthy active men [208], [209]. A previous study in male rugby players during pre-season claimed there was a 'likely beneficial' effect of FO plus protein supplementation on CMJ peak force [121]. Although, it should be noted this was based on magnitude-based inferential

statistics, whose use has been heavily criticised [210], [211]. Regardless, CMJ height and force may not have been the most optimal test of changes in muscle function following ER, as participants only completed three jumps for the determination of maximal height and force. As such, no fatiguing would have taken place. Dietary n-3 PUFA have previously been suggested to specifically reduce muscle fatigue during exercise through changes in oxygen consumption, heart rate and neuromuscular function [111], [120], [212]. On the other hand, studies in older adults with FO supplementation have noted improvements in peak torque, rate of torque development, chair rising performance [45], handgrip strength, 1RM strength [46] and maximal isometric torque [47]. Thus, there are indications that n-3 PUFA supplementation may improve performance in isolated muscle function tests. Like changes in energy metabolism, the changes in muscle function appear to be more pronounced in females compared to males [47]. In addition to functional changes in muscle (oxygen consumption, neuromuscular, strength, fatigue), dietary n-3 PUFA also have been suggested to improve recovery from exercise. This effect is believed to be driven by reduced inflammation and muscle damage following exercise, although evidence in this field is limited [54], [121]. As such, it is hypothesised that dietary n-3 PUFA may benefit exercise performance during periods of high frequency exercise training. However, in the current study, participants were instructed not to undertake strenuous exercise in the two days leading up to testing sessions, and therefore their muscle would not be in a damaged state. Our ability to detect changes in muscle function during ER using CMJ performance was further limited by several factors. Participants received instructions on how to perform the test and were allowed 3 practice jumps before recording at each session. However, not all participants may have been fully familiarised with the test before measurement, and there was a large range of athletic ability. While some participants were athletes partaking in structured team sport, others were recreationally active gym-goers. Therefore, our ability to detect an effect of n-3 PUFA on the change in muscle function following ER may have been impaired by inter-individual variability in the ability to perform CMJ. In addition,

although participants were instructed to maintain habitual exercise intensity and duration throughout the ER period, we were unable to monitor training activity in most participants. Therefore, there may have been considerable intra- and inter-individual variability in exercise training intensity and frequency during ER. In addition, the 14-day duration of ER may not have been sufficient to impair muscle function to a detectable degree [149], [203]. As we observed no detriments in muscle function with ER of a high severity that individuals are unlikely to undergo voluntarily, the need for nutritional strategies to preserve muscle function during ER can perhaps be called into question. Based on our observation, it would seem the maintenance of habitual exercise training during ER may be enough to prevent a loss of muscle function, despite significant loss of lean mass. However, future studies may wish to investigate the influence of dietary n-3 PUFA on muscle function during ER using more controlled exercise tests and in a more homogenous sample. Further, it remains unclear whether dietary n-3 PUFA may preserve muscle function during ER when exercise levels are not maintained.

Some considerations should be made around factors that may have affected our ability to detect an effect of n-3 PUFA on physiological changes during ER. In terms of study design, we utilised a previous model that achieved rapid weight loss over a 2-week duration, based on a study by Mettler et al. (2010). Athletes preparing for competition in weight-restricted categories may use rapid weight loss strategies, potentially even more severe than ours [213]. However, it is unlikely individuals in the general population would voluntarily undertake a weight loss programme as severe as in our study. Given high protein diets have previously been shown to attenuate loss of lean mass during ER, any voluntary weight loss programme would be recommended to include a higher relative contribution of protein to the diet than was the case in our study. Interestingly, we noticed some participants reported very high amounts of habitual protein consumption on their food diaries (>200g/d). As the relative contribution of protein to the diet was set at 15% across the

last 3 weeks of the study, this would have represented a large shift in the macronutrient composition of their diet. It is not clear what the short-term effects are of such a shift in the macronutrient composition of the diet. Further, although participants were provided with their entire diet for the last 3 weeks of the study, the pattern of dietary intake was not controlled. As such, given limited protein was provided, and the pattern of daily protein intake influences total rates of MPS [214], [215], differences in the dietary habits of participants may have limited their ability to maintain muscle mass during ER.

Further, in terms of the design of the 2-week 40% ER period, the overall rate of weight loss and the ratio of fat to lean mass loss appeared to be highly variable. Due to the nature of the weight loss model employed, participants likely spent the majority of ER in 'phase I' weight loss [146]. That is, a period of rapid weight loss characterised by a decrease in both fat- and lean mass, which includes glycogen and protein. As glycogen and protein associate strongly with water (~1-2g water per g of glycogen; ~1.5-3g water per g of protein), much of the weight lost during this initial phase of weight loss comprises body water [146]. We attempted to standardise the hydration of participants by instructing participants to consume 500mL water before coming to the laboratory in the morning of testing, and then emptying their bladder before DEXA measurement [176]. Further, glycogen is mostly stored in muscle and liver, and therefore is included in measurements of lean mass (e.g. via DEXA). Considerable variations in water and glycogen may have occurred, which limit the accurate detection of changes in lean mass. In addition, we cannot rule out that measurement errors in the DEXA scans resulted in inconsistent observations across time points. There were several instances where participants were too tall or broad to fit on the DEXA scan field, resulting in parts of their feet and/or arms to be excluded from measurement. Although the DEXA software allows estimation of missing body parts based on values from the opposite side, this was not possible for the feet. When participants do not fully fit on the scan field, it is

recommended separate scans are made for top and bottom or left and right half, even if this poses additional challenges [192]. Therefore, our ability to observe changes in lean and fat mass were likely compromised by measurement error in the DEXA scans. In addition, recordings of body mass were only made at the start and end of ER, and therefore we were unable to ascertain the trajectories of lean and fat mass change throughout these 2 weeks.

In terms of participant assignment to supplemental groups, this was done on a true random basis. Therefore, the baseline characteristics of the placebo and fish oil supplement groups were not matched. The possibility that the discrepancy in baseline weight influenced weight loss during the ER period cannot be ruled out. Furthermore, despite controlling energy intake of our participants, inter-individual differences in weight loss were high. While participants were instructed to maintain their habitual EE levels throughout the 6-week study, we were unable to reliably confirm if they were successful. Due to logistical issues, we only managed to collect complete energy expenditure data for a limited subset of participants (n = 8). While data from these individuals does show they maintained their AEE and TEE from EB to ER, it is unclear if this was the case for others who were not monitored. In addition, while participants were provided with their entire diet for weeks 4-6, compliance was not monitored other than through verbal confirmation. Therefore, we cannot confirm if participants were all in a 40% energy deficit throughout the ER period.
In terms of the timing of measurements, our study was limited by the lack of a 'true baseline'. That is, measurements were not made until day 7 of supplementation ("baseline"). Thus, limited conclusions can be drawn about analyses of the effects of dietary n-3 PUFA on body composition, metabolism and muscle function, during EB. Additionally, physiological measurements were only made immediately pre (end of week 4) and post (end of week 6) the 14d ER period. As such, it is possible certain short-term effects associated with ER were missed and we are thus unable to report about any intermediate changes. Finally, while measurements of energy metabolism and

muscle function were made in laboratory settings, we were limited by the short time window that these measurements capture. Several studies have recently employed measurement techniques that allow a more dynamic capture of physiological function in free living conditions, such as using doubly labelled water to measure energy expenditure. As such, future studies may wish to employ these types of measurements to capture a more accurate representation of the potential effects of n-3 PUFA on energy metabolism and muscle function during ER in free-living conditions.

In conclusion, supplementation with 4g/d fish oil does not influence changes in body composition during severe short-term energy restriction in normal weight healthy active males. Participants lost an average of 3.2 kg total body mass, with approximately equal rates of fat and lean mass regardless of supplemental group. The weight loss period was relatively severe, with exercise habits maintained and energy intake restricted by 40% during the 2-week period. Nevertheless, dietary n-3 PUFA did not ameliorate the loss of lean mass despite previous suggestions that they may increase muscle anabolism and reduce muscle catabolism. Dietary n-3 PUFA also did not appear to influence changes in energy metabolism, substrate utilisation and muscle function during energy restriction. Therefore, at this time FO supplementation cannot be recommended for the maintenance of lean mass in healthy normal weight adults undergoing energy restriction. However, as the current study contained several limitations, further research is required to corroborate these findings.

Chapter 3 – randomised controlled trial of n-3 PUFA supplementation shows no influence on muscle anabolic signalling and expression of muscle developmental genes during energy restriction

3.1 - Introduction

In this chapter we set out to expand on previous research into the molecular effects of n-3 PUFA on muscle anabolism and catabolism at the proteome and transcriptome levels. At a molecular level, n-3 PUFA or their oxidised metabolites, eicosanoids, interact with G-protein coupled receptors, nuclear receptors and transcription factors [27], [216]. Additionally, n-3 PUFA are incorporated into phospholipid membranes, which alters membrane structure, function and lipid-protein interactions [1], [198]. N-3 PUFA have previously been observed to influence the regulation of muscle anabolism and catabolism, which may result in reductions in muscle atrophy [43], [44]. During energy restriction (ER), reductions in the availability of energy and amino acids (AA), result in changes in the activity of the AMPK/mTOR/p70s6k pathway which regulates muscle protein turnover [96]. ER therefore typically leads to undesirable losses in skeletal muscle, which may impair metabolic and physical function [90]. Short-term ER has been observed to reduce resting MPS by ~20-30% [164], [165], [217]. Additionally, MPB has been observed to increase by ~60% during short-term ER, which frees up amino acids and energy for novel protein synthesis [166]. Despite this, some have argued the dominant mechanism for the loss of lean mass during short-term ER and muscle disuse is a reduction in MPS, as these authors observed limited contribution of MPB [217], [218]. However, this is still a contested hypothesis as others did observe significant contributions of MPB [219]. In the context of muscle disuse, n-3 PUFA has been shown to reduce muscle atrophy, through improvements in myofibrillar MPS, alongside suppression of catabolic gene expression [106]. In cultured muscle cells, and rodents subjected to catabolic situations, n-3 PUFA have been shown to benefit both muscle anabolic and catabolic processes [91]–[94], [105].

Similarly, n-3 PUFA have been shown to improve muscle mass and function in older adults [45]–[47]. Aging muscle is thought to experience 'anabolic resistance', thereby limiting the activity of anabolic signalling in response to stimulation [40]. Although n-3 PUFA have already been shown to improve aspects of muscle anabolism and catabolism in different contexts, their potential in this regard, and in the catabolic situation of ER, has not been studied in humans.

The mechanisms responsible for n-3 PUFA-mediated improvements in muscle anabolism have been suggested to involve potentiation of insulin-related pathways. As such, several studies have found improvements in the activity of proteins along the Akt-mTOR-p70s6k signalling axis, that is stimulated by insulin through the growth factor pathway, IRS1-PI3K [49], [87], [103], [104], [220]. In young and middle-aged, and older adults, n-3 PUFA supplementation was demonstrated to potentiate the mixed muscle MPS response to infusion of AA and insulin [87], [220]. However, n-3 PUFA did not affect MPS in the postabsorptive condition. To determine the effect of n-3 PUFA on a fraction of MPS suggested to be specific to muscle hypertrophy, McGlory et al. (2016) measured myofibrillar MPS at rest and following unilateral resistance exercise (REx) and 30g protein feeding [49]. As such, 5g/d n-3 PUFA supplementation for 8 weeks increased MPS to a greater degree than placebo, following protein feeding, and also following protein feeding plus REx, although these increases did not reach statistical significance. However, alongside minor (non-significant) increases in MPS, they also observed suppression of p70s6k activity 3 hours post feeding and REx, and of Akt activity post REx. In the resting condition, n-3 PUFA supplementation did not alter postabsorptive MPS, although Akt activity also was suppressed. Therefore, it was suggested that the suppression of anabolic signalling protein activity, combined with no reduction in MPS, may reflect an improved efficiency of the protein synthetic machinery with n-3 PUFA [49]. The ability for protein synthetic machinery to maintain activity despite reductions in anabolic stimulation would seem very useful for the maintenance of muscle mass during catabolic situations. However,

it should be noted that in the study by McGlory et al. (2016), the provision of 30g protein may have maximally stimulated the muscle protein synthetic machinery, due to a concept known as the 'muscle full' effect [221]. Therefore, the effects of n-3 PUFA on MPS in this study were not fully clear. It may suggest that stimulation of muscle anabolism by n-3 PUFA may be especially potent in situations where anabolic stimulation is sub-optimal, such as during ER with reduced amino acid availability. Furthermore, although most studies have not observed changes in postabsorptive MPS with n-3 PUFA supplementation, Lalia et al. (2017) did report improvements in specific fractions of postabsorptive MPS following 3.9g/d n-3 PUFA supplementation for 16 weeks [48]. In older adults, n-3 PUFA significantly increased rates of mitochondrial and sarcoplasmic MPS, but did not increase mixed muscle and myofibrillar MPS. However, in young people n-3 PUFA had no effect on postabsorptive MPS. In the context of ER, the ability for n-3 PUFA to influence postabsorptive MPS has not been studied.

In addition to observed increases in muscle anabolism with n-3 PUFA, they have also been demonstrated to attenuate muscle catabolism in various contexts. In mice undergoing 24h starvation, EPA treatment reduced ubiquitin-proteasome pathway (UPP)-mediated proteolysis [92]. In cachectic mice, 1g/kg/d EPA for 4 days also was demonstrated to reduce MPB [93]. In addition, in cultured mouse skeletal muscle cells, 50 μM EPA for 24h also reduced MPB [91]. Furthermore, n-3 PUFA has been demonstrated to suppress expression of catabolic genes during muscle disuse, and cancer cachexia, in rodents [94], [105]. Specifically, n-3 PUFA suppresses the increase in expression of MAFbx and MuRF1 typically seen during catabolic situations. This effect was recently also shown in young women supplemented with 5g/d n-3 PUFA before and during 2-week leg immobilisation [106]. As these genes code for key components of the UPP, responsible for targeted degradation of proteins, their downregulation by n-3 PUFA may be a pivotal target in the prevention of muscle atrophy [222], [223].

Besides the UPP, autophagy also plays a vital role in the turnover of muscle protein [101], [224]. Autophagy is regulated by mTOR through phosphorylation of ULK1. As such, autophagy is induced when mTOR is inactive, which may serve as a mechanism to free up energy, and materials for protein synthesis [101], [225]. In cancer cell lines, n-3 PUFA induces autophagy through the mTOR pathway, potentially via reactive oxygen species (ROS)-mediated mechanisms [226], [227]. However, to our knowledge, the effects of n-3 PUFA on autophagy in muscle from healthy adults has not been studied. Given ER limits mTOR activation, autophagy may be an important contributor to muscle health during conditions of energy stress. Finally, gene pathway analysis in older adults supplemented with 4g/d n-3 PUFA for 6 months demonstrated significant reductions in expression of proteolysis and inhibition of mTOR signalling-related genes [228]. However, they did not detect differences in the expression of individual genes related to muscle development. Therefore, although there are indications that n-3 PUFA may positively influence expression of muscle developmental genes, further study is required.

Cellular regulation of muscle protein turnover is also regulated by cytokines and hormones, whose production and function may be influenced by n-3 PUFA status [152], [229]–[231]. Although AA are known to stimulate mTOR-p70S6k directly, studies which have shown increased muscle anabolism with n-3 PUFA utilised infusion of both AA and insulin [87], [103], [220]. On the other hand, McGlory et al. (2016) only provided AA (in the form of a protein drink) and did not detect a significant potentiating effect of n-3 PUFA on muscle anabolism [49]. As such, there may be a role of insulin in the potential anabolism-enhancing effects of n-3 PUFA. It has also been suggested that n-3 PUFA supplementation increases muscle insulin sensitivity, due to increased fatty acid oxidation [103], [231], [232]. In rodents, n-3 PUFA-rich diets improved insulin sensitivity during ER, which was associated with improved body composition [233]. Therefore, factors regulating insulin action may contribute to the altered regulation of muscle protein turnover by n-3 PUFA. Leptin, an

'adipokine' produced primarily by adipocytes, is one such factor, which is suggested to modulate the actions of insulin [231], [234]. Dietary energy restriction reduces leptin concentrations, primarily due to loss of fat mass, although independent changes may also occur [235]. As high leptin concentrations are associated with insulin resistance and inflammation, downregulation of leptin by ER or n-3 PUFA may be protective in terms of metabolic health [137]. In rodent skeletal muscle, leptin stimulates lipolysis, FA oxidation and inhibits FA storage, uptake and transport [236]–[238]. Therefore, the effects of n-3 PUFA on muscle protein metabolism may be in part due to changes in production and secretion of adipokines from adipose tissue, a relationship which has yet to be investigated in humans.

Although there are indications from various catabolic contexts that n-3 PUFA may improve muscle anabolism, no study to date has investigated the influence of n-3 PUFA on muscle anabolic signalling during ER in humans. Therefore, the primary aim of this study was to investigate the influence of 4g/d n-3 PUFA for 6 weeks on skeletal muscle anabolic signalling during a 2-week 40% ER in healthy active males. We hypothesised that n-3 PUFA would be able to potentiate the anabolic signalling response to REx and protein, thereby preserving muscle anabolism during energy restriction. Furthermore, we aimed to investigate the influence of n-3 PUFA on the expression of genes related to muscle anabolism, catabolism and development, during ER. Finally, we also aimed to investigate the influence of n-3 PUFA on leptin levels in ER, to probe the potential link between whole-body hormonal regulation and muscle protein metabolism.

3.2 - Methods

3.2.1 - Participants

Muscle biopsy and blood samples from a subset of 16 participants from the weight loss study (reported in Chapter 2) were analysed in this study. From the initial 20 participants who had

volunteered to take part, two experienced a vasovagal reaction to cannulation and withdrew their consent to further sampling. Additionally, analysis of biopsy samples from 4 participants was not possible for technical reasons. Therefore, blood sample (n = 16, FO = 8, PLA = 8) and muscle sample analysis (n = 14, FO = 7, PLA = 7) were completed with a limited sample size. Participants were male, resistance trained (≥ 2 times/week for at least 6 months prior to commencement of the study), did not already consume a diet high in n-3 PUFA acids and were healthy as determined by our pre-participation health questionnaire. The study conformed to the principles set out by the Declaration of Helsinki and was approved by the NHS West of Scotland Research Ethics Committee 4 (16/WS/0248) and the University of Stirling Research Ethics Committee. All volunteers provided written informed consent prior to taking part in the study.

3.2.2 - Experimental design

Participants consumed supplements containing 4g/d n-3 PUFA (FO) or placebo (PLA) for a total of 6 weeks, as detailed previously (Chapter 2). Participants attended trials in the morning of three different days spread over the 6-week period. During the first visit (supplementation day 7), participants completed leg extension and leg press 1RM protocols, following completion of other testing reported in chapter 2. The position of the seat and knee angle were recorded for replication on subsequent visits. Participants completed a unilateral 1RM protocol [239], with the dominant leg, on leg press and leg extension machines with fixed resistance (Cybex International, Medway, MA, United States). In brief, participants initially warmed up with a light load that allowed ten repetitions. Following a 1-minute rest, weight was increased by 10-20%, and participants were asked to complete three to five repetitions. A near-maximal load was estimated, by adding another 10-20% of weight. Following a 2-minute rest, participants were asked to complete two to three repetitions. Following another 2-minute rest, weight was increased by 10-20%, and participants attempted a 1RM. If this attempt was successful, participants rested another

2 minutes and completed another 1RM attempt at 10% added weight. If successful, the previous step was repeated. If a 1RM attempt was failed, the load was decreased by 5-10% and an attempt at the lower weight was made after a 2-minute rest. Following this visit, participants were instructed to maintain habitual activity levels. In addition, they were instructed to remain in energy balance until week 4, when they were provided with a 100% energy diet, and weeks 5-6, when they were provided with a 60% energy diet, as described previously (Chapter 2). Visits 2 and 3 (days 28 and 43) were identical to each other and took place immediately before and after the 2-week 40% energy restriction period. Participants were instructed not to undertake strenuous exercise in the 24h before each trial, and to attend in the morning after an overnight fasted state. Participants were provided with the same diet in the 3 days leading up to visits 2 and 3.

3.2.3 - Acute exercise trial

As the anabolic potential for n-3 PUFA is believed to stem from modulated responses to resistance exercise and protein, we assessed muscle molecular responses to an acute bout of resistance exercise and protein consumption at pre and post-ER. The acute exercise trial protocol is summarised in figure 3.1. Participants consumed a meat-free diet in the three-day lead up to both trials, to enable analysis of plasma 3-methylhistidine as a marker of muscle protein breakdown (MPB) [240], [241]. On day 28 (pre-ER) and day 43 (post-ER) of supplementation, participants reported to the laboratory at 7:00 am in a fasted state. A cannula was inserted into an antecubital vein, from which an initial blood sample was drawn. Prior to the exercise bout, the vastus lateralis (VL) of the dominant leg was prepared for muscle biopsy. First, the site was anaesthetised using 2% lidocaine injected subcutaneously, followed by a small incision. In a different room, participants then completed leg press and leg extension (Cybex International, Medway, MA) exercise with their dominant leg. Following a warm-up, consisting of 12 repetitions at 40%, 10 repetitions at 50% and 8 repetitions at 60%, participants completed 3 sets of 10 repetitions at 70% of 1RM, resting 1

minute between sets. Participants returned to the biopsy room, and immediately approximately 150mg of muscle tissue was collected using the suction-modified Bergström technique [242], [243]. Muscle tissue was rinsed with ice-cold saline, blotted dry and any visible fat or connective tissue was removed before being snap-frozen in liquid nitrogen and stored at -70 °C until later analysis. Within the 5 minutes following the biopsy procedure, participants consumed a protein shake containing 12.77g whey protein isolate (78.3% / 10g protein; Maximuscle, UK) with 500mL water. Participants rested until a second biopsy was taken from a proximal site on the same muscle at 90 minutes post-exercise[244]. They continued to rest until 120 minutes post exercise. During the post-exercise resting period, blood samples were drawn at 0, 15, 30, 45, 60, 90 and 120 minutes post exercise. Blood was collected into one 6 mL clot activator Vacutainer (BD, UK), allowed to clot at room temperature; and one 6 mL lithium-heparin Vacutainer (BD, UK) which was immediately placed on ice. All samples were centrifuged at 3000 rpm, 4°C for 15 minutes at the end of the trial. Serum and plasma, respectively, were transferred into Eppendorf tubes and placed in storage at -70°C until later analysis.

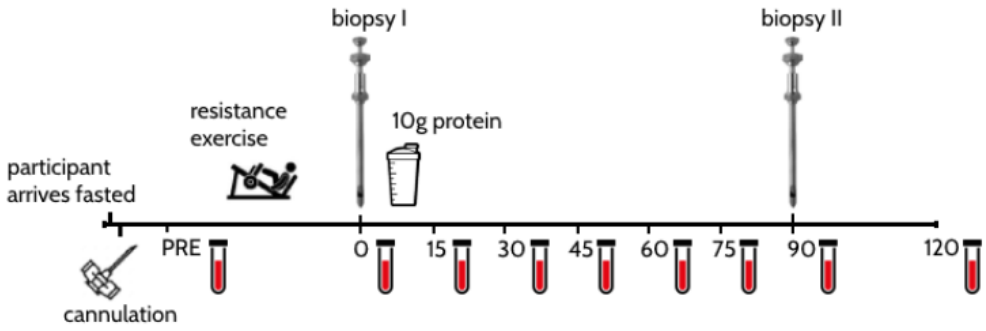

Figure 3.1 - Acute exercise trial day, which took place on days 28 (wk 4) and 43 (wk 6), immediately prior to and at the end of the 2-week ER period. Numbers are time in minutes relative to the end of exercise. Muscle biopsies were taken immediately after, and 90 minutes after the completion of a bout of unilateral leg resistance exercise. A resting blood sample was drawn before exercise, and further blood samples were drawn afterwards.

3.2.4 - Analytical procedures

Lipid extraction of whole blood samples

Samples of whole blood were placed onto four collection spots on Whitman 903 blood collection cards (GE Healthcare Ltd, UK). The cards were allowed to dry at room temperature and then stored at -70°C until analysis at a later date. The blood spots were analysed by the Institute of Aquaculture at the University of Stirling, as previously described [183]. In brief, the blood spots were placed into vials containing 1.25M methanol-HCl and placed in a hot block at 70°C for 1h. They were allowed to cool to room temperature, after which 2mL distilled water and 2mL of saturated HCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 2 x 2 mL isohexane. FAME were quantified by gas-liquid chromatography (GLC).

Preparation of protein sample

30-50mg of muscle sample was homogenised in ice-cold PKB lysis buffer consisting of 1mM/L TrisHCl pH7.5, 0.1mM/L EGTA, 1mM/L EDTA, 1% TX100, 50mM/L sodium fluoride, 5mM/L sodium pyrophosphate, 0.27M/L Sucrose, 0.1% beta-mercaptoethanol, 1mM/L sodium orthovanadate and 1 protease inhibitor cocktail tablet (Sigma Aldrich; #11836170001). After incubation on ice for 20 min, the homogenate was centrifuged at 14800 rpm for 20 min to remove the insoluble portion. The protein concentration of the supernatant was determined using DC^{TM} protein assay using the manufacturers' instructions (#500-0112, BioRad, Watford, UK). Equal amounts of protein were added to 100 µL Laemmli sample buffer, containing 10% BME, and PKB lysis buffer, after which samples were boiled for 5 min at 100 °C. Finally, the samples were placed in storage at -70°C until analysis at a later date.

Western blotting

Ten micrograms of protein were loaded into pre-cast 4-20% Criterion™ TGX™ 18-well gels

(#5671094; BioRad, UK), which underwent SDS-PAGE at 150V for 1.5h (PowerPac HC, BioRad, UK) in 1X Tris/Glycine/SDS (#161-0732; Biorad, UK). Following electrophoresis, proteins were transferred to an Amersham™ PVDF membrane (#GE10600023, Sigma Aldrich, Gillingham, UK) at 100V for 1h (PowerPac HC, BioRad, UK). Membranes were blocked with 5% bovine serum albumin (BSA; #11413164, Fisher, Loughborough, UK) in Tris-buffered saline (#1706435, BioRad, Watford, UK) with 0.1% Tween™ 20 (TBS-T; #10485733, Fisher, Loughborough, UK) at room temperature for 1 hour. Membranes were then incubated overnight at 4°C in primary antibody diluted 1:5000 in TBS-T, with gentle agitation on a Cole-Palmer™ Stuart ™ see-saw rocker (Fisher Scientific, Loughborough, UK) set to a speed of 40 rpm. Following 3 x 5 min washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-linked secondary antibodies diluted 1:10000 in TBS-T. Membranes were again washed 3 x 5 min in TBS-T. Following incubation in Cytiva Amersham™ ECL™ Prime Western Blotting Detection Reagent (#10308449, Fisher Scientific, Loughborough, UK) for 5 min, HRP activity was visualised on a ChemiDoc XRS+ device (BioRad, Watford, UK). Protein bands were quantified on Image Lab™ software (version 6.0, BioRad, Watford, UK). Phosphorylated and total protein concentrations were normalised to a pooled protein sample on each gel, and the phosphorylated:total protein ratio was analysed.

Antibodies

The rabbit monoclonal antibodies for phospho-mTOR (Ser2448; #2971), total mTOR (#2983), phospho-rpS6 (Ser 235/236; #2211), and total rpS6 (#2217) were purchased from Cell Signaling Technology (London, UK). Anti-rabbit horse radish peroxidase (HRP) linked secondary antibodies were also purchased from Cell Signaling Technology (#7074; London, UK).

Quantitative polymerase chain reaction (qPCR)

RNA was extracted from frozen vastus lateralis muscle using Invitrogen™ TRIzol™ reagent

(#15596026, Fisher Scientific, Loughborough, UK) in accordance with the manufacturer's instructions. The concentration and purity of total RNA were determined based on their absorbance at 260 and 280 nm using a DS-11 FX Spectrophotometer (DeNovix, Wilmington, DE, USA). 1 µL of oligo$(dT)_{18}$ was added to 100 ng of template RNA and H_2O was added to final volume of 12 µL. Next, 4 µL of reaction buffer, 1 µL of RiboLock RNase inhibitor, 2 µL dNTP mix and 1 µL reverse transcriptase were added to final volume of 20 µL. The reverse transcriptase (RT) reaction for complementary DNA (cDNA) synthesis consisted of 60 min at 42°C followed by 72°C for 5 min. Before use in quantitative PCR (qPCR), cDNA was diluted 1:5. The qPCR programme was completed on a Lightcycler 480 II (Roche, Burgess Hill, UK), and consisted of UDG treatment at 55°C for 2 min, DNA denaturing at 95°C for 10 min, followed by 40 quantification cycles of 95°C for 15s, 60°C for 30s and 72°C for 30s. Genes of interest were quantified relative to the geometric mean of four reference genes: GAPDH, ACTB, B2M and YWHAZ [245]. Quantified genes and their primer sequences are listed in table 3.1. Results are expressed as linearised expression values (ΔCt), normalised to the geometric mean of the four reference genes.

Table 3.1 – Accession numbers and primer sequences for muscle development, autophagy and reference genes.

Gene	Accession number	Forward primer sequence (5' -> 3')	Reverse primer sequence (5' -> 3')
MuRF1 (TRIM63)	NG_033268.1	GACTACAGATGGGCAACACTA	CCTGGGCAACATAGTGAGATA
MAFbx (FBXO32)	NM_058229.3	TACTCCAGACCCTCTACACATC	GAATCGTCTCCATCCGATACAC
COX2 (PTGS2)	NM_000963	TACTGGAAGCCAAGCACTTT	GGACAGCCCTTCACGTTATT
NFκB	NM_003998.3	CTCCACAAGGCAGCAAATAGA	ACTGGTCAGAGACTCGGTAAA
PIK3C3 (hVps34)	NM_002647.3	CTCAGCAGAGAGATCCAAAGAC	GCCAGCAAAGAACGCATAAC
MYOG	NM_002479.6	CCCTGATGCTAGGAAGCCAG	CTGGTCCCCTGCTTTACCTC
MYOD	NM_002478.5	TCCTGAAACCCGAAGAGCAC	AGGCCCTCGATATAGCGGAT
BECN1	NM_001313998.2	TCCATGCTCTGGCCAATAAG	ACGGCAGCTCCTTAGATTTG
BNIP3	NM_004052.3	AGCTCACAGTCTGAGGAAGA	CCGACTTGACCAATCCCATATC
BNIP3L	NM_001330491.1	TTCCACTTCAGACACCCTAAAC	AGGAAGAGAGATGGAATGAACAC
GABARAPL1	NM_031412.4	CCACCGCAAGGAGACAGAAG	GAAAATGTGATGGTGTGT
MAP1LC3B	NM_022818.5	GCAGCTTCCTGTTCTGGATAA	GAGCTGTAAGCGCCTTCTAAT
GAPDH	NM_001256799.3	GGCATGGACTGTGGTCATGAG	TGCACCACCAACTGCTTAGC
ACTB	NM_001101.5	AATGTGGCCGAGGACTTTGATTGC	AGGATGGCAAGGGACTTCCTGTAA
B2M	NM_004048.4	TCTCTGCTCCCCACCTCTAAGT	TGCTGTCTCCATGTTTGATGTATCT
YWHAZ	NM_001135699.2	TGTAGGAGCCCGTAGGTCATC	GTGAAGCATTGGGGATCAAGA

3-Methylhistidine analysis

Participants consumed a meat free diet for three days leading up to blood sampling to minimise the contribution of dietary 3-methylhistidine to subsequent measurements. Serum from day 28 (end of week 4 EB) and day 43 (end of ER) was analysed for 3-methylhistidine concentrations by Heartland Assays (Ames, Iowa, US). 3-methylhistidine analysis was completed via gas chromatography/mass spectrometry (GC/MS) as previously described [241].

Leptin analysis

Leptin concentrations were determined in serum from day 28 (EB) and day 43 (ER), collected in the resting and overnight fasted condition. Using a magnetic bead panel (ThermoFisher, UK; #EPX01A-12039-901), leptin concentrations were analysed using the Luminex 200 instrument (Luminex Corp, Austin, TX, United States) according to the manufacturer's instructions.

3.2.5 - Cell culture

To determine the influence of n-3 PUFA-rich plasma on skeletal muscle development, human skeletal muscle myoblasts (Lonza, Walkersville, US, #CC2580) were cultured in SkGM^{TM}-2 growth medium (GM; Lonza, Walkersville, US, #CC3244) at 5% CO_2 and 37°C. At 70% confluence, cells were passaged to 300,000 per well for differentiation in Gibco™ 1:1 DMEM/F12 (DM; Fisher Scientific, UK, #11320-074) supplemented with Gibco™ 2% horse serum (Fisher Scientific, UK, #26050070). After 7 days, differentiated cells were serum starved for 1 hour in Gibco™ 1:1 DMEM/F12. Then, cells were conditioned with n-3 PUFA-rich or placebo ex vivo human serum for 4 hours. This model was previously shown to successfully condition medium and regulate MPS in *in vitro* skeletal muscle cells [246]. Serum from three fish oil supplemented participants was pooled and matched as closely as possible with serum from three placebo supplemented participants. Matching was

based on the relative amount of lean mass participants lost during the weight loss period. To determine the effect of n-3 PUFA supplementation alone, rather than changes in lean mass, one relatively low, one medium and one high loser of lean mass under ER were selected and matched between groups. Due to ethics requirements, only samples from participants who consented to additional analysis was included (FO n = 3, PLA n = 3). Average total weight and lean mass loss of participants whose samples were selected is displayed in table 3.2. Ex vivo human serum from FO and PLA in the postabsorptive, resting condition (Rest/Fast) and 90 minutes post-REx and 10g protein (REx/Fed) was compared between pre-ER (day 27) and post-ER (day 42).

Table 3.2 – Mean weight loss characteristics of participants whose serum was pooled for ex vivo analysis.

	Fish oil (n = 3)	Placebo (n = 3)
Total weight (kg)	2.3	2.6
Lean mass (kg)	1.0	0.7
Lean mass (%)	40.5	29.8

Table 3.3 – n-3 PUFA profiles of pooled serum. EPA and DHA values are as % of total fatty acids, while total n-3 PUFA is presented as a % of total PUFA (i.e. n-3 + n-6).

	Pre-ER				Post-ER			
	Rest/Fast		REx/Fed		Rest/Fast		REx/Fed	
	FO	PLA	FO	PLA	FO	PLA	FO	PLA
EPA (% total FA)	4.2	0.41	4.3	0.40	4.8	0.40	4.7	0.41
DHA (% total FA)	4.0	1.4	4.4	1.4	4.1	1.6	4.0	1.6
Total n-3 PUFA (% PUFA)	60	23	60	23	57	23	57	23
EPA:ARA	1.2	13.1	1.2	13.3	1.3	15.7	1.3	15.3

Following serum conditioning, the medium was removed and cells were washed 2x in ice cold Gibco™ PBS (ThermoFisher, UK, #14190-094). 350 µL Buffer RLT (Qiagen, UK, #79216) was added per well and cells homogenised with a rubber policeman. Detachment was checked under microscope. 350 µL 70% ethanol was added per well and mixed via pipetting. Cells were transferred to a 1.5 mL Eppendorf before RNA harvest and qPCR analysis as previously described above (section 3.2.4/qPCR).

Additionally, we determined the influence of n-3 PUFA on insulin-mediated glucose uptake to

further evaluate the potential for n-3 PUFA to improve insulin sensitivity in human muscle cells. A Glucose-GLO™ assay (Promega, UK) was completed with the eight different serum conditions listed in table 3.3. Cells were plated onto a 96-well plate at 8000 cells/well with 100 µL GM, incubated at 37°C and 5% CO_2. After 1 day, GM was replaced with DM. Two days before the assay, DM was removed and replaced with serum-free Gibco™ 1:1 DMEM/F-12 (ThermoFisher, UK, #1132007). One day before the assay, the medium was replaced with 100 µL serum- and glucose-free DMEM with 1mM insulin (Sigma Aldrich, UK, #I9278). On the day of the assay, cells were incubated for 5 hours in 100 µL DMEM with 1mM insulin and the different ex vivo human serum pooled conditions. Treatment serum was added at 20% of total volume, which is a quantity that was previously determined not to impair cell viability [246]. 1 hour before the assay, the medium was refreshed with the same amounts of DMEM, insulin and serum treatments. Finally, medium was removed, and cells were washed with PBS. PBS was then removed, and cells were assayed according to the manufacturer's instructions (Glucose-GLO™ Assay kit, Promega, UK). That is, cells were incubated for 30 minutes at 25°C in 1mM 2DG in PBS. 25 µL stop buffer, 25 µL neutralisation buffer and 100 µL 2DG6P detection reagent were added sequentially, with brief shaking following each step. Cells were then incubated at 25°C for 1 hour before 1 second luminescence was recorded on a GloMax® Explorer Multimode Microplate Reader (Promega, Madison, WI, USA).

3.2.6 - Data presentation and statistical analysis

All statistical analysis was performed on Windows 10 using R (version 4.0.5) and RStudio (version 1.4). Western Blot data were analysed using Welch's t-tests for unequal variance [247]. RpS6 Western Blot data was log base 2 transformed due to non-normality. Gene expression data from muscle biopsy samples were normalised using the dCt method and consequently analysed using Welch's t-test [247], [248]. Leptin and 3-methylhistidine were analysed using mixed effects linear modeling with participant ID as random factor, and time and group as fixed factors. A type III

analsyis of variance (ANOVA) was then performed using the Kenward & Roger approach for approximation of degrees of freedom, as is recommended for small sample sizes [184], [185]. Where significant effects were found, post-hoc analysis was performed using Tukey's method [186]. Effect sizes were expressed as Hedges' *g*, which includes a correction for small sample size [188], [189]. Data are presented as mean ± standard deviation (SD) unless otherwise stated.

3.3 - Results

3.3.1 – Anabolic signalling

To address whether dietary fish oil preserves anabolic signalling during energy restriction, we examined the change in the ratio of phosphorylated mTOR ^{Ser2448}:total mTOR from EB to ER in the postabsorptive (0 min post REx) and anabolically stimulated states (90 min post REx plus 10g protein) (Figure 3.2 & 3.3). In the postabsorptive state, the change in the ratio of phosphorylated mTOR^{Ser2448}:total mTOR from EB to ER was not different between FO (-0.105 ± 0.294 AU) and PLA (-0.100 ± 0.279 AU) (p = 0.97). After anabolic stimulation, the change in the ratio of phospho:total mTOR from EB to ER was not greater in FO (0.177 ± 0.306 AU) compared to PLA (-0.126 ± 0.262 AU), as this did not reach statistical significance (p = 0.071). The effect size of n-3 PUFA for the change in basal mTOR ratio from EB to ER was *g* = 0.017 (95% CI: -1.073 – 1.107). The effect size of n-3 PUFA for the change in mTOR ratio in response to anabolic stimulation from EB to ER was *g* = 0.995 (95% CI: -0.161 – 2.151).

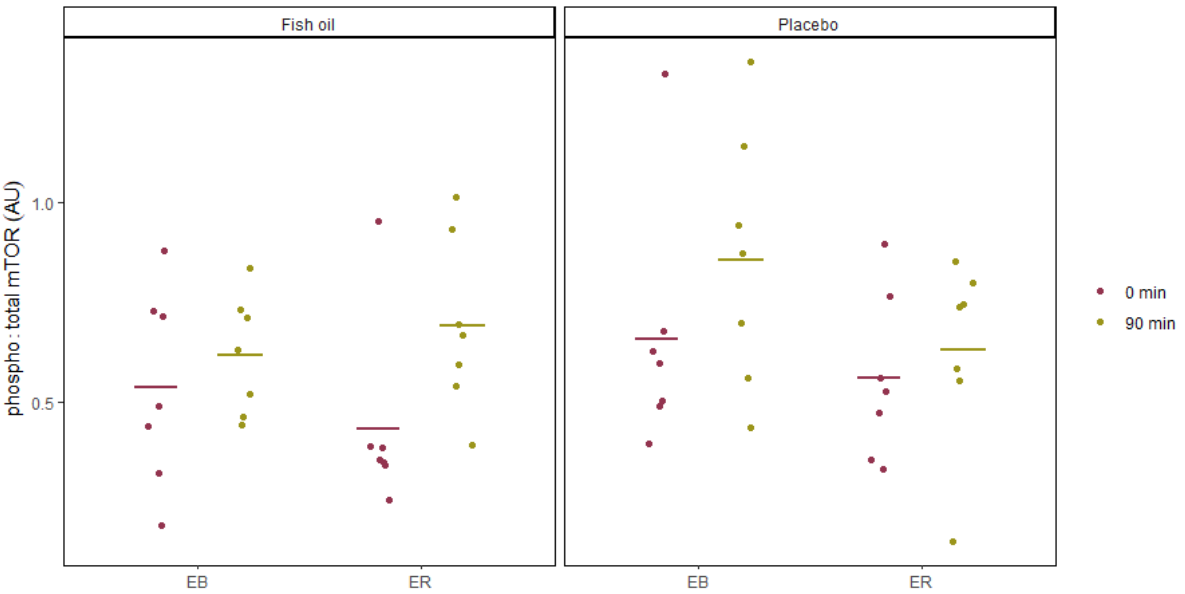

Figure 3.2 – Ratio of phosphorylated mTOR^{Ser2448}:total mTOR during energy balance (week 4) and after 2 weeks of energy restriction (week 6). Muscle biopsies were taken from participants in the postabsorptive state immediately post-exercise (0 min) and 90 minutes after exercise and consumption of 10g protein (90 min).

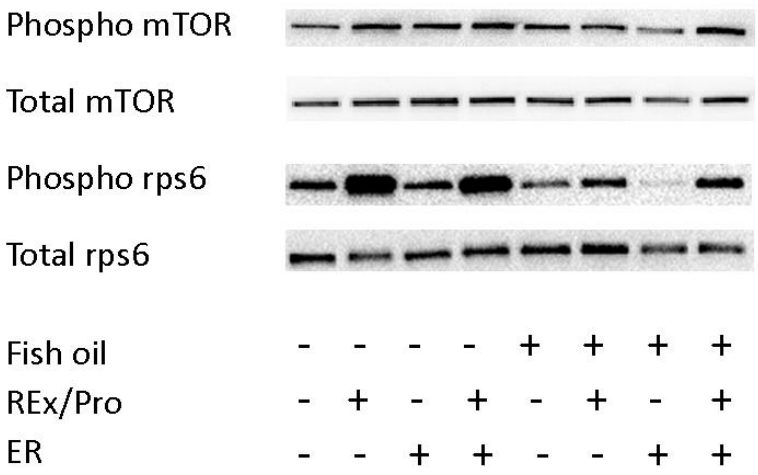

Figure 3.3 – Representative blot images of phosphorylated mTOR^{Ser2448}, total mTOR, phosphorylated rpS6$^{Ser235/236}$ and total rpS6.

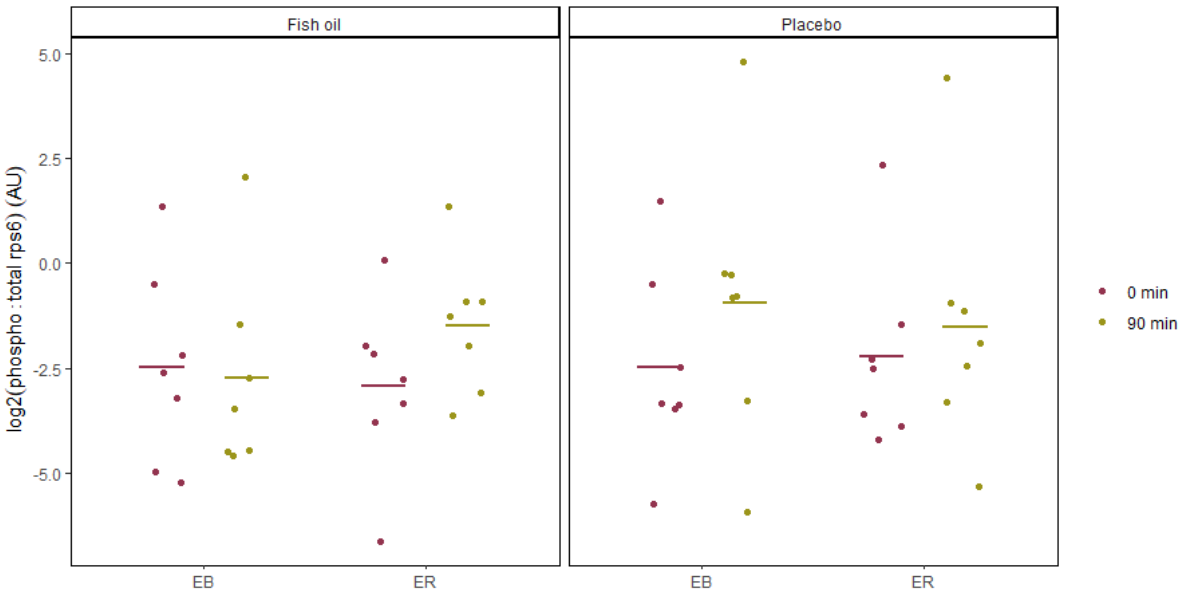

Figure 3.4 – Ratio of phosphorylated rpS6$^{Ser235/236}$:total rpS6 during EB (week 4) and at the end of 2 weeks ER (week 6). Ratio values were log base 2 transformed.

Further, to address whether dietary fish oil preserves anabolic signalling downstream from mTOR, we examined the ratio of phosphorylated rpS6$^{Ser235/236}$:total rpS6 in the postabsorptive state and after anabolic stimulation (Figure 3.3 & 3.4). RpS6 data was log2 transformed due to non-normality. The change in the ratio of postabsorptive phospho:total rpS6 from EB to ER was no different between FO (-0.462 ± 1.64 AU) and PLA (0.264 ± 1.275 AU) ($p = 0.375$). After anabolic stimulation, the change in the ratio of phospho:total rpS6 from EB to ER was significantly greater in FO (1.71 ± 1.81 AU) compared to PLA (-0.849 ± 0.835 AU) ($p = 0.0087$). The effect size of n-3 PUFA for the change in basal rpS6 ratio from EB to ER was $g = 0.462$ (95% CI: -0.642 – 1.567). The effect size of n-3 PUFA for the change in rpS6 ratio in response to anabolic stimulation, from EB to ER, was $g = 1.698$ (95% CI: 0.426 – 2.970).

3.3.2 – Gene expression response to energy restriction

To determine the influence of n-3 PUFA status on ER-induced changes in expression of genes related to muscle hypertrophy and atrophy, the difference in the change in normalised linearised gene expression values from pre- to post-ER were compared between FO and PLA (figure 3.5).

Several genes related to muscle development presented divergent, although non-significant, changes during ER. The ER-induced increase in expression of MAFbx/FBXO32 was not different between FO and PLA (t = 1.369, p = 0.222). Similarly, the ER-induced increase in expression of MuRF-1/TRIM63 was not different between FO and PLA (t = 0.671, p = 0.543). During ER, the change in NF-κB expression was not different between FO and PLA (t = -0.834, p = 0.424). The change in expression of MyoD was also not significantly different between FO and PLA (t = -1.486, p = 0.173). Similarly, the change in expression of MyoG was not significantly different between FO and PLA (t = -0.970, p = 0.366). Finally, the change in expression of COX2/PTGS2 also was not significantly different between groups (t = 0.448, p = 0.664).

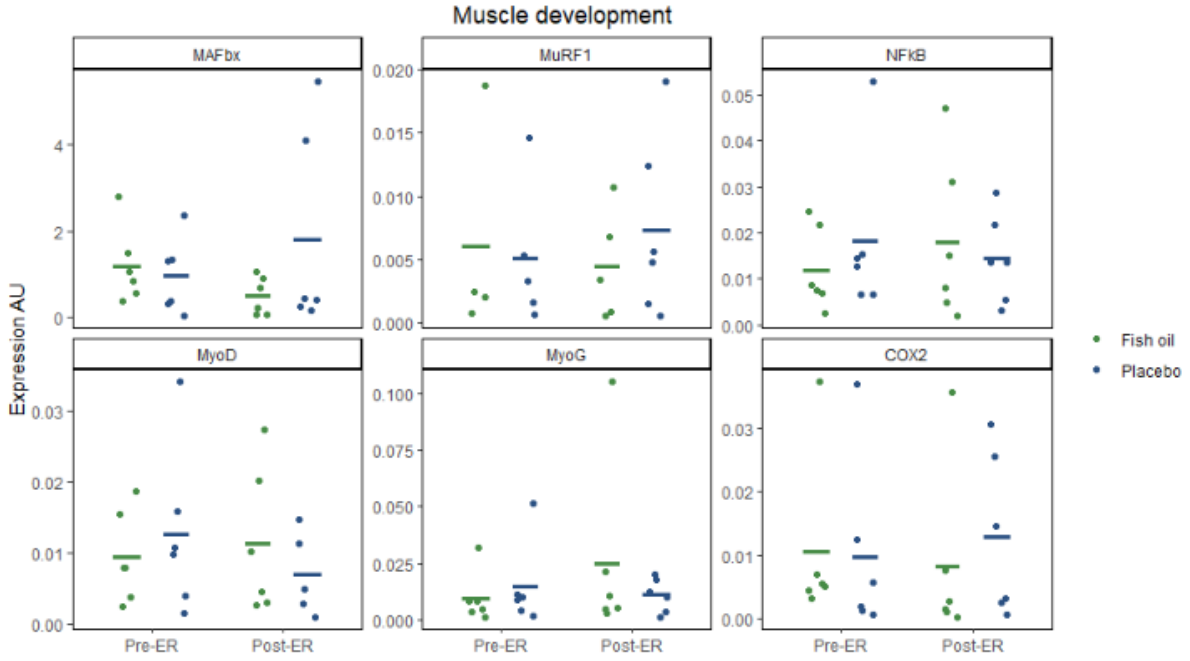

Figure 3.5 – Scatterplots of normalised muscle and atrophy related gene expression pre-ER (week 4 of supplementation) and post-ER (week 6 of supplementation).

To further understand the role of n-3 PUFA on autophagy, we also assessed the influence of n-3 PUFA status on changes in expression of key autophagy genes during ER (figure 3.6). Although the expression patterns of several genes appeared divergent between FO and PLA, there were no significant differences in any genes. As such, there was no difference in the change in expression of BNIP3L/NIX during ER in FO and PLA (t = 0.704, p = 0.503). Similarly, the change in expression of

GABARAPL1 was not significantly different between groups (t = 0.404, p = 0.698). The change in MAP1LC3B expression also was not different between FO and PLA (t = 0.480, p = 0.648). The change in expression of PIK3C3/hVps34 was not different between FO and PLA (t = 0.522, p = 0.613). Finally, expression of BECN1 and BNIP3 did not change during ER in FO or PLA, and there was no difference between groups (p > 0.95).

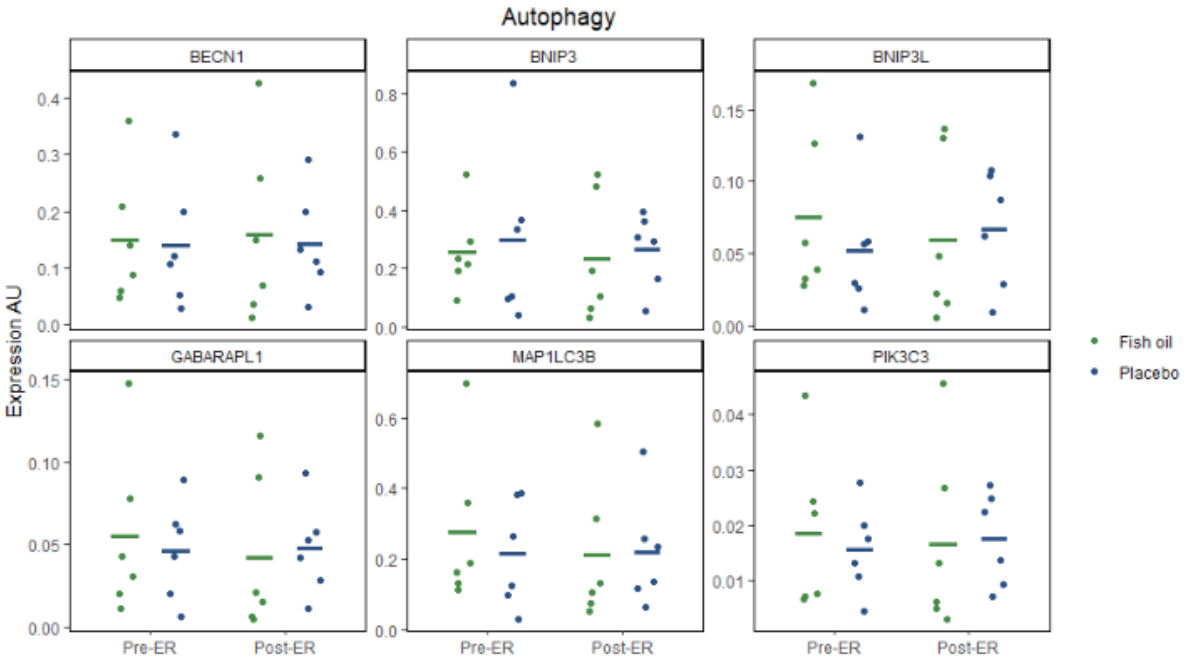

Figure 3.6 – Scatterplots of autophagy-related normalised gene expression pre-ER and post-ER.

3.3.3 – Whole-body protein breakdown

To determine whether n-3 PUFA influenced changes in whole-body protein breakdown, we examined serum 3-methylhistidine (3MH) concentrations pre- to post-ER (Figure 3.7). 3MH appeared to increase by 0.38 ± 0.68 nmol/mL from pre-ER to post-ER, although this did not reach statistical significance (t = 4.46, p = 0.0547). There was no difference in 3MH between FO and PLA (t = 0.421, p = 0.528).

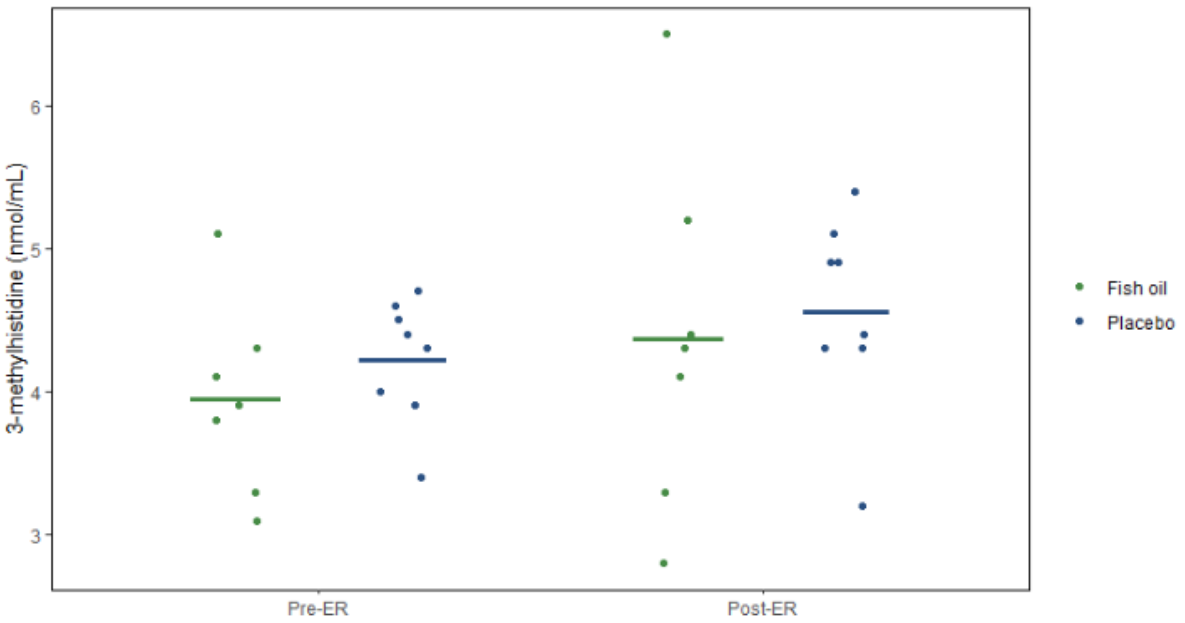

Figure 3.7 – Scatterplot of postabsorptive serum 3-methylhistidine concentrations pre (week 4) and post-ER (week 6).

3.3.4 – Leptin

To assess the influence of n-3 PUFA status on ER-induced changes in leptin, serum leptin was examined pre- and post-ER (figure 3.8). Leptin data was natural log-transformed due to non-normality. Although log(leptin) appeared higher in FO (7.54 ± 0.72 pg/mL) compared to PLA (6.68 ± 0.99 pg/mL), this difference did not reach statistical significance (F = 4.56, p = 0.051; Figure 3.12). As such, there was no significant difference between FO and PLA during EB (p = 0.077) or ER (p = 0.057). The decrease in log(leptin) with ER (-0.31 ± 0.70 pg/mL) also did not reach statistical significance (F = 2.83, p = 0.115).

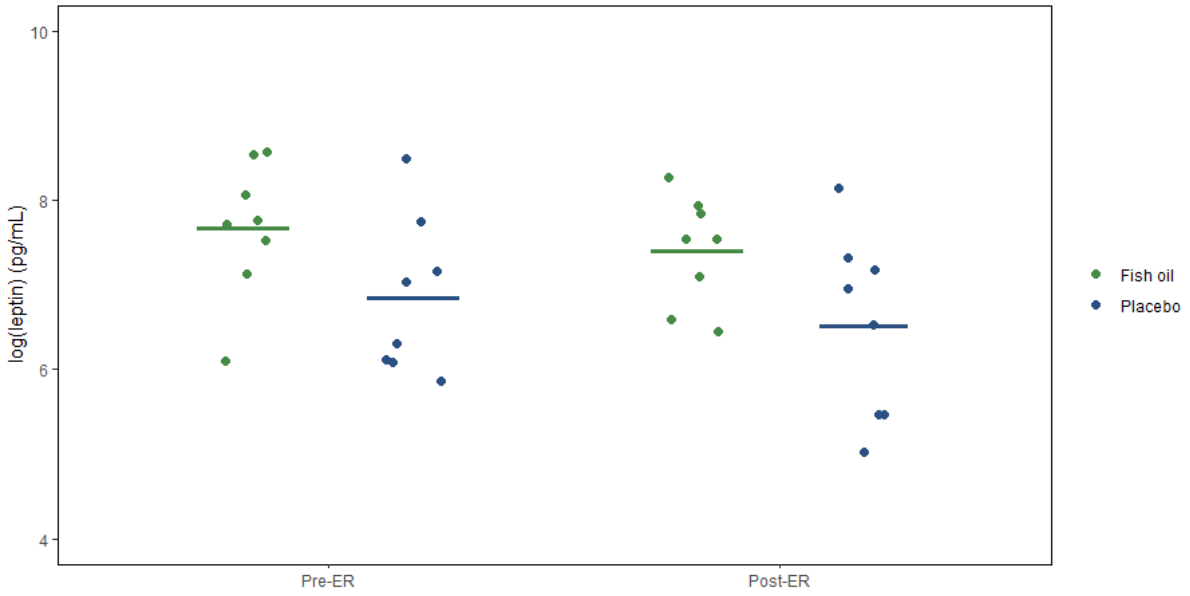

Figure 3.8 – Scatterplot of natural log-transformed postabsorptive serum leptin concentrations pre-ER (week 4) and post-ER (week 6).

As supplemental groups were not matched for fat mass (as detailed in chapter 2), we also examined log(leptin) concentrations normalised for whole-body fat mass (as determined via DEXA scans – chapter 2.3.3). Consequently, there was no difference in normalised leptin between groups (F = 0.085, p = 0.775; Figure 3.9). However, there was a significant effect of time (F = 7.574, p = 0.0156), such that normalised leptin was higher post-ER compared to pre-ER (+0.0359 ± 0.0511 pg/mL/g).

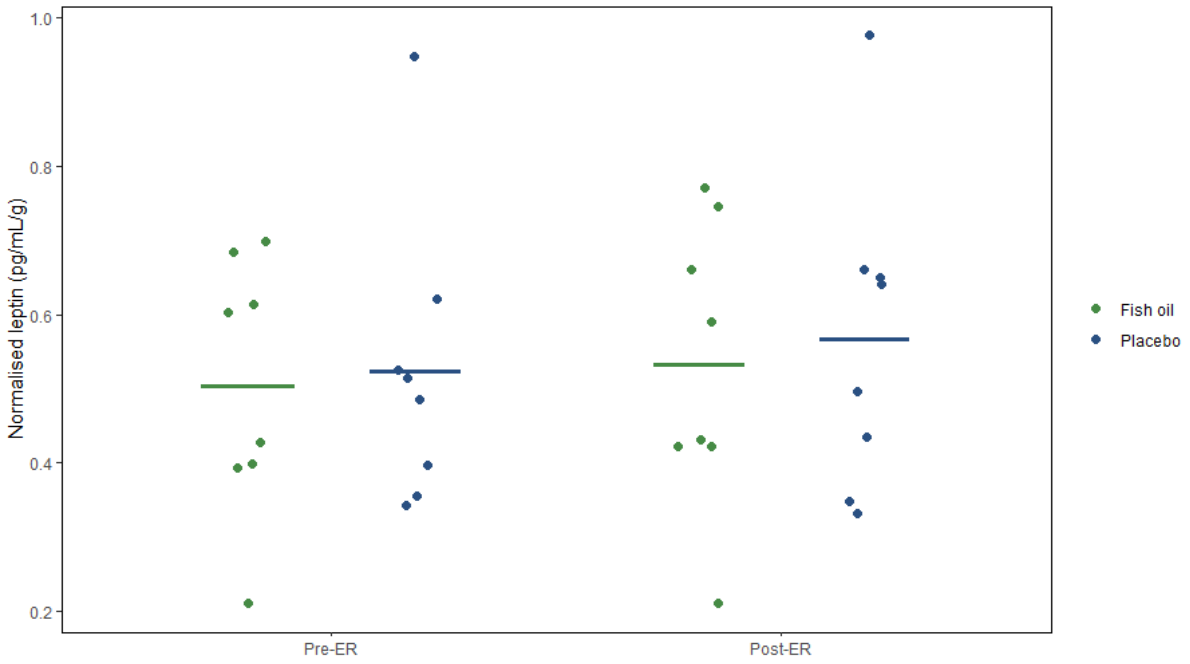

Figure 3.9 – Scatterplot of fat mass-normalised, natural log-transformed serum leptin concentrations pre- and post-ER.

3.3.5 – Gene expression in skeletal muscle cells conditioned with ex vivo human serum

To further understand the role of n-3 PUFA status on genes related to muscle development and autophagy, the expression of 12 genes was assessed in cultured human skeletal muscle cells conditioned with ex vivo human serum. As the results are only from one experiment with three technical replicates, no statistical analysis was undertaken, and results are presented as mean difference ± standard error. The full table of differences in gene expression, including 95% confidence intervals, is available in appendix A3.1. First, we assessed the influence of pre- and post-ER serum collected from individuals in the fasting, resting condition.

Expression of genes related to muscle development are shown in figure 3.10. MAFbx expression was not different between FO and PLA pre-ER, but appeared higher in PLA than FO post-ER (0.0178 ± 0.0053 AU). Similarly, NFkB expression was not different pre-ER, but appeared higher in PLA than FO post-ER (0.0281 ± 0.0105 AU). Expression of MyoD was not different between FO and PLA pre-ER, but was higher in FO than PLA post-ER (0.0099 ± 0.0050 AU). MyoG expression was lower in FO

than PLA pre-ER (-0.124 ± 0.016 AU), but this was reversed post-ER (0.181 ± 0.024 AU). COX2 expression was higher in FO than PLA pre-ER (0.0119 ± 0.0032 AU), but no different between FO and PLA post-ER.

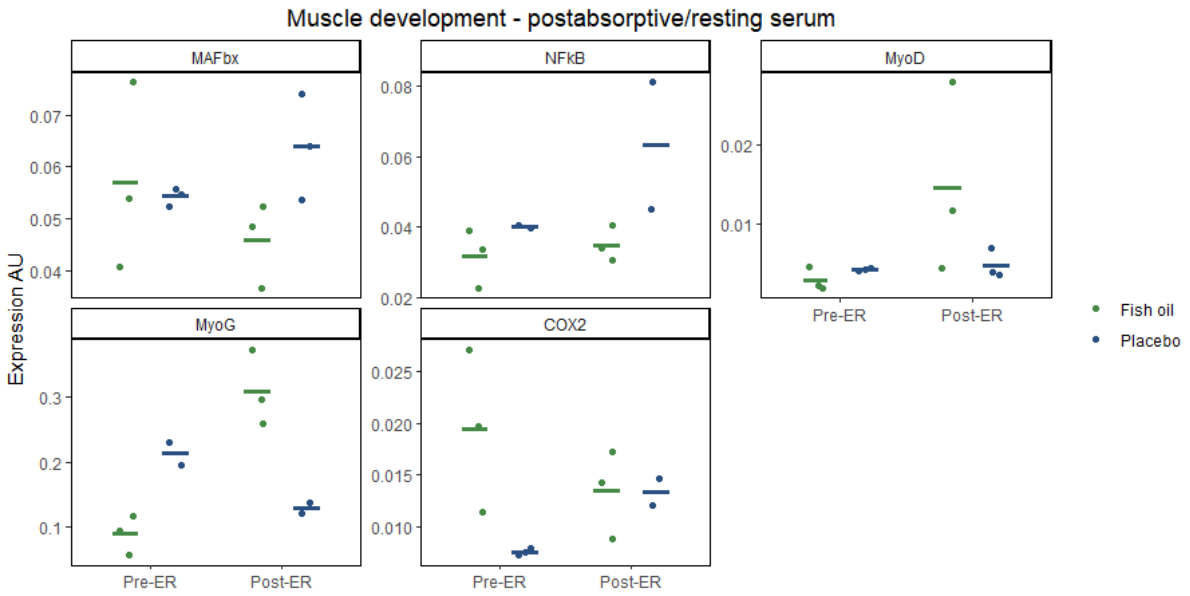

Figure 3.10 - Gene expression in primary skeletal muscle cells conditioned with ex vivo human serum. Serum was collected in the morning following an overnight fast. Individual dots within each condition represent technical replicates.

Expression changes of genes related to autophagy and cellular trafficking are displayed in figure 3.11. Expression of BECN1 was higher in FO than PLA pre-ER (0.0194 ± 0.0086 AU), but lower in FO than PLA post-ER (-0.0298 ± 0.0156 AU). Expression of BNIP3 was higher in FO than PLA pre-ER (0.036 ± 0.025 AU), but lower in FO than PLA post-ER (-0.067 ± NA AU; no SE due to only 1 observation). BNIP3L expression was higher in FO than PLA pre-ER (0.439 ± NA AU; no SE due to only 1 observation), and lower post-ER (-0.071 ± 0.009 AU). GABARAPL1 expression was higher in FO than PLA pre-ER (0.0115 ± 0.0023 AU) and post-ER (0.0112 ± 0.0030 AU). MAP1LC3B expression was no different between FO and PLA pre-ER, but was higher in PLA than FO post-ER (0.0055 ± 0.0196 AU). Finally, expression of PIK3C3 was no different between FO and PLA pre-ER, but was higher in PLA than FO post-ER (0.0033 ± 0.0053 AU).

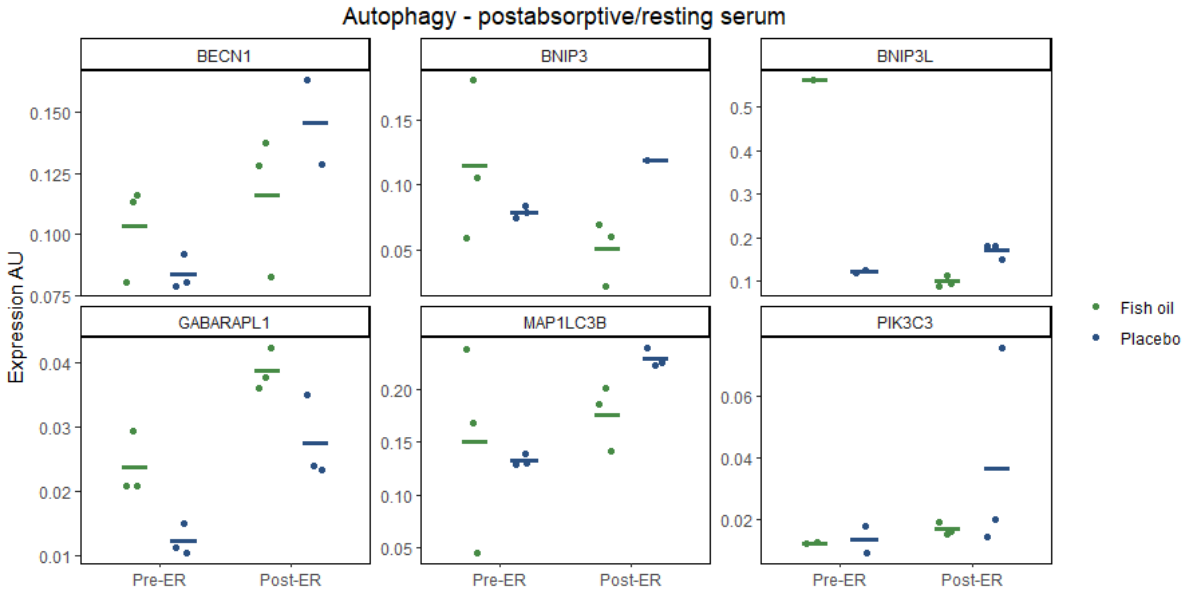

Figure 3.11 – Gene expression in skeletal muscle cells conditioned with ex vivo human serum. Serum was collected in the morning following an overnight fast. Individual dots within each condition represent technical replicates.

In addition, we examined gene expression in skeletal muscle cells conditioned with ex vivo human serum from an anabolically stimulated condition (90-min post-REx and 10g protein feeding; Figure 3.12 & 3.13). Again, no statistical analysis was undertaken, and results are presented as mean difference ± standard error (see appendix table A3.1 for full table).

With serum from the anabolically stimulated state, expression of MAFbx was higher in PLA than FO pre-ER (0.0173 ± 0.0070 AU), but this was reversed post-ER (0.0102 ± 0.0075 AU). NFkB expression was lower in FO than PLA both pre-ER (-0.0144 ± 0.0037 AU) and post-ER (-0.0027 ± 0.0020 AU). MyoD expression was not different between FO and PLA, pre-ER or post-ER. MyoG expression was higher in PLA than FO pre-ER (-0.0943 ± 0.012 AU), but no different post-ER. COX2 expression was no different between FO and PLA pre-ER, but higher in FO than PLA post-ER (0.0084 ± 0.0053 AU).

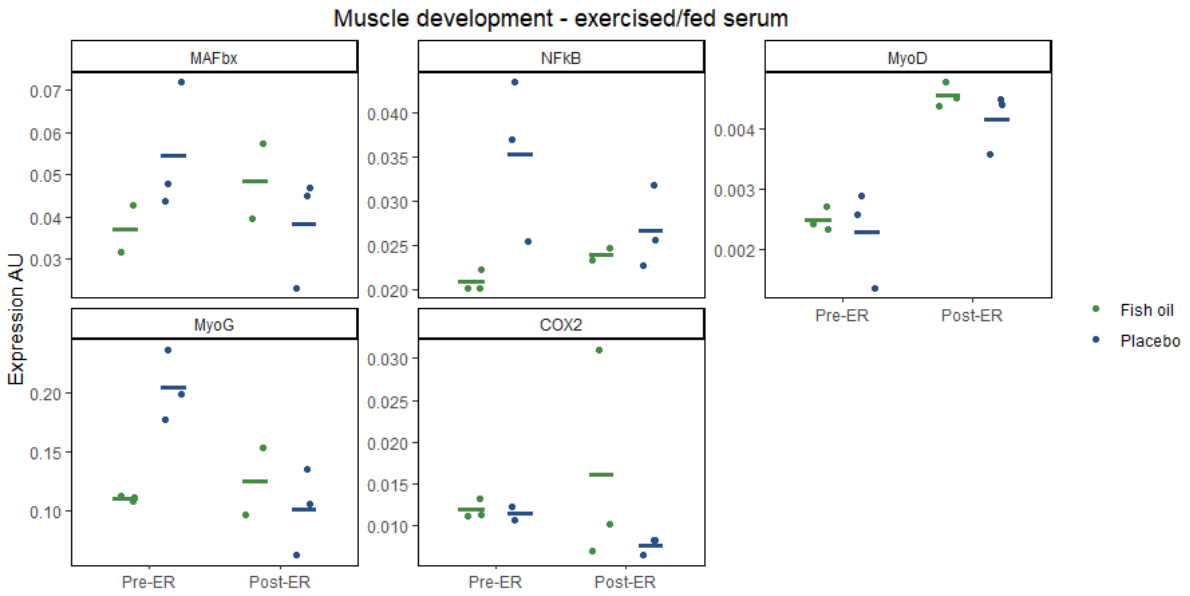

Figure 3.12 – Gene expression in skeletal muscle cells conditioned with ex vivo human serum. The serum was collected 90 minutes following unilateral leg resistance exercise and consumption of 10g protein. Individual dots within each condition represent technical replicates.

Finally, in the anabolically stimulated state, expression of BECN1 was lower in FO than PLA pre-ER (-0.0579 ± 0.0072 AU), but no different post-ER. Expression of BNIP3 was lower in FO than PLA pre-ER (-0.0603 ± 0.0052 AU), but higher in FO than PLA post-ER (0.0233 ± 0.0285 AU). BNIP3L expression was lower in FO than PLA, both pre-ER (-0.0990 ± 0.0049 AU) and post-ER (-0.0615 ± 0.0173 AU). GABARAPL1 expression was not different between FO and PLA pre-ER, but higher in FO than PLA post-ER (0.0081 ± 0.0020 AU). MAP1LC3B expression was lower in FO than PLA pre-ER (-0.1018 ± 0.0277 AU), but no different between FO and PLA post-ER. Finally, expression of PIK3C3 was lower in FO than PLA pre-ER (-0.1018 ± 0.0277 AU), but no different between FO and PLA post-ER.

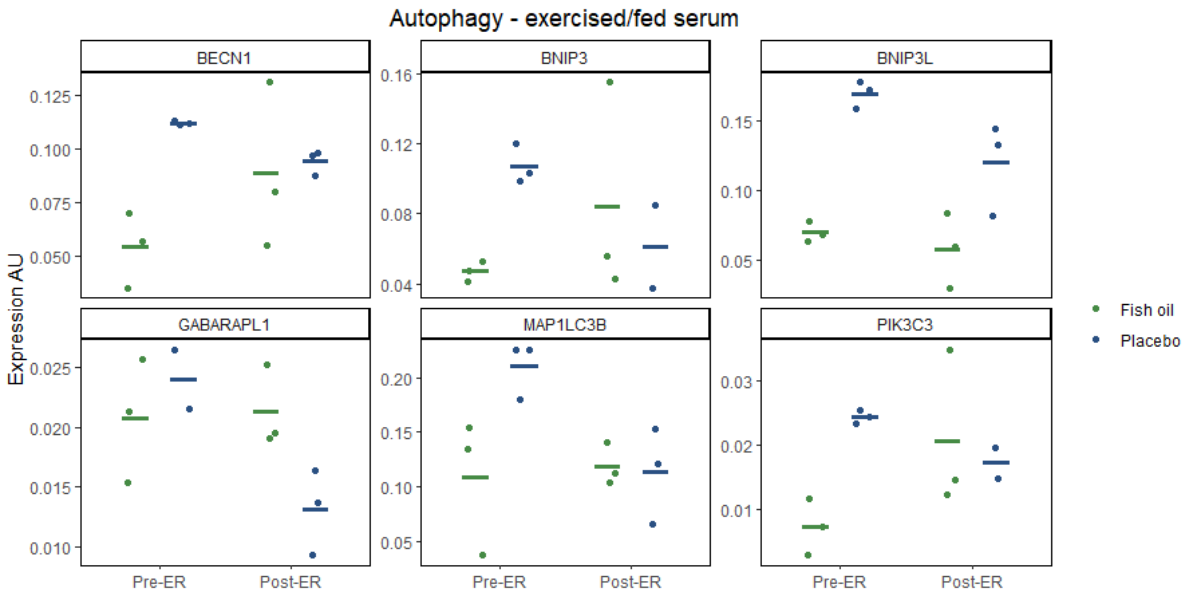

Figure 3.13 – Gene expression in skeletal muscle cells conditioned with ex vivo human serum in an anabolically stimulated state. Individual dots within each condition represent technical replicates.

3.3.6 – Insulin sensitivity of skeletal muscle cells cultured with ex vivo human serum

To determine the influence of n-3 PUFA in human serum on insulin-mediated glucose uptake, serum conditioned skeletal muscle cells were assayed by Glucose-Glo™ assay (figure 3.14). In muscle cells conditioned with serum collected under resting, and fasting conditions (figure 3.14 Fasted), there was a significant ER by group interaction ($t = -3.45$, $p = 0.00547$). As such, there was no difference in glucose uptake between FO and PLA in cells conditioned with pre-ER serum ($t = -0.126$, $p = 0.902$). However, glucose uptake was significantly higher in FO (388767 ± 204783 RLU) than PLA (66866 ± 18342 RLU) in post-ER serum cells ($t = 4.85$, $p < 0.001$). In muscle cells conditioned with serum collected 90 minutes after REx and consumption of 10g protein (Ex/Fed; figure 3.14 Exercise + 10g Protein), glucose uptake was significantly higher ($t = 4.76$, $p < 0.001$) in FO (300688 ± 146902 RLU) than PLA (67073 ± 24664 RLU). However, there was no difference in glucose uptake between cells conditioned with Ex/Fed pre-ER and post-ER serum ($t = 0.55$, $p = 0.594$).

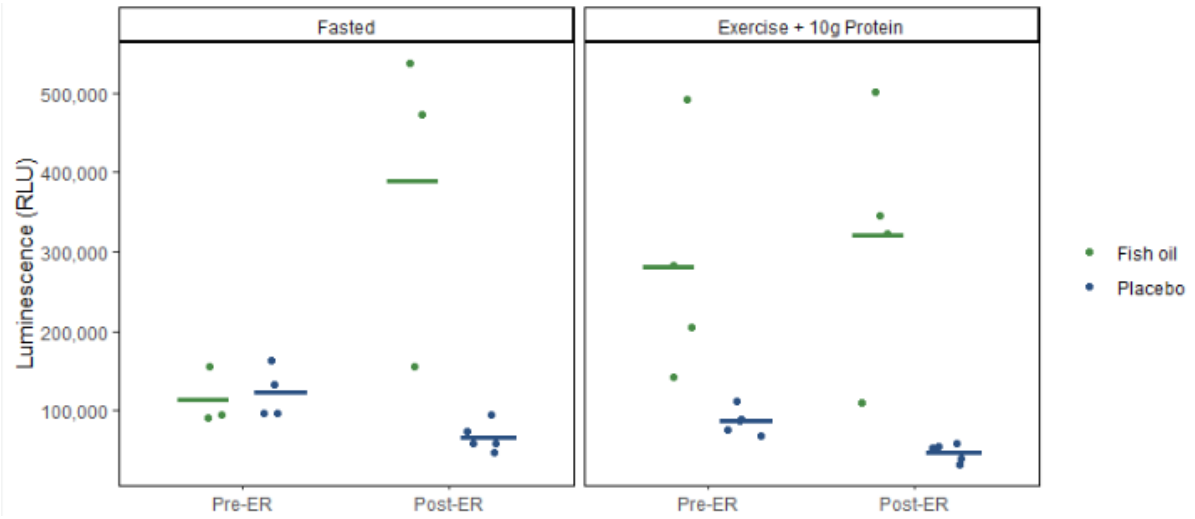

Figure 3.14 – Scatterplot of relative light unit (RLU) luminescence values for skeletal muscle cells conditioned with ex vivo human serum containing high (FO) or low (PLA) amounts of n-3 PUFA. Individual dots within each condition represent biological replicates.

3.4 - Discussion

The primary aim of this study was to investigate the role of n-3 PUFA supplementation on changes in anabolic signalling in muscle of energy restricted healthy young males. We hypothesised that n-3 PUFA would attenuate the reduction in skeletal muscle anabolism typically seen in humans during short-term energy restriction [164], [165], and potentiate signalling responses to anabolic stimulation. Although we were unable to measure muscle protein synthesis directly, we observed the phosphorylation status of proteins regulating MPS with n-3 PUFA as a proxy measurement. While these do not directly reflect the endpoint measurement of MPS, acute changes in phosphorylation status are known to be indicative of overall muscle anabolic activity [249], [250]. Nevertheless, due to technical issues with analysis and sample size, we were unable to detect any convincing effects of n-3 PUFA on anabolic signalling, if there were any. In addition, we hypothesised that n-3 PUFA would increase the expression of muscle developmental genes and reduce expression of genes related to muscle catabolism and inflammation. Although changes in individual genes were small and not statistically significant, the direction of ER-induced changes in expression of genes related to muscle anabolism and catabolism may indicate improvements in the regulation of muscle protein metabolism with n-3 PUFA, which warrants further investigation. Additionally, conditioning of primary skeletal muscle cells with n-3 PUFA rich ex vivo human serum appeared to benefit expression of genes related to muscle anabolism and catabolism, although this also was not statistically significant. Finally, we did observe improved glucose uptake in skeletal muscle cells conditioned with n-3 PUFA-rich serum. Nevertheless, as we observed no overall influence of n-3 PUFA on lean mass (Chapter 2), and did not detect convincing effects of n-3 PUFA on aspects of muscle turnover and development, their use for improving muscle mass in healthy active young males cannot be recommended.

We first set out to examine whether n-3 PUFA may preserve postabsorptive muscle anabolism

during short-term energy restriction in healthy active males. Using phosphorylation status of mTOR^{Ser2448} and rpS6$^{Ser235/236}$ as a proxy for the anabolic state of muscle (i.e. MPS), there appeared to be a reduction in postabsorptive muscle anabolism during ER. Although we did not test this change for statistical significance due to the limited sample size, such an effect would be consistent with previous studies that have investigated muscle protein metabolism during short-term ER [164], [165]. In men and women undergoing 20% ER for 10d, MPS at rest was decreased by 19% alongside significant decreases in Akt and 4EBP1 phosphorylation [164]. In young men and women undergoing ~33% ER (as 30 kcal/kg FFM/d) for 5d, postabsorptive, resting MPS was reduced by 27% from EB [165]. However, ER did not alter the phosphorylation status of Akt, mTOR, p70S6K or rpS6 in that study (values not reported). In contrast however is a study in men and women undergoing 40% ER for 21d, which observed a decrease in postabsorptive p70s6k phosphorylation, but no changes in basal rates of MPS and phosphorylation status of Akt, rpS6, eEF2 and eIF4E [151]. As such, the authors suggested the reductions in anabolic signalling and protein synthesis resulting from ER may be limited to the short-term (i.e. ≤ 2 weeks), as eventually the anabolic machinery adapts to the altered energy state. Nevertheless, in our study, supplementation with n-3 PUFA for 4 weeks prior to, and during 2 weeks of ER, did not significantly influence the change in the postabsorptive ratio of phosphorylated:total mTOR and rpS6, during 40% ER for 14d.

The majority of previous studies investigating postabsorptive MPS and activation of anabolic signalling with n-3 PUFA also did not find an effect of supplementation [48], [49], [87], [220]. However, these groups assessed different fractions of MPS, as Smith et al. investigated mixed MPS, while McGlory et al. investigated myofibrillar MPS, which was suggested to be more specific to muscle hypertrophy. Conversely, one study in older men and women observed an increase in postabsorptive mitochondrial and sarcoplasmic MPS with n-3 PUFA for 16 weeks, but no change in mixed or myofibrillar MPS [48]. While this may indicate that the effects of n-3 PUFA on MPS are

different across muscle fractions, differences in study population, length of supplementation and timing of MPS measurement and muscle sampling must be considered. Additionally, it has been suggested that basal MPS is regulated by a different pathway from the increases in MPS we see following exercise and AA availability, which is regulated through mTOR [102]. Therefore, further research is required to determine how n-3 PUFA may affect specific isolated fractions of MPS in different energy states.

In contrast to a lack of observed changes in postabsorptive muscle anabolism, n-3 PUFA have been observed by others to amplify muscle anabolic signalling in response to stimulation through REx and/or AA availability [48], [49], [87], [93], [220]. In our study, FO appeared to reverse the blunting of mTOR signalling which was observed in PLA, although this difference did not reach statistical significance. Similarly, rpS6, which is phosphorylated by p70S6K1 directly downstream from mTOR, followed a similar pattern to mTOR in response to anabolic stimulation. That is, while the response to anabolic stimulation appeared to be blunted during ER in PLA, this effect was reversed by FO, which represented a significant difference between groups. Others have shown that during ER, decreases in postabsorptive, resting muscle anabolism can be 'rescued' by REx and consumption of protein [151], [165]. Our data suggest n-3 PUFA can potentiate the activity of rpS6, but not mTOR, during ER, a context in which their activity has previously been observed to be diminished [164]. This was the first study to investigate the influence of n-3 PUFA on anabolic signalling during ER. However, the finding that n-3 PUFA can amplify anabolic signalling following anabolic stimulation is consistent with studies during EB. Infusing steers 4% of their diet as n-3 PUFA for 35d resulted in significantly increased phosphorylation of Akt and p70S6K1, and a trend for an increase in mTOR and 4EBP1, during hyperinsulinaemic-euglycaemic-euaminoacidaemic clamp [103]. In young and middle-aged men and women, and older adults, supplementation with FO for 8 weeks significantly amplified MPS and phosphorylation of mTOR and p70S6K1 during hyperinsulinaemic-

hyperaminoacidaemic clamp [87], [220]. In C2C12 myotubes, EPA significantly increased protein synthesis and p70S6K1 phosphorylation, while DHA did not. However, phosphorylation of Akt, mTOR, 4EBP1 and rpS6 was not significantly altered by EPA or DHA [91]. As such, while responses of specific anabolic signalling proteins to anabolic stimulation may be inconsistent across studies, there appears to be a pattern of promotion of muscle anabolism with n-3 PUFA. Nevertheless, although our data support a promotion of muscle anabolic signalling by n-3 PUFA, ultimately this did not result in a preservation of muscle mass (Chapter 2).

Interestingly, in our study, the ratio of phosphorylation:total protein of mTOR and rpS6 in response to anabolic stimulation appeared subdued in FO compared to PLA during EB (although this was not subjected to statistical testing). That is, REx and 10g protein seemed to increase mTOR and rpS6 phosphorylation above postabsorptive rates in PLA during EB. However, the response to this anabolic stimulation appeared blunted in FO. Thus, if our observation is accurate, a suppression of anabolic signalling with FO compared to PLA at rest and following anabolic stimulation would be consistent with the findings by McGlory et al. (2016), although they did observe this effect in different proteins along the same axis (Akt & p70s6k vs. mTOR and rpS6). The increase in the efficiency of anabolic signalling protein activity and MPS with n-3 PUFA, suggested by these authors, would be highly valuable during periods of reduced energy availability, as it implies suppression of redundant signals, and therefore preservation of energy. This interpretation should be considered with caution however, as the combination of REx and consumption of a 30g dose of whey protein likely led to maximal stimulation of MPS in the study by McGlory et al. (2016). To address whether fish oil can potentiate the signalling response to a suboptimal dose of anabolic stimulation, participants in our study completed a similar exercise protocol, but only consumed 10g protein. Although we did not measure MPS, the amplitude of the change in mTOR and rpS6 phosphorylation in response to stimulation appeared to be smaller in PLA compared to FO,

indicating muscle anabolic signalling was not at full capacity. Therefore, n-3 PUFA may be especially potent in situations where anabolic stimulation is suboptimal, such as during ER or in states associated with anabolic resistance. As our data represent a limited sample size, and we did not measure MPS directly, further research into the influence of n-3 PUFA on anabolic signalling and MPS during ER is warranted.

The discrepancy in the findings of McGlory, et al. (2016) and our observations, and those of others may be explained by the methodology used to assess anabolic signalling activity. Most studies, including ours, have used Western Blotting (WB) to assess the phosphorylation status of specific threonine and serine kinases on proteins of interest, as a proxy for overall protein activity. The phosphorylation of downstream proteins allows the targeted protein to change its activity, such as disassociation from or association with other proteins, or translocation. However, there seems to be a disconnect in the relationship between the amount of phosphorylation and protein activity [249]. That is, while there might be a large fold change in the phosphorylation of a protein, this may not induce an equivalent change in the rate of activity [251]. For example, p70s6k is known to only become phosphorylated at Thr389 once other parts of the protein are already highly phosphorylated [99], [252]. Changes in phosphorylation status of specific protein residues embody a dynamic relationship between signal input and protein activity. We, and others, attempted to quantify the phosphorylation of specific threonine or serine residues on anabolic signalling proteins via WB. In comparison, McGlory, et al. (2016) assessed the activity of p70S6K and Akt via quantitative kinase assay, using labelled ATP [251]. As such, they were able to examine the relationship between anabolic signalling protein activity and muscle protein synthesis directly. In addition to methodological differences, sample sizes of studies investigating signalling proteins in humans are typically low, leading to issues with statistical power. Methodological differences in the assessment of protein concentrations via WB also are known to lead to divergent statistical

outcomes [253]. As such, methodological issues likely contribute to the ambiguous profile of outcomes from studies investigating the role of n-3 PUFA on anabolic signalling proteins. In our study, we attempted to lessen the issue of statistical power by addressing only two specific hypotheses: 1) n-3 PUFA reduces the loss of postabsorptive anabolic signalling during ER; 2) n-3 PUFA preserves/amplifies the increase in anabolic signalling in response to anabolic stimulation during ER. Therefore, while sample size was low, the potential for type-II error was limited. However, further research should aim to repeat our investigation with direct measurement of both anabolic protein kinase activity and MPS in a larger sample population. It should also be noted that, in our study, muscle samples were taken immediately after REx lasting approximately 20 minutes. As such, it may be that differences in the acute response to REx influenced our observations of mTOR and rpS6 phosphorylation levels at the '0 min' time point. In addition, the measurement of anabolic signalling protein phosphorylation/activity at specific time points only represents a 'snapshot', and not the overall response to stimulation, which may span up to 48 hours [254], [255]. Therefore, further research using a more robust study design is required to substantiate our findings.

The long-term effects of supplementation with n-3 PUFA may, for an unknown but substantial part, be due to changes in expression of genes related to muscle anabolism and catabolism. The mechanism for these changes is suggested to be the binding of PUFA and PUFA-derived lipid peroxidation products (eicosanoids) to transcription factors [43], [256], [257]. As participants had been supplementing with FO or PLA for 4 weeks prior to sampling, we were able to examine the influence of n-3 PUFA status on the change in expression of genes during ER. Although we did not find a significant effect of FO on the change in expression of any genes, some of the patterns we observed are consistent with previous studies that have analysed the effect of n-3 PUFA on expression of anabolic and catabolic genes. Firstly, we examined the expression of two key muscle

developmental genes, myogenin (MyoG) and myogenic differentiation 1 (MyoD). Although not significant, the expression of these genes appeared to be increased following ER in FO, while unchanged in PLA. MyoG and MyoD are growth regulatory factors that are believed to play important roles in muscle regeneration and mTOR-pathway mediated MPS [258]. Their expression has previously been shown to increase with n-3 PUFA [94], [95]. Although we did not measure change in gene expression from baseline, our data suggest n-3 PUFA may modulate the change in expression of MYOG and MYOD during ER. Therefore, further research into the effects of n-3 PUFA on myogenic differentiation factors is warranted.

As muscle catabolism plays a crucial role in muscle protein turnover, we also examined expression of genes related to muscle protein breakdown. The majority of muscle protein breakdown is mediated by the ubiquitin-proteasome pathway (UPP), which uses ubiquitination to target proteins for degradation by proteasomes [225]. Although not significant, expression of genes encoding key ubiquitin ligases, MuRF1 and MAFbx, appeared to increase in PLA in response to ER. However, this pattern was reversed in FO. This observation is consistent with previous studies on the effects of n-3 PUFA on muscle atrophy in rodent models of cancer cachexia, energy restriction, arthritis and immobilisation, in which n-3 PUFA was able to attenuate increases in MuRF1 and MAFbx expression [92], [94], [105], [259]. In young women undergoing leg immobilisation for 2 weeks, n-3 PUFA significantly blunted the increased expression of MuRF1 at 3d, although this was not the case at 7d or 14d [106]. In this same study, MAFbx expression followed a similar pattern, with the increase in expression at 3d being lower in fish oil compared to control, although this difference was not significant. These authors suggest n-3 PUFA may blunt the early UPP response to a catabolic situation (in their case immobilisation). Our study only analysed gene expression pre-to-post 14d ER, and therefore we may have (partly) missed the window for the effects of n-3 PUFA on

ER-induced changes in UPP expression. Additionally, in our study, MAFbx and MuRF1 expression were lower at 14d post-ER compared to pre-ER, which is in contrast with the findings from McGlory et al. In their study, while FO seemed to attenuate the increase in expression of these genes, it was still elevated from baseline at 14d. Nevertheless, MuRF1 and MAFbx remain interesting targets for potential attenuation of muscle atrophy during energy restriction, and further research should aim to investigate the effects of n-3 PUFA on their gene expression, protein levels and activity.

MuRF1 and MAFbx are regulated by a variety of transcription factors, one of which is nuclear factor kappa B (NFκB). NFκB is a major protein complex that regulates a variety of functions, and its expression has been shown to be influenced by DHA and EPA [260], [261]. Interestingly, NFκB expression appeared to increase during ER in FO, while decreasing in PLA, although this was not significant. This was surprising, as we observed a (non-significant) reduction in the expression of downstream targets of NFκB, in MuRF1 and MAFbx, with FO. However, as NFκB subunits require translocation through the nuclear membrane to become active, changes in NFκB expression do not necessarily result in changes in activity [30]. On the proteome level, EPA has been shown to inhibit NFκB through IκBα, which prevents NFκB translocation [30]. As such, EPA inhibits NFκB-induced inflammation, resulting in reduced levels of inflammatory cytokines such as TNFα and IL-6 [257], [262]. Increases in inflammatory cytokines are common in disease-induced muscle atrophy, such as cancer cachexia and muscular dystrophy [262]. In C2C12 myoblasts, EPA has been observed to reduce TNF-α and IL-6 gene expression, indicating reduced activation of NFκB [257], [263]. However, information on gene expression of NFκB during energy restriction is limited. In one study, Carbone, et al. (2014) observed no changes in NFκB gene expression following 21d 40% ER in healthy men and women [166]. Given NFκB is involved in a plethora of processes related to cell

death and survival, it is unclear to what extent changes in NFκB expression during ER may influence muscle anabolic and catabolic processes. Therefore, further research is required to determine whether n-3 PUFA can significantly influence NFκB expression and activity during ER, and what effects these changes in expression may have on muscle anabolism and catabolism.

A previous study in energy restricted mice suggests downregulation of UPP-mediated muscle atrophy is mediated in part by lipoxygenase (LOX) enzyme activity[92]. Lipoxygenases are a family of enzymes, alongside cyclooxygenase (COX) and CYP450 cytochromes, that facilitate oxidation of PUFA [16]. By altering the availability of substrate (e.g. increase in EPA & DHA), these enzymes produce different eicosanoids that have varying effects on inflammation and other cellular functions. In myotubes, EPA reduced proteasome activity (i.e. UPP-mediated protein breakdown) through changes in LOX-associated eicosanoids, which in turn influence NFκB activity [264]. Similar to LOX, the cyclooxygenase (COX) enzymes also oxidise PUFA, resulting in different subclasses of eicosanoids. In our study, FO appeared to reverse the increase in COX2 gene expression during ER observed in PLA. This is consistent with a previous study in rats, in which n-3 PUFA blocked the increase in COX2 gene expression during muscle immobilisation, which was associated with reduced muscle atrophy [105]. In vitro cancer models also demonstrate that reductions in COX2-derived prostaglandin synthesis positively influence cellular development and exert anti-tumour effects [265]. Therefore, besides changes in eicosanoids produced by COX and LOX enzymes, n-3 PUFA also may influence expression of the enzymes themselves. Consequently, eicosanoids and eicosanoid-related enzymes are promising targets for n-3 PUFA-mediated modulation of cellular health. However, as eicosanoids perform important regulatory functions in terms of metabolic stress and inflammation, the consequences of changes in gene expression of eicosanoid production-related enzymes in varying conditions are still unclear.

Besides the UPP, autophagy is another key mediator of muscle protein breakdown. Autophagy occurs at a basal level, and greater activity can be induced in response to energy stress, such as during energy restriction [101]. During low energy availability, AMPK promotes autophagy through phosphorylation of ULK1. However, mTOR activity also regulates autophagy through ULK1, such that when mTOR is activated, autophagy is inhibited [100]. It has been suggested that despite being a catabolic process, autophagy should mainly be considered as beneficial for myofiber function and muscle health as it recycles damaged proteins and generates energy and materials for novel protein synthesis [224]. Therefore, autophagy may play a vital role in the maintenance of (healthy) muscle mass during energy restriction. N-3 PUFA have been suggested to influence apoptosis and autophagy in cancer cells, potentially by mediating responses to mitochondrial reactive oxygen species (ROS) in association with changes in Akt and mTOR signalling [227], [266], [267]. In our study, we attempted to investigate for the first time the influence of n-3 PUFA on expression of autophagy-related genes in muscle from healthy adults. Alongside ULK1, the phosphatidylinositol 3-kinase class III (PIK3-III) complex is another regulator of autophagy initiation [268]. Gene expression of beclin-1 (BECN1) and PIK3C3 (hVps34), two components of the PIK3-III complex, did not appear to change during ER in our study. PIK3C3 has been suggested to act as a nutrient-sensitive activator of mTOR, and it has been shown to be activated in response to REx and AA availability [269]–[271]. Nevertheless, n-3 PUFA did not affect expression of BECN1 or PIK3C3 in muscle of healthy individuals in our study. We also observed no effect of ER or n-3 PUFA on BNIP3 and BNIP3L (NIX), which regulate the breakdown of mitochondria in a process known as mitophagy [272]. We hypothesised that n-3 PUFA may influence the expression of these genes, as previous research suggests n-3 PUFA may alter mitochondrial function and biogenesis [273]. Finally, we also observed no changes in GABARAPL1 or MAP1LC3B (LC3B) expression during ER, and no effect of n-

3 PUFA. GABARAPL1 and LC3B regulate the formation of autophagosomes, and therefore changes in their expression may play an important role in regulation of total protein turnover [274]. In muscle wasting conditions, short-term energy restriction, and denervation, expression of PIK3C3, BNIP3, BNIP3L, BECN1 and GABARAPL1 have previously been shown to be upregulated [272], [275]. The discrepancy in changes in expression of these genes during ER in our study compared to other studies may be due to the timing of observations. Skeletal muscle mRNA was analysed pre-to-post a 14d ER, which may have been too large an interval to observe significant changes in the autophagy response. In addition, muscle was sampled immediately after REx, and therefore acute changes in gene expression may have occurred that obscured the effects of n-3 PUFA and ER. Finally, in a general sense, it must be considered that the proteins involved in autophagy themselves may experience increased breakdown when protein breakdown is upregulated [101]. Therefore, it is difficult to grasp the full picture of autophagy in muscle without measuring protein levels and their activity alongside changes in gene expression (i.e. by measuring autophagic flux) [276].

To gain further understanding on the role of n-3 PUFA status on muscle gene expression, we assessed the influence of ex vivo human serum on in vitro human skeletal muscle cells. In these cultured cells, conditioning with n-3 PUFA-rich serum appeared to differentially affect muscle developmental and autophagy-related gene expression. However, due to low sample size, this was a qualitative assessment only. Nevertheless, for some genes, n-3 PUFA appeared to induce similar gene expression responses in cultured skeletal muscle cells as noted in biopsied muscle (both in resting/fasting condition). Specifically, the pattern of differences between FO and PLA were the same for expression of MAFbx, between muscle tissue and cell culture, with an apparent suppression of activation by FO during ER. Differences in the expression of MyoD, MyoG and COX2

between FO and PLA also were similar between muscle tissue and cell culture, although the pattern did not match as closely. However, expression of the other genes (NFkB, BECN1, BNIP3, BNIP3L, GABARAPL1, MAP1LC3B and PIK3C3) did not follow the same pattern in cell culture as biopsied muscle. Interestingly, the expression of NFkB appeared suppressed by FO in cell culture, which matches our original hypothesis, unlike its expression in biopsied muscle. In muscle tissue, changes in expression of autophagy-related genes were very minor. In cultured muscle cells, the amplitude of differences between FO and PLA was larger relative to biopsied muscle. These observations suggest that n-3 PUFA-rich serum may suppress expression of BECN1, BNIP3, BNIP3L, MAP1LC3B and PIK3C3 during ER, compared to PLA. In our study, individuals were exposed to supplementation with FO or PLA for 4 weeks prior to pre-ER muscle biopsy sampling. On the other hand, cultured muscle cells were conditioned with FO or PLA serum only for 4 hours. Although n-3 PUFA may incorporate into leukocytes and platelets within hours, the time course of their incorporation into muscle is unknown [125], [126]. Therefore, differences in the expression of genes between biopsied muscle and cultured cells may relate to the effects of incorporated n-3 PUFA. On the other hand, similarities in the expression of some genes may indicate that circulating factors, such as eicosanoids or cytokines, may play a role in the changes in gene expression observed with n-3 PUFA supplementation. In addition, the different patterns of expression in skeletal muscle cells conditioned with postabsorptive/resting serum and exercised/fed serum may support an effect of circulating factors on the regulation of gene expression post-REx and protein feeding, and these interactions may further be influenced by the presence of n-3 PUFA. However, caution should be taken with these interpretations, as they concern a very small sample size, especially in the case of the cultured cells. Therefore, further research is required to determine the effects of n-3 PUFA on skeletal muscle developmental and autophagy-related gene expression, potential differences between the effects of incorporated and circulating n-3 PUFA, and potential interactions between n-3 PUFA and gene expression during ER.

In chapter 2, we observed considerable ambiguity in weight loss responses during ER. To better understand driving factors behind changes in fat mass during ER, and the potential influence of n-3 PUFA thereon, we investigated leptin concentrations in serum from pre-ER and post-ER. Leptin, which is secreted by adipose tissue, is known to be positively correlated with fat mass [137]. In our participants, those with high n-3 PUFA status (i.e. FO group) appeared to have higher leptin concentrations overall (although non-significant). However, this was to be expected, as participants in FO had higher total body and fat mass (chapter 2.3.3). When leptin concentrations were normalised for fat mass, there no longer was a difference between FO and PLA. However, the amount of serum leptin per gram of fat mass (normalised leptin) was significantly increased post-ER. The observed lack of effect of n-3 PUFA on serum leptin concentrations is consistent with most previous studies [137]. Of the previous studies that examined the effect of n-3 PUFA supplementation on leptin, only two studies using a long duration (10 and 24 weeks) observed a significant change. In myocardial infarction patients, 3g/d EPA+DHA for 10 weeks significantly lowered leptin compared to placebo [277]. At the same time, adiponectin was significantly increased by n-3 PUFA in this group. Although leptin may be a good reflection of adipose tissue status, the leptin-to-adiponectin (LAR) ratio has been suggested to be a better indicator of health, as it better represents the relationship with inflammatory markers [137]. Inflammation may seem primarily applicable to contexts of disease, which are associated with excessive levels of inflammatory markers such as TNF-α and IL-6 [58], [137]. However, inflammation also plays a key role in the regulation of muscle protein turnover, as it influences activity of TSC2/Tuberin, a key regulator of mTOR [96]. Therefore, changes in the concentrations of adipokines, resulting from changes in fat mass or n-3 PUFA supplementation may contribute to altered regulation of muscle protein turnover. The only other study that observed a decrease in leptin concentrations with n-3

PUFA, did so using a supplementation period lasting 24 weeks in overweight women, who also underwent 10% weight loss during the first 12 weeks[278]. In this study, the weight loss-induced decrease in leptin was greater in the n-3 PUFA group. Thus, n-3 PUFA may provide additive benefits to the regulation of leptin in overweight individuals undergoing weight loss. The observed ER-induced increase in normalised leptin (i.e. leptin per gram of fat mass) in our cohort of healthy active males is consistent with the notion that leptin concentrations reflect a relatively long-term response to changes in energy state[137], [279]. That is, the non-linear reduction in leptin relative to fat mass may reflect a natural delay between changes in serum leptin concentration and changes in fat mass. At the same time, the lack of an influence of n-3 PUFA on leptin over a duration of 14d ER is consistent with studies of similar duration[137]. The mechanisms for the effect of n-3 PUFA on leptin concentrations have been suggested to include changes in gene expression, changes in adipocyte size and improvements in insulin sensitivity[137]. Therefore, our findings and those of others suggest an influence of n-3 PUFA on leptin might require a longer timeframe. Nevertheless, it is possible that improvements in metabolic control with n-3 PUFA may in part be mediated by changes in the regulation of adipokines.

Besides the methodological shortcomings mentioned earlier, the experimental protocol that participants were subjected to also had several limitations. Muscle samples were only taken at the end of weeks 4 and 6, following the start of supplementation. Therefore, we were unable to examine the influence of increasing n-3 PUFA in muscle on molecular changes. Rather, we examined the influence of n-3 PUFA on the changes in signalling and gene expression from EB to ER. Thus, we are unable to assert whether molecular changes in muscle anabolic signalling and gene expression may improve in the initial period when individuals start increasing their omega-3 intake. Thus, we essentially assessed whether n-3 PUFA status influences the change in muscle

anabolic signalling during ER. In a wider context, it is therefore unclear if n-3 PUFA intake may improve muscle anabolism acutely. Individuals who wish to preserve muscle during involuntary energy restriction or other catabolic situations such as disease or immobilisation due to injury are unlikely to have undertaken such a 'loading' period. Although n-3 PUFA has mostly been investigated in the context of prolonged supplementation (4-12+ weeks), they also may influence cellular signalling and gene expression acutely, through interactions with G-protein coupled receptors and transcription factors[27], [280]. Therefore, further research is required to elucidate separate acute and chronic effects of dietary n-3 PUFA. Furthermore, the sample size used in this study was relatively small, which limits our ability to draw robust conclusions from our observations. For Western Blotting, a sample size of 14 was used (7 per group), and the results obtained were from single experiments without initial optimisation protocols. Recommendations for Western Blotting suggest experiments should include initial optimisation steps for protein loading and dynamic range[281], [282]. Besides mTOR and rpS6, we also set out to examine the phosphorylation status of p70s6k1 and 4EBP1, as additional key components of the mTOR pathway that are often assessed when examining muscle anabolic signalling changes in response to intervention. Unfortunately, due to further methodological issues we were unable to gather enough data for these proteins.

This was the first study to examine the effects of 4g/d n-3 PUFA for 6 weeks on the activity of anabolic signalling proteins in muscle from energy restricted healthy males. As such, we demonstrated a potentiating effect of n-3 PUFA supplementation on the ratio of phosphorylated:total protein of rpS6, but not mTOR, during ER. Our findings suggest n-3 PUFA supplementation may amplify some muscle anabolic signalling responses to anabolic stimulation through resistance exercise and protein feeding, during ER. Additionally, we did not observe any

significant changes in the expression of genes related to muscle development and autophagy, in muscle from energy-restricted healthy males. As this may have been due to limited sample size and technical limitations of the study, others may wish to study these genes for potential benefit of n-3 PUFA in the future. Furthermore, in cultured muscle cells, we were unable to show effects of n-3 PUFA-rich ex vivo human serum on expression of some genes related to muscle development. In these cells, n-3 PUFA-rich serum did appear to induce increases in insulin sensitivity, measured through the uptake of glucose. On the other hand, we observed no effect of n-3 PUFA supplementation on the increase in whole-body protein breakdown and fat mass-normalised leptin levels. Together, these findings implicate that there may be a beneficial role for n-3 PUFA in maintenance of healthy muscle protein turnover during ER. However, due to shortcomings of our study, we are unable to make robust conclusions. In addition, although n-3 PUFA may improve specific localised aspects of muscle metabolic health, these effects may not be large enough to induce changes on a whole-body scale. As our study concerned a small sample size, further research is required to establish whether n-3 PUFA may influence anabolic signalling and gene expression responses during ER in healthy muscle.

Chapter 4 – randomised controlled trial of n-3 PUFA supplements shows no acute influence on time trial performance in trained cyclists

4.1 - Introduction

Omega-3 polyunsaturated fatty acids (n-3 PUFA) have been observed to reduce oxygen consumption, heart rate and ratings of perceived exertion during exercise [111], [113], [114], [117]. Besides these changes in whole-body parameters of exercise function, improvements in overall muscle function [45], [46], [153], muscle fatigue resistance [111], [112], [114] and neuromuscular function [120] have also been observed with chronic n-3 PUFA supplementation. However, there are also reports of no observed changes in these same measurements with n-3 PUFA supplementation. And, despite some studies observing improvements in various individual aspects of exercise function, no studies have been able to demonstrate a beneficial effect of n-3 PUFA on exercise performance (reviewed in Chapter 1 – Table 1.2). As regular consumption of n-3 PUFA leads to their incorporation into phospholipid membranes of smooth and skeletal muscle, most studies investigating potential benefits on exercise function have employed prolonged supplementation periods. As such, the incorporation of n-3 PUFA, and particularly docosahexaenoic acid (DHA), has been suggested as the primary mechanism responsible for muscular improvements in efficiency of oxygen use and fatigue resistance [112]. However, the effects of n-3 PUFA on exercise function may not necessarily be dependent on their chronic incorporation into muscle cell membranes. Several studies showing improvements in parameters of vascular function within hours of their ingestion [283]–[286], suggest n-3 PUFA are able to exert acute effects. Nevertheless, studies investigating the influence of n-3 PUFA on exercise function have typically supplemented individuals with n-3 PUFA for ~4-8 weeks, while performing exercise tests in the fasted state or following a standard meal.

As vascular function is crucial for delivery of substrates to and clearance of metabolites from working tissue, acute increases in n-3 PUFA may aid exercise efficiency, that is, the demand placed on working tissues to perform a set amount of work. In addition, improvements in vascular function with n-3 PUFA may benefit exercise performance, and these effects may be independent from changes in muscle membrane incorporation [287]. N-3 PUFA have been observed to improve parameters of vascular function, with chronic supplementation [74], [111], [288]–[291], but also acutely following their ingestion (0-5 hours) in healthy adults [283]–[286], [292]. In resting conditions, n-3 PUFA-rich meals improve endothelium-dependent vascular reactivity and arterial stiffness relative to control meals. In addition, as one study observed, they may also be able to improve these markers of vascular function on an absolute basis compared to baseline [135], [283], [284], [292]. In the context of exercise, one study studied the effects of EPA and DHA-rich meals on vascular function during light exercise. In healthy men, a DHA-rich meal reduced systemic vascular resistance during light cycle ergometer exercise (25W + 25W/3min for 12 min), 5 hours following its consumption, although an EPA-rich meal did not [285]. However, as this was very low intensity exercise, further investigations in athletic settings may show further benefit of acute n-3 PUFA. Improvements in vascular function mediated by nitrate supplementation have been observed to result in improved exercise performance [293], [294]. Potentially, improvements in vascular function with n-3 PUFA could mimic those seen with nitrate supplementation.

The link between n-3 PUFA and improvements in vascular function is likely to be driven for a large part by eicosanoids. Eicosanoids, produced through free radical oxidation or in association with CYP450, COX or LOX enzymes, influence a wide range of physiological responses, including vascular tone, bronchial tone, leukocyte and platelet aggregation, and inflammation [16]–[18]. The increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS) with exercise promotes

release and oxidation of PUFA [72], [73]. In individuals with low n-3 PUFA status, eicosanoids derived from arachidonic acid (ARA) are dominant [14]. However, n-3 PUFA are known to compete with n-6 PUFA for binding with lipid peroxidation enzymes, thereby altering the profile of eicosanoids produced in contexts of oxidative stress [16], [74]. Separately, increased presence of n-3 PUFA may alter non-enzymatic eicosanoid synthesis through reduction of free radical production or scavenging of free radicals [295], [296]. Therefore, changes in eicosanoid production induced by the presence of n-3 PUFA may alter regulation of metabolic functioning [297]–[299]. This has been shown in a clinical long-term supplementation study, where n-3 PUFA-induced alterations in the eicosanoid profile were associated with improved endothelial function in patients with acute myocardial infarction [300]. However, n-3 PUFA administration may also alter eicosanoid production acutely. As such, one acute study demonstrated acute changes in production of eicosanoids LTB_4, TXB_2, 6-keto-PGF1α after 12-hour n-3 PUFA administration [301]. Additionally, changes in the production of eicosanoids have been observed within a 6-hour timeframe following the consumption of n-3 PUFA-rich meals [298]. In addition, concentrations of circulating ARA, and TXB_2, PGE_2, and PGF1α, have been observed to increase acutely during and after exercise in healthy males [73]. Therefore, increasing the availability of n-3 PUFA in circulation may alter the formation of eicosanoids acutely. However, whether acute changes in the eicosanoid profile, induced by n-3 PUFA supplementation, may result in improvements in exercise function remains unclear.

Although several studies have previously attempted to investigate the influence of n-3 PUFA on exercise performance, these have typically used chronic supplementation. Although this allows substantial incorporation into tissue such as skeletal and cardiac muscle, it does not allow for investigation of independent effects of circulatory n-3 PUFA. In addition, several studies investigating n-3 PUFA and exercise performance have used time to exhaustion/fatigue (TTE)

protocols [83], [115], [288], [302]. However, this type of protocol has been criticised as TTE is not a valid representation of real-world exercise performance [303]. Sporting events are typically time or distance based and therefore require a pacing strategy rather than simply exercising until exhaustion. In addition, TTE protocols typically have coefficients of variation (CV) >25%, likely due to differences in psychological factors [303], [304]. Real-world repeat performance is expected to vary by ~3-5% within any given athlete within the same season, although elite athletes may reach lower values of 1-2% [305]. Thus, any interventions which seek to improve exercise performance will need to control factors affecting the reliability of measurements to within reasonable margins of error. Additionally, as the size of the effect of nutritional interventions on exercise performance is typically very small (<10%), it is crucial to determine if any potential effects exceed the expected variation [306]–[308]. By calculating the typical error of the measurement, the smallest worthwhile change can be calculated as a threshold that needs to be exceeded for there to be a meaningful effect of the intervention [309].

The results of scientific studies in sport have traditionally been analysed using null hypothesis significance testing (NHST). In NHST, the null hypothesis of 'no difference' is evaluated using p-values, with 'significance' conventionally set at 0.05. By comparing an intervention group to a control group, a significant p value ($p < 0.05$) is often interpreted as there being an effect of the intervention. However, this is incorrect as p-values merely represent the probability of the observed data (or more extreme values) given the null hypothesis of 'no difference/effect', if the study was repeated an infinite number of times [210], [310]. A p-value does not actually inform us whether there is an effect, what size that effect may be, and how sure we can be that there is an effect. Thus, the ambiguity of the evidence surrounding the effects of n-3 PUFA on exercise function and performance may in part be due to statistical methods employed. As intra-individual changes in sporting performance, particularly with nutritional interventions, are small, a Bayesian

approach has been suggested to be better suited [210], [311]. Bayesian techniques use prior evidence or knowledge to parameterise distributions of probable outcomes, based on observed data [312]. Thus, we can use our knowledge of physiologically relevant outcomes to shape model posterior estimates. Probabilistic inferences can then be made from these posterior distributions. As such, we can determine the probability of an intervention effect, the size of such a potential effect and the degree of certainty in our observation.

Numerous studies have investigated the effects of chronic n-3 PUFA supplementation on exercise function and performance. However, n-3 PUFA may also alter various aspects of exercise function acutely, within hours of their ingestion. As such, n-3 PUFA may lower the physiological demand placed on the body during fatiguing exercise, and therefore also may lower psychological strain. However, there have been no randomised controlled studies on the acute influence of n-3 PUFA on exercise function and performance. Therefore, the primary aim of this study was to investigate the influence of fish oil supplements containing 5g EPA and 3.3g DHA, ingested 1 hour before the start of exercise, on time trial performance following a fatiguing 2-hour submaximal pre-load in trained male cyclists. Secondary aims were to assess oxygen consumption, respiratory exchange ratio, heart rate, mean arterial pressure, rate pressure product and rate of perceived exertion as parameters of exercise function during the submaximal pre-load cycle. Previous studies suggest pre-emulsification of PUFA improves their bioavailability [313]. Thus, we also aimed to compare the bioavailability of n-3 PUFA between standard fish oil and emulsified fish oil supplements.

4.2 - Methods

4.2.1 - Participants

We aimed to recruit 10 male trained cyclists for this study, however due to COVID-19 restrictions taking effect in March 2020, only the 7 individuals that had completed full testing were included. Participants trained in cycling at least twice weekly for 6 months prior to participation and were healthy as determined by our pre-participation health questionnaire. In addition, they did not already consume omega-3 fish oil supplements and did not regularly eat oily fish. Participant baseline characteristics are displayed in table 4.1. The study was approved by the University of Stirling NHS, Invasive or Clinical Research (NICR) ethics committee (NICR 17/18-039) and conformed to the regulations set out by the Declaration of Helsinki.

4.2.2 - Pre-screening

During an initial visit, participants provided written informed consent and low recent n-3 PUFA consumption was confirmed using a validated food frequency questionnaire [314]. Stature and mass were recorded before participants completed a two-stage lactate threshold (LT) and VO_{2max} testing protocol on a cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). During the first stage, participants cycled at 120W, with 30W added after every 3 min step. At the end of each step, blood lactate concentration from finger-prick blood samples was measured using a handheld lactate meter (Lactate Pro 2, Arkray Europe, The Netherlands). The LT test was terminated when an increase in blood lactate of 2 mmol/L relative to the previous step was observed. The LT was then estimated as the workload of the penultimate stage. After a 10-minute rest, a VO_{2max} test was started at the workload of the penultimate step from the LT test. The workload was increased by 30W every minute until failure. Participants were verbally encouraged to exert maximal effort. Oxygen consumption (VO_2), CO_2 production (VCO_2) and respiratory exchange ratio (RER) were determined using a face mask connected to an automated metabolic

gas analysis system (Oxycon Pro, BD, UK). Heart rate (HR) was monitored using a heart rate monitor worn around the chest (Polar, Kempele, Finland). VO_{2peak} was determined as the maximum average VO_2 recorded over a 30 second period. Finally, to determine baseline omega-3 levels, blood from finger-prick was placed onto two circular collection spots on Whatman™ 903 cards (Cytiva, Sheffield, UK). The cards were allowed to dry at room temperature for two hours before storage at -70°C until later analysis.

Table 4.1 – Participant characteristics at baseline. Anthropometrics were recorded during a pre-testing session. Fatty acid analysis was performed on samples of whole blood obtained during the pre-testing session. Abbreviations: oxygen consumption (VO_2), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), fatty acids (FA), polyunsaturated fatty acids (PUFA; i.e. omega-3 + omega-6), arachidonic acid (ARA).

Participants (n = 7)			
Age (years)	25 ± 4	**EPA (% total FA)**	0.61 ± 0.10
Stature (cm)	183.1 ± 5.6	**DHA (% total FA)**	2.02 ± 0.49
Mass (kg)	79.5 ± 9.2	**Total n-3 PUFA (% total PUFA)**	23.34 ± 2.12
VO_{2peak} (ml/min)	4905 ± 539	**ARA (% total FA)**	9.99 ± 0.60
VO_{2peak} (ml/kg/min)	62.0 ± 5.6	**ARA:EPA**	16.9 ± 2.9
PPO (W)	378 ± 70		

4.2.3 - Experimental protocol

Participants completed a familiarisation session and three supplementation trials on separate occasions, each separated by at least 7 days. Participants recorded their dietary intake in the 3 days prior to each visit using a food diary. In addition, they were requested not to undertake strenuous exercise in the 2 days prior to each visit. Participants arrived for testing in an overnight fasted state and completed a 2-hour submaximal cycle pre-load, which was followed by a work target-based time trial (TT). The workload for the pre-load was set at 95% of their pre-determined LT minus 10 Watts. During the pre-load, VO_2, VCO_2, RER, HR and rate of perceived exertion (RPE) were recorded every 15 minutes after the start of exercise. For RPE, participants were asked to rate their perceived exertion using the 6-20 Borg scale (Borg, 1982). In addition, every 30 minutes,

blood pressure (BP) was measured in duplicate using a manually inflated blood pressure cuff connected to a Greenlight 300 Sphygmomanometer (Accoson, Irvine, UK). From this data, rate pressure product (RPP) and mean arterial pressure (MAP) were calculated:

systolic blood pressure x heart rate = rate pressure product

*(systolic blood pressure + 2*diastolic blood pressure) / 3 = mean arterial pressure*

Participants consumed water ad-libitum during the pre-load. Following the pre-load, participants were allowed to rest for 5 min and to use the toilet if necessary. The time trial task required participants to complete a set amount of work in kJ. The formula to determine the target work to be completed was:

1800 seconds x PPO x 0.7 = work target in kJ

Where PPO is peak power output from the VO_{2max} test in Watts. As such, participants were expected to complete the time trial in 30 minutes (1800 seconds) if they cycled at 70% of their PPO. The ergometer was set up with a constant resistance factor α, which was determined by the formula:

$$0.7 \times PPO / rpm^2 = \alpha$$

Thus, participants were able to control their workload by altering their pedal cadence (rpm). Only the amount of work left to be completed was visible on a screen in front of the participants and they received no other inputs. Time to complete the work target was recorded as time trial performance in seconds.

4.2.4 - Supplement trials

Participants completed three supplement trials in double-blind randomised order. During these trials, overnight fasted participants initially rested while a cannula was inserted into an antecubital vein. Blood samples were drawn at the following time points relative to ingestion of the

supplement: immediately before (PRE), immediately after (0), start of exercise (60), 90, 120, 150, 180 minutes and after the time trial (POST; variable time ~5-10 min). Blood samples were drawn into K2E-EDTA tubes and were immediately placed on ice. At the end of the trial, blood samples were centrifuged at 3500 rpm, 4°C for 15 min and plasma was separated and stored at -70°C until further analysis. Following PRE blood sampling, participants consumed the test supplement in the form of 2 x 42g gel sachets with lemon flavouring to disguise the taste of the fish oil. The standard fish oil (FO-ST) and emulsified fish oil (FO-EM) supplements contained 2.5g EPA and 1.65g DHA per sachet, for a total dose of 5g EPA and 3.3g DHA, a dose previously shown to induce significant acute increases in plasma n-3 PUFA [313]. The placebo (PLA) supplement was designed not to induce any acute alterations in the plasma fatty acid (FA) profile of participants. As such, it consisted of a mix of palm and soybean oil (4:1 ratio) to represent the typical FA profile of the UK diet [315]. Participants were allowed to drink water with the supplement. Following ingestion of the supplement, participants rested until 1 hour after ingestion of the supplement, before commencing the pre-load cycle. The temperature in the laboratory was set to 19°C during the exercise trials.

4.2.5 - Analytical procedures

Whole blood on Whatman™ 903 collection cards and plasma samples were analysed by the Institute of Aquaculture at the University of Stirling, as previously described [183]. In short, the blood spots were placed into vials containing 1.25M methanol-HCl and placed in a hot block at 70°C for 1h. They were allowed to cool to room temperature, after which 2mL distilled water and 2mL of saturated HCl solution were added. Fatty acid methyl esters (FAME) were then extracted using 2 x 2 mL of isohexane. FAME were quantified by gas-liquid chromatography (GLC). Concentrations of individual FA are reported as a percentage of total FA, while concentrations of total n-3 PUFA are reported as a percentage of total PUFA (total n-3 plus n-6 PUFA).

4.2.6 – Data presentation and statistical analysis

All statistical analysis was performed using R (version 4.1.0) and Rstudio (version 1.4) for windows 10. We used Bayesian regression modelling with Stan [316] via the *rstanarm* package [317] to analyse the differences in exercise performance and efficiency parameters between supplemental groups. We made the reasonable assumption that each parameter was (approximately) normally distributed, and that the differences between supplemental trials were also (approximately) normally distributed. For each parameter, a linear model was created with weakly informative priors based on data from previous literature. Although the individual components of our exercise trials (i.e. submaximal pre-load and work-target time trial) matched previous research, the specific combined design was unique. Therefore, we opted for weakly informative priors to reflect a reasonable level of uncertainty in each parameter. Previous studies that have investigated the acute effects of nutritional interventions (e.g. β-alanine, nitrate) have observed effect sizes between 0-1. As the size of the effect of acute n-3 PUFA on each parameter is unknown, we used an effect size of 3 as a conservative estimate to inform our prior choices. The prior choices for each parameter are explained below. Effect sizes are expressed as Hedges' *g*, which applies a correction for small sample size [188], [189]. We also calculated the 90% highest density interval (HDI) for each parameter, which describes the most credible area of a distribution [318]. As such, the HDI encompasses the range of values containing 90% of the probability in the density plots.

Mean time trial time to completion (TT; in seconds) was compared between supplemental groups with a random factor for participant (id) to account for the repeated measurements:

$$TT = group + (1|id)$$

Parameters recorded during the 2-hour pre-load (VO_2, RER, HR, MAP and RPP) were modeled with fixed factors for time, group and their interaction, and with a random factor for participant (id)

using the *stan_lmer* function:

$$Outcome = group * time + (1|id)$$

For TT, the intercept prior was a normal distribution centered on the mean of all data (arithmetic mean of all TT times), with the standard deviation (SD) set as the SD of all TT times. The slope prior was set as a normal distribution centered on 0, with SD set as 3 times the estimated CV (3.35% from previous literature [304]) times the observed mean of all trials. Adjusting the width of the slope prior allows us to incorporate a certain degree of uncertainty in the expected effect of supplementation. However, as there is no information on the expected effect size of acute n-3 PUFA supplementation on TT, it was set to allow for uncertainty 3 times as great as the CV for work-target based time trial performance [304]. The prior for the residual variation (auxiliary) and the covariance were set as the default generated by *rstanarm*.

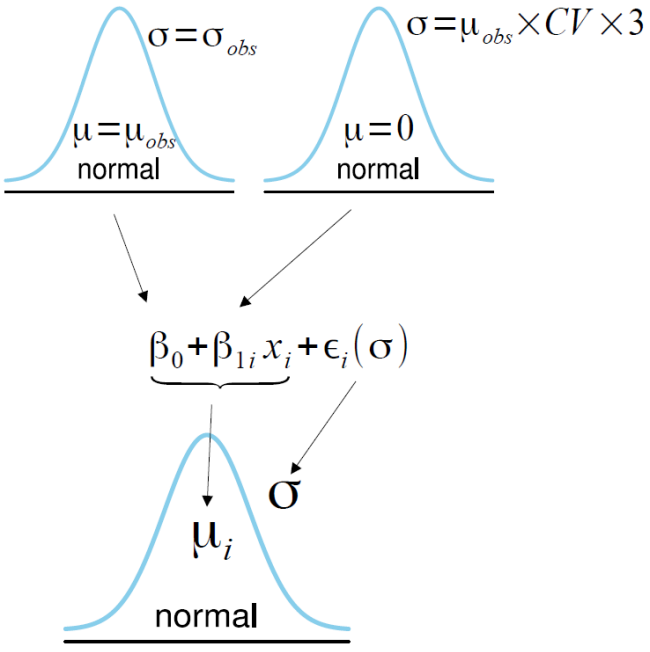

Figure 4.1 – Graphical representation of the Bayesian model used in this study. The physiological parameters (TT, VO2, RER, HR, MAP and RPP) are modeled by a normal distribution with a mean for each group composed of the intercept and slope coefficient from a linear model (β0 + β1). β0 and β1 have normal priors parameterised as in the figure. The prior for the intercept (β0) is set on the mean of all observed data (μ_{obs}) and the standard deviation of all observed data (σ_{obs}). The prior for slope (β1) allows for increases or decreases in each physiological parameter, with a mean set on 0 (no difference) and a standard deviation 3 times the expected CV (from previous literature) times the observed mean for each parameter.

The priors for the residuals of the linear model were set to the default generated by *rstanarm*. Note these distributions are not to scale. The graphical representation follows the style of Kruschke (https://doingbayesiandataanalysis.blogspot.com/2018/02/make-model-diagrams-for-human.html) and were made available by R. Bååth (http://www.sumsar.net/blog/2013/10/diy-kruschke-style-diagrams/).

For all parameters assessed during the 2-hour submaximal pre-load, informative priors were selected based on our own observations and previous literature. For VO_2, RER, HR, MAP and RPP, the intercept priors were set as normal distributions centered on the mean of all observed data, with SDs as the SD of all observed data. The slope prior was set as a normal distribution centered on 0, with SD 3 times the observed mean times the estimated CV (from previous literature). As the expected effect size of acute n-3 PUFA on VO_2, RER, HR, MAP and RPP is unknown, a variability 3 times as large as the expected CV was selected as a conservative estimate. Therefore, the wide variability in the slope prior accounted for the uncertainty in the expected effect size of acute n-3 PUFA supplementation on each parameter. The prior for the residual variation (auxiliary) and covariance were set to the default setting generated by *rstanarm*.

RPE data was treated as ordinal and therefore analysed using the *stan_polr* function, using only supplemental group, time and their interaction as factors:

$$RPE = group * time$$

RPE is a subjective measure of how hard someone feels they are working, which is loosely based on heart rate [319]. As such, the 6-20 scale reflects the range of heart rates from resting (~60) to maximum effort (~200). However, the relationship between RPE and HR is not linear, and may depend on exercise intensity and duration [320]. Although RPE data was collected as numerical values on the 6-20 point Borg scale, they were analysed using the descriptive categories within the range of reported values: *light (11-12)*, *somewhat hard (13-14), hard (15-16)* and *very hard (17-18)*. Thus, as the range between categories is not necessarily equal, but merely reflects the order of responses, RPE data was analysed using an ordered-logit model [321]. Using the *stan_polr*

function, the R^2 prior was set as a Dirichlet distribution with jointly uniform priors (all categories equiprobable), and with a mean of 0.5. As such, this reflected the default setting for ordinal models with weakly informative priors.

Following model parameterization, Markov Chain Monte Carlo (MCMC) sampling was used to establish posterior distributions of probable values for each parameter. Bayesian analyses require diagnostic checks that MCMC chains converge to the same value. The recommendations are to check the Rhat values and to plot the trace plots [322]. Rhat values were checked (all converged on 1.0), and trace plots are presented in appendix 4.1. To determine whether n-3 PUFA supplementation elicited a worthwhile change compared to placebo, the smallest worthwhile change was calculated for each parameter. For each parameter, the typical error for that type of measurement was calculated using the following formula [309]:

$$TE = CV * \mu_{PLA} \div 100$$

Change was therefore deemed 'worthwhile' when an improvement was seen that exceeded the TE of the control group (i.e. $\mu_{PLA} - TE$).

The relative concentrations of EPA, DHA and ARA (in % of total FA), and total n-3 PUFA (in % of total PUFA) were also modelled via the *stan_lmer* function. For each type of FA, the intercept prior was set as a normal distribution centered on the mean of all data, with the SD set as the SD of all trials. As there was no prior information on the expected CV of changes in the relative concentration of each FA, the empirical Bayes method was applied to set the slope priors [323], [324]. Empirical Bayes uses the observed data to inform priors. As such, each slope prior was set as a normal distribution centered on 0 with the SD set as 3 times the observed mean times the observed SD of all trials. To determine whether FO-ST and FO-EM elicited worthwhile changes in EPA, DHA, ARA and total n-3 PUFA, at each time point their values were compared to the 95th

quantile of PLA. As such, the probability of a worthwhile change in their relative concentration was determined with 95% certainty.

The CVs used in the selection of the slope priors and calculation of SWC were as follows:

VO_2 – 5.7% [325]

RER – 1.5% [326]

HR – 2.5% [327]

MAP – 8.4% [328]

RPP – 5.1% [328]

EPA – σ of observed data

DHA – σ of observed data

Total n-3 PUFA – σ of observed data

ARA – σ of observed data

4.3 - Results

4.3.1- Palatability of supplements

Two participants reported gastrointestinal distress during the placebo trial. One of these participants completed their trial whilst the other aborted their trial but completed it on another day. However, due to a blinding error, the participant consumed the wrong supplement on the repeat trial and no data was collected for the placebo supplement in this instance. Thus, data is reported for PLA (n = 6), FO-EM (n = 7) and FO-ST (n = 7).

4.3.2 - Time course of n-3 PUFA bioavailability

The time courses of appearance in plasma for EPA, DHA, total n-3 PUFA and ARA are displayed in figures 1-4. Baseline EPA, DHA, total n-3 PUFA and ARA were not different between trials.

The relative concentration of EPA, DHA, total n-3 PUFA and ARA did not change during the PLA trial. The relative concentration of EPA increased in both FO-ST and FO-EM (figure 4.2), but this occurred earlier for FO-EM than FO-ST. In addition, the peak of EPA was much higher in FO-EM than FO-ST. DHA also increased in both n-3 PUFA conditions, but this occurred earlier and to a much greater degree in FO-EM (figure 4.3). Total n-3 PUFA increased in both FO-ST and FO-EM, but this also occurred earlier and to a greater degree in FO-EM compared to FO-ST (figure 4.4). The probability of a difference in the relative concentration of ARA with FO-EM or FO-ST, compared to PLA, was very small (figure 4.5).

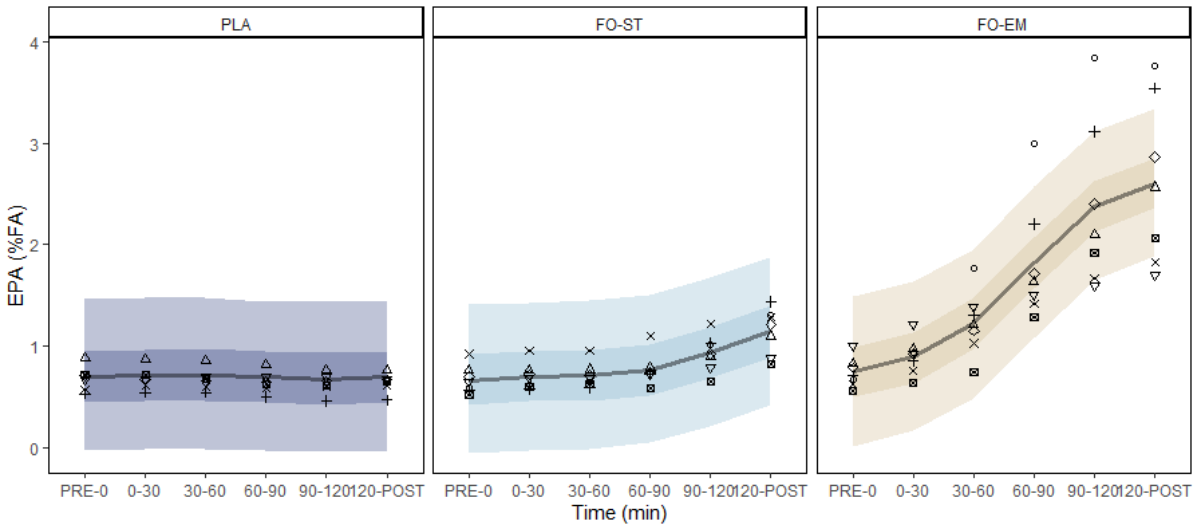

Figure 4.2 – Time course of eicosapentaenoic acid (EPA) appearance in plasma. Values are as a % of total fatty acids. PLA, placebo; FO-ST, fish oil standard oil; FO-EM, fish oil emulsion.

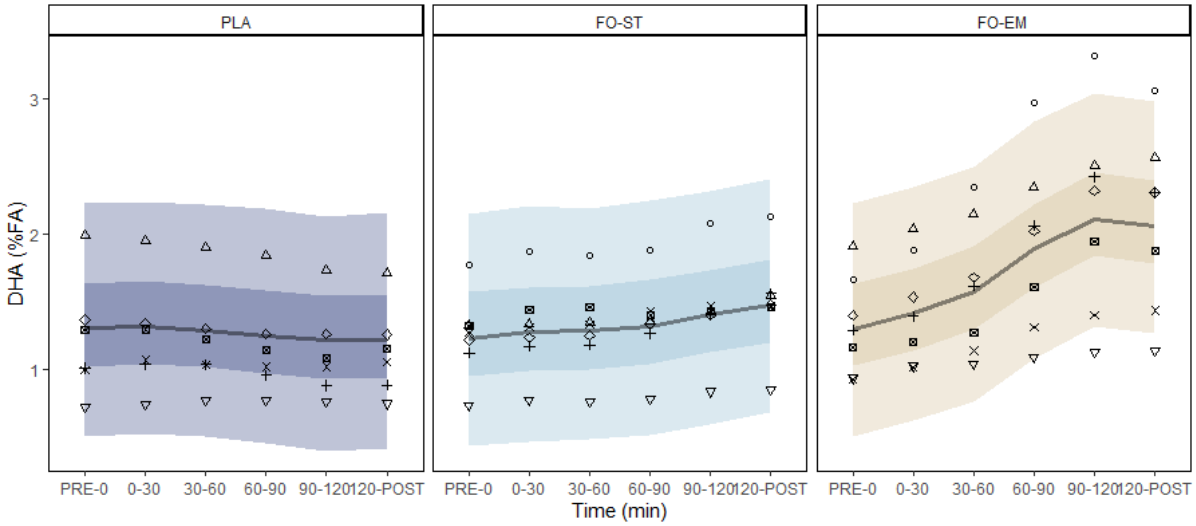

Figure 4.3 – Time course of docosahexaenoic acid (DHA) appearance in plasma. Values are as a % of total fatty acids.

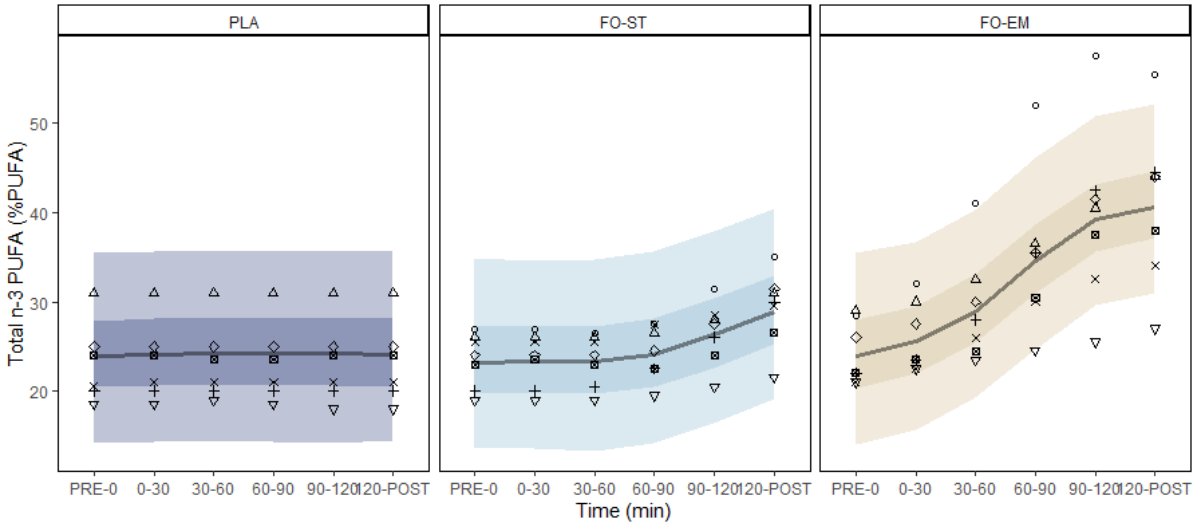

Figure 4.4 – Time course of total n-3 PUFA appearance in plasma. Values are as a % of total PUFA (n-3 + n-6).

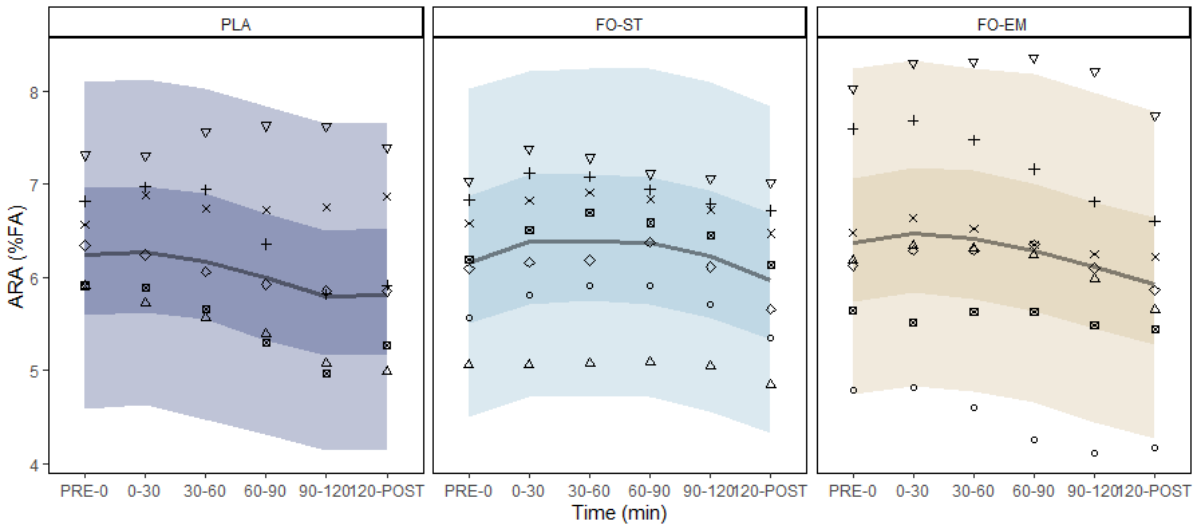

Figure 4.5 – Time course of arachidonic acid (ARA) appearance in plasma. Values are as a % of total fatty acids.

To determine the probability of a difference between the n-3 PUFA conditions and PLA, the posterior distributions of FO-EM and FO-ST were compared to the 95th quantile of PLA for each time point. As such, the probability of a difference between n-3 PUFA conditions and PLA was determined with 95% certainty (Table 4.2).

Table 4.2 – Proportion of posterior distributions for FO-ST and FO-EM exceeding the 95th quantile of PLA at each time point. Plasma fatty acid concentrations were determined before supplementation (PRE), immediately after ingestion of the supplement (0 min), every 30 min thereafter until the end of the pre-load (30, 60, 90, 120) and following completion of the time trial (POST). Abbreviations: eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (ARA) and omega-3 polyunsaturated fatty acids (total n-3 PUFA)

FO-ST	PRE - 0	0 - 30	30 - 60	60 - 90	90 - 120	120 - POST
EPA	0.02	0.04	0.04	0.11	0.52	0.93
DHA	0.02	0.03	0.04	0.11	0.28	0.38
ARA	0.03	0.08	0.13	0.28	0.33	0.11
Total n-3 PUFA	0.03	0.02	0.02	0.03	0.22	0.71

FO-EM	PRE - 0	0 - 30	30 - 60	60 - 90	90 - 120	120 - POST
EPA	0.09	0.33	0.97	1.00	1.00	1.00
DHA	0.05	0.12	0.45	0.97	1.00	1.00
ARA	0.10	0.12	0.14	0.21	0.20	0.08
Total n-3 PUFA	0.05	0.15	0.69	1.00	1.00	1.00

4.3.3 - Food diaries

To determine if participants successfully replicated their diet in the 3 days leading up to each trial, they were requested to complete food diaries. However, 3/7 participants were unable to provide complete food diaries for all trials. In total, 14/21 food diaries were returned by participants. Self-reported mean daily caloric intake was 2496 ± 937 kcal (range: 1506 – 3474 kcal). Self-reported mean carbohydrate intake was 51% of total caloric intake. Some participants (n =3) reported consumption of caffeine-containing beverages (coffee, tea) within 24 hours of testing days.

4.3.4 - Exercise performance

The mean workload completed by participants during the time trials was 505.8 ± 71.8 kJ, at a mean time of 2061 ± 348 seconds. The arithmetic means of time trial completion were 2037 ± 503 s for PLA, 2077 ± 322 s for FO-ST and 2064 ± 261 s for FO-EM (figure 4.6). The smallest worthwhile change (SWC) in TT was set as a 68 s improvement from the mean of PLA (2037 s). The probability that the improvement in TT exceeded the SWC with FO-ST was 0.217. The effect size of the *predicted* change in TT with FO-ST was *g* = -0.11 (95% CI: -0.15 – -0.06). The probability that the improvement in TT exceeded the SWC with FO-EM was 0.249. The effect size of the *predicted* change in TT with FO-EM was *g* = -0.35 (95% CI: -0.39 – -0.30).

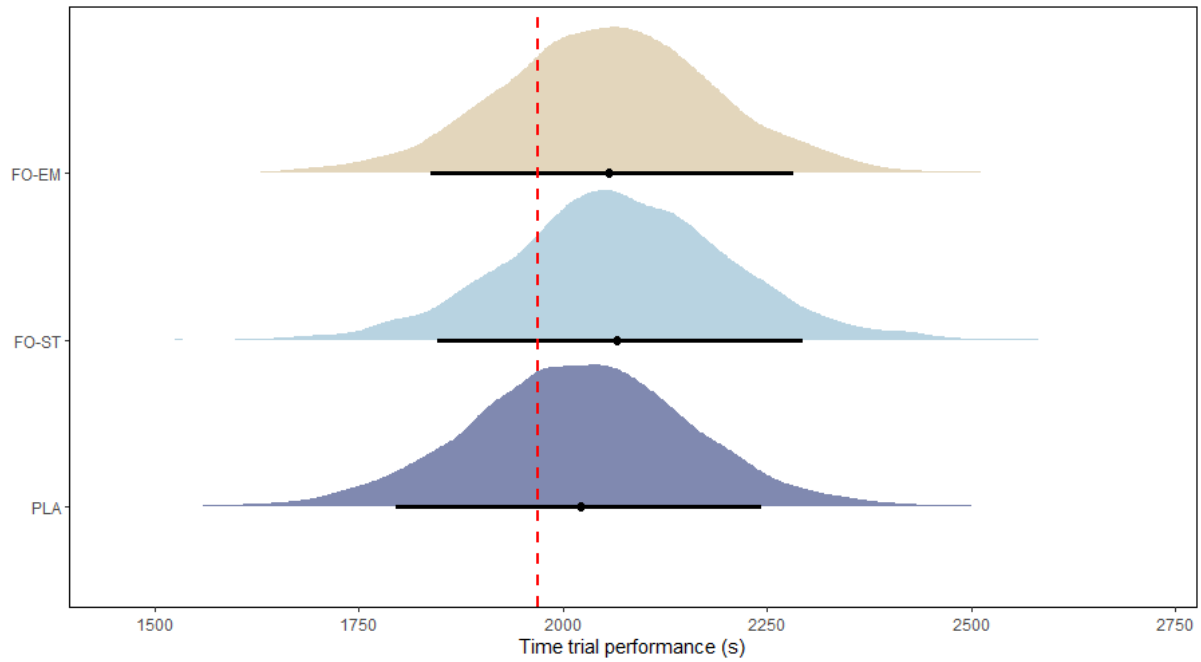

Figure 4.6 – posterior densities of time trial performance. The dashed line represents the smallest worthwhile change (SWC) in TT.

Table 4.3 – Posterior estimates, standard deviation and 90% highest density intervals (HDI) for exercise performance and efficiency parameters. The HDI describes the range of values encompassing 90% of the probability of the density plots. Estimates for FO-ST and FO-EM are relative to the estimate for PLA.

Parameter	Group	Estimate	Standard dev.	90% HDI
TT (s)	PLA	2021.7	137.3	1794.8 – 2243.8
	FO-ST	47.5	110.9	-141.6 – 226.2
	FO-EM	33.8	111.8	-152.3 – 219.2
	sigma	243.1	56.5	168.4 – 350.6
VO_2 (mL/min)	PLA	2901.5	158.9	2636.2 – 3155.2
	FO-ST	-20.6	64.3	-126.8 – 83.9
	FO-EM	-8.1	70.1	-120.5 – 108.7
	sigma	122.7	8.1	110.2 – 136.8
RER	PLA	0.925	0.020	0.893 – 0.957
	FO-ST	-0.018	0.012	-0.038 – 0.002
	FO-EM	-0.019	0.013	-0.040 – 0.002
	sigma	0.038	0.002	0.034 – 0.042
HR (bpm)	PLA	130.3	4.8	122.1 - 138.0
	FO-ST	0.202	2.25	-3.52 - 3.89
	FO-EM	-0.336	2.29	-4.03 - 3.43
	sigma	4.91	0.30	4.45 - 5.43
MAP (mmHg)	PLA	97.3	3.7	91.3 – 103.5
	FO-ST	-1.6	2.9	-6.4 – 3.3
	FO-EM	-0.8	2.9	-5.6 – 4.0
	sigma	4.8	0.5	4.1 – 5.6
RPP (mmHg x bpm)	PLA	19721.2	880.2	18293.2 – 21152.4
	FO-ST	-164.2	586.6	-1124.2 – 789.9
	FO-EM	353.6	572.2	-577.1 – 1303.5
	sigma	973.3	96.7	824.9 – 1143.1

4.3.5 – Oxygen consumption

To determine the influence of n-3 PUFA supplements on acute changes in exercise function, participants cycled at a mean constant workload of 197 ± 35W (48 ± 4% W_{max}) for 2 hours prior to TT. Oxygen consumption as a percentage of pre-testing maximum (VO_2peak) was 60 ± 8% in PLA, 60 ± 7% in FO-EM and 59 ± 5% in FO-ST.

The arithmetic means of VO_2 during the pre-load were 3051 ± 460 mL/min in PLA, 2917 ± 444 in FO-EM and 2891 ± 410 in FO-ST (figure 4.7A). The SWC for mean VO_2 was set at 2877 mL/min, a

reduction of 174 mL/min from the mean of PLA. The probability that the change in oxygen consumption exceeded the SWC with FO-ST was 0.455. The effect size of *predicted* change in VO_2 with FO-ST was *g* = 0.33 (95% CI: 0.32 – 0.35). The probability that the change in oxygen consumption exceeded the SWC with FO-EM was 0.334. The effect size of *predicted* change in VO_2 with FO-EM was *g* = 0.01 (95% CI: -0.00 – 0.03). There was no effect of FO-ST or FO-EM on the change in VO_2 over time (Figure 3B).

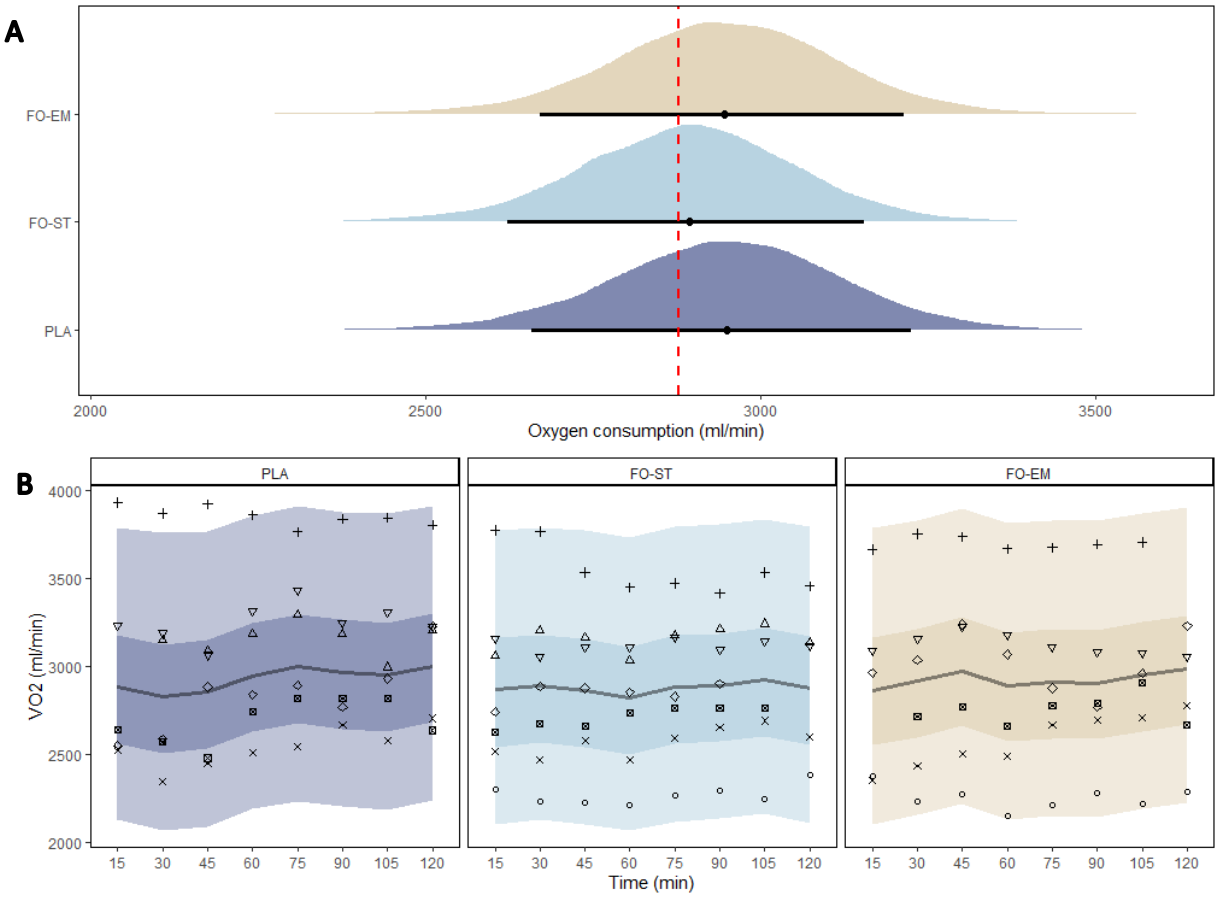

Figure 4.7 – **A)** Posterior densities of VO_2. The dashed line represents the smallest worthwhile change (SWC). **B)** predicted VO_2 by time, with original data points shown. Darker shaded regions represent 50% CI, while lighter regions represent 95% CI. Different shapes represent repeat data points from individual participants.

4.3.6 – Respiratory exchange ratio

Mean RER during the 2-hour pre-load was 0.915 ± 0.068 in PLA, 0.891 ± 0.046 in FO-ST and 0.898 ± 0.052 in FO-EM (figure 4.8A). The SWC for mean RER was set at 0.901, a 0.014 reduction from the mean of PLA. The probability that the change in RER exceeded the SWC with FO-ST was 0.630. The effect size of predicted change in RER with FO-ST was *g* = 0.92 (95% CI: 0.90 – 0.94). The probability that the change in RER exceeded the SWC with FO-EM was 0.546. The effect size of predicted change in RER with FO-EM was *g* = 0.74 (95% CI: 0.72 – 0.75).

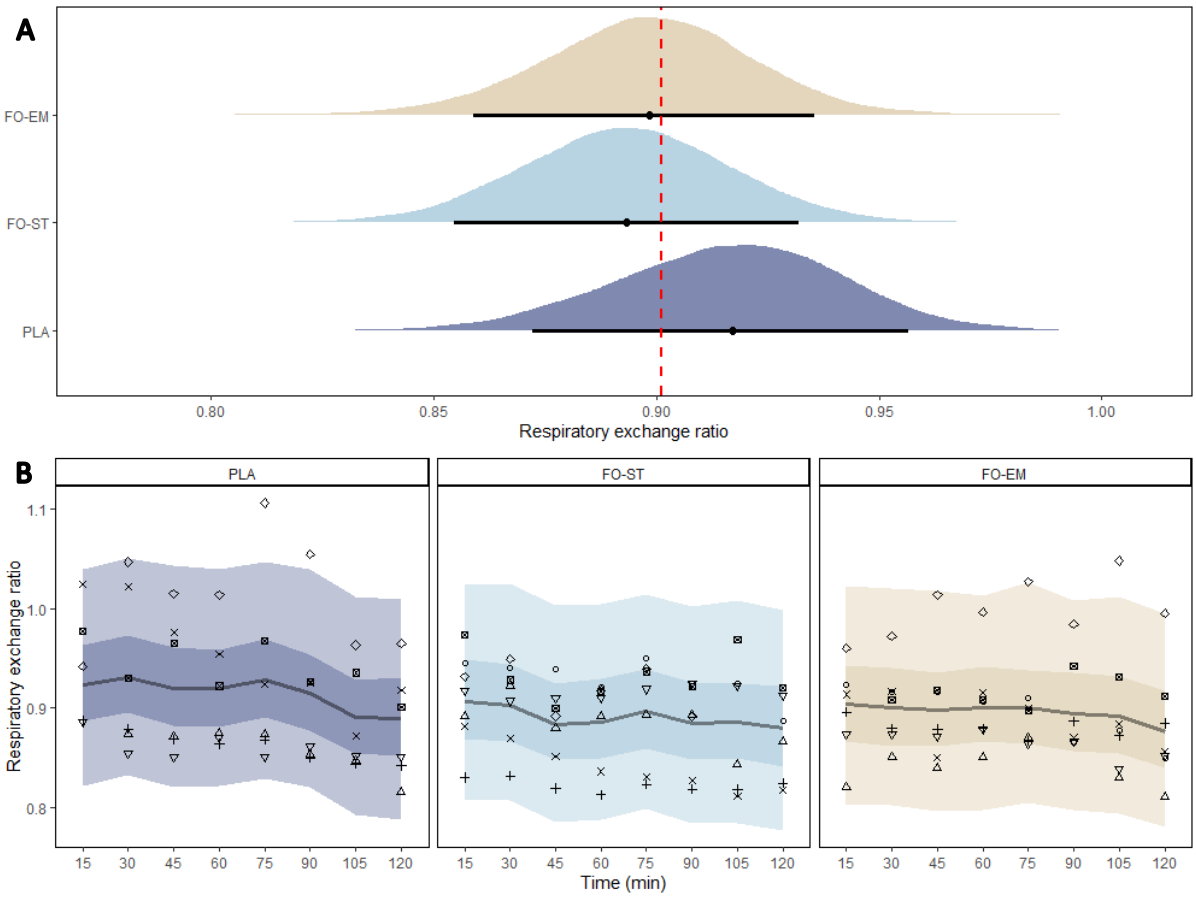

Figure 4.8 - **A)** Posterior densities of RER. The dashed line represents the smallest worthwhile change (SWC). **B)** predicted RER by time, with original data points shown. Darker shaded regions represent 50% CI, while lighter regions represent 95% CI. Different shapes represent repeat data points from individual participants.

4.3.6 - Heart rate

Mean heart rate during the 2-hour preload was 133 ± 11 bpm in PLA, 136 ± 14 bpm in FO-ST and 136 ± 15 bpm in FO-EM (figure 4.9A). The SWC for HR was set at 129 bpm, a 4 bpm reduction from the mean of PLA. The probability that the decrease in HR exceeded the SWC in FO-ST was 0.117. The effect size of the predicted change in HR with FO-ST was *g* = -0.03 (95% CI: -0.05 – 0.02). The probability that the decrease in HR exceeded the SWC in FO-EM was 0.123. The effect size of the predicted change in HR with FO-EM was *g* = -0.14 (95% CI: -0.16 – 0.13). There was no effect of FO-ST or FO-EM on the change in HR over time (figure 4.9B).

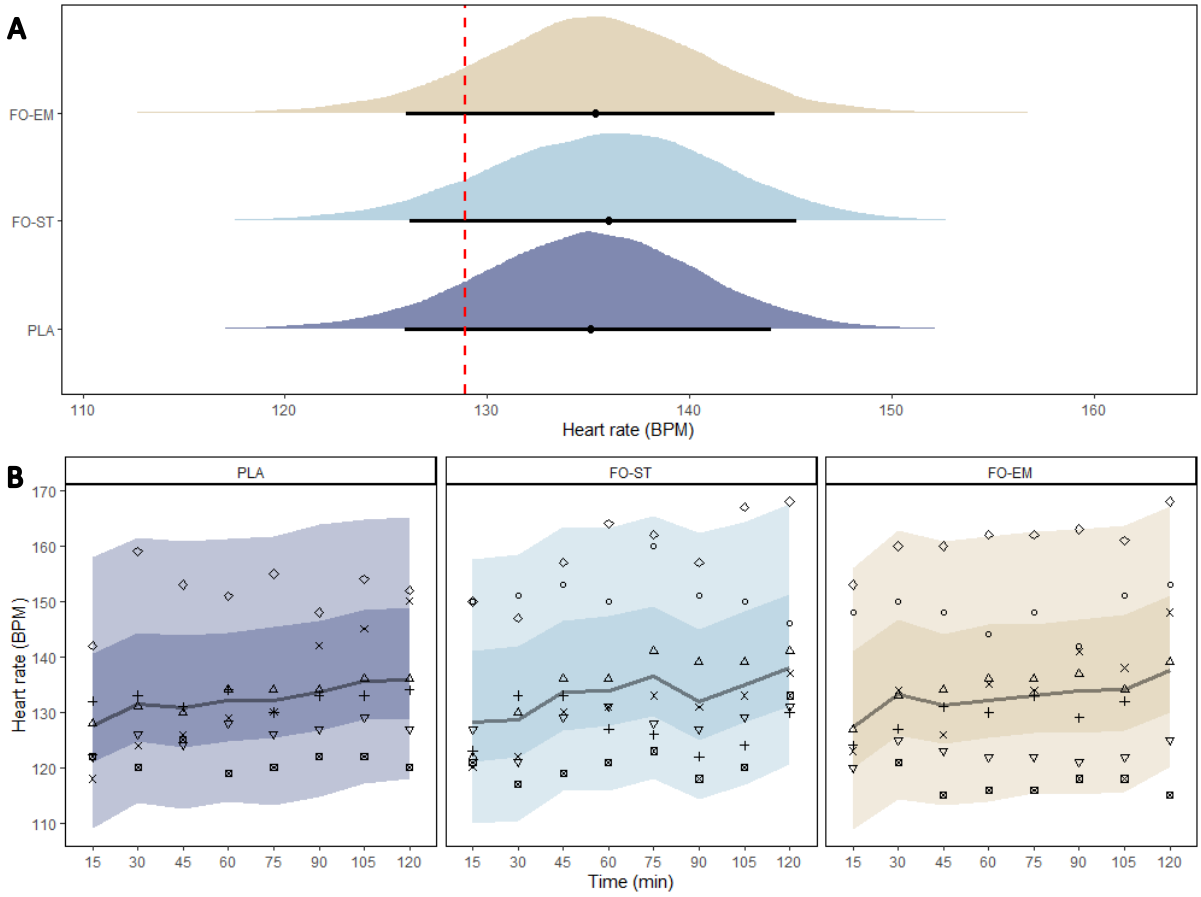

Figure 4.9 – **A)** Posterior densities of heart rate. The dashed line represents the smallest worthwhile change (SWC). **B)** predicted heart rate values by time, with original data points shown. Darker shaded regions represent 50% CI, while lighter regions represent 95% CI. Different shapes represent repeat data points from individual participants.

4.3.7 – Rate of perceived exertion

Mean RPE during the 2-hour pre-load was 13 ± 1 in PLA, 14 ± 2 in FO-ST and 14 ± 1 in FO-EM. Observed RPE values (11-18) were divided into categories *light* (11-12), *somewhat hard* (13-14), *hard* (15-16) and *very hard* (17-18), in line with Borg scale classifications. Over the duration of the pre-load, the probability of reporting a *light* RPE was no different between groups (figure 4.10).

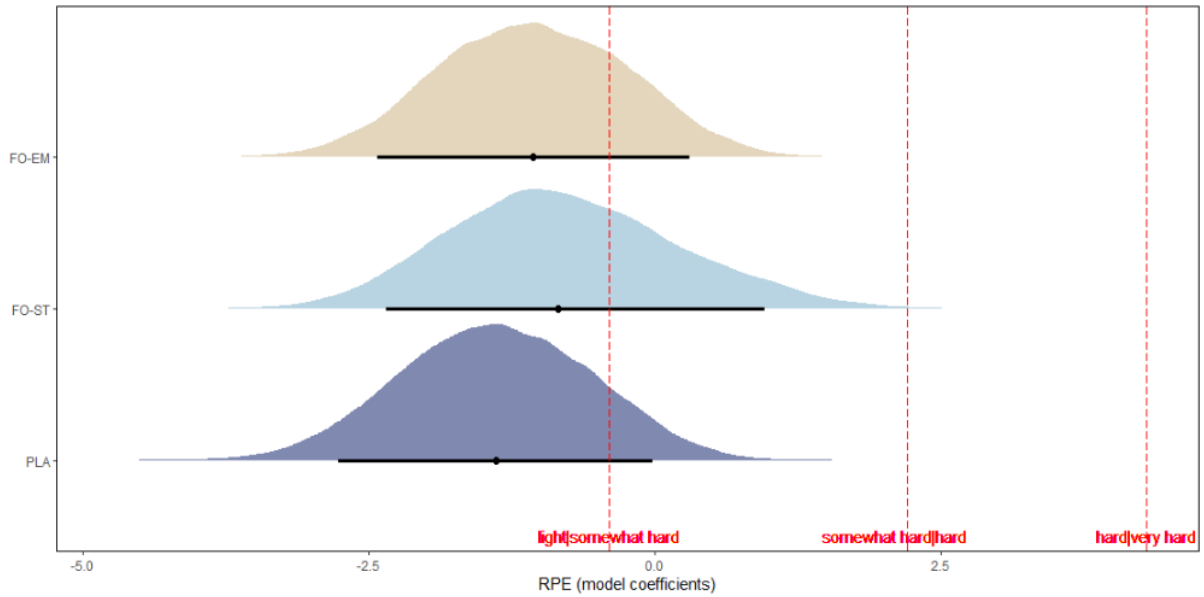

Figure 4.10 – Posterior densities of RPE. The x-axis represents the ordinal model coefficients. The dashed lines represent the model estimate of the transition boundary between different RPE categories.

Figure 4.11 displays the probabilities of participants reporting RPE values in *light*, *somewhat hard*, *hard* or *very hard* categories. During the initial stages of the pre-load cycle, the probability of participants reporting *light* or *somewhat hard* is high in all groups. The changes over time in the probability of reporting different RPE categories was similar between PLA and FO-EM. That is, the reduction in the probability of reporting *light*, the lack of change in *somewhat hard*, the increase in *somewhat hard* and the minor increase in *very hard* are all approximately equal and occur at the same time points in PLA and FO-EM. However, the probability of reporting higher RPE categories increases at earlier time points in FO-ST, compared to PLA and FO-EM. As such, the probability of reporting *light* or *somewhat hard* decreases at earlier time points, with concomitant increases in the probability of *hard* or *very hard*.

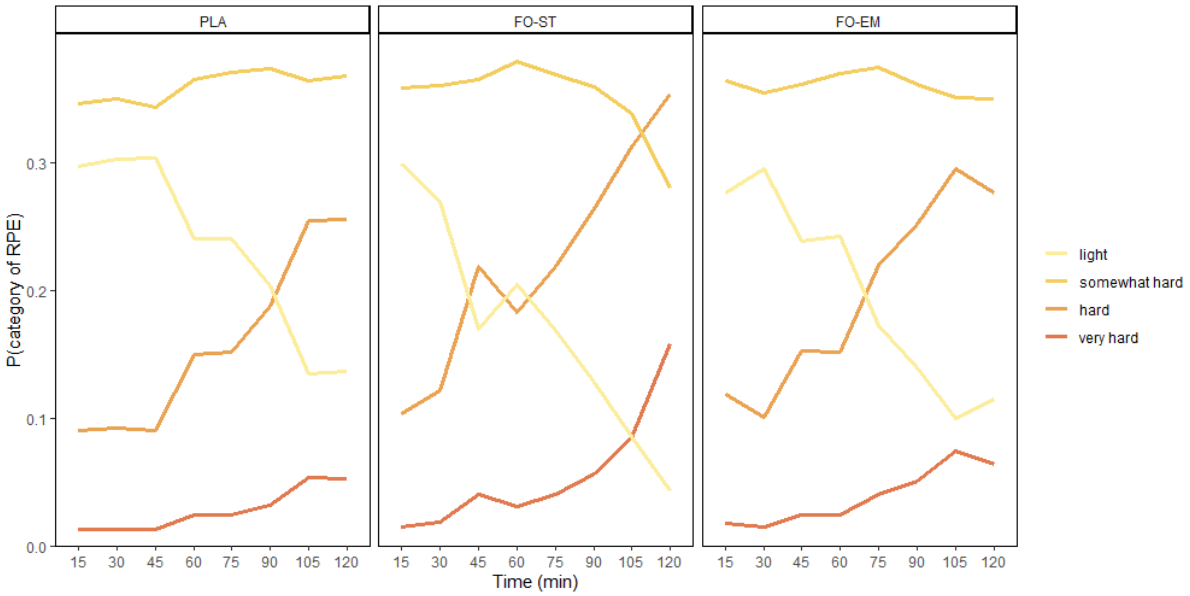

Figure 4.11 – Probability of participants reporting each RPE category during the 2-hour pre-load.

4.3.8 – Mean arterial pressure

Mean MAP was 98.8 ± 9.6 mmHg during PLA, 94.7 ± 8.6 during FO-ST and 95.0 ± 8.3 during FO-EM (figure 4.12A). The SWC for mean MAP was set at 90.7 mmHg, an 8.1 mmHg reduction from the mean of PLA. The probability that the decrease in MAP exceeded the SWC with FO-ST was 0.08. The effect size for the predicted change in MAP with FO-ST was *g* = 0.51 (95% CI: 0.49 – 0.54). The probability that the decrease in MAP exceeded the SWC with FO-EM was 0.10. The effect size for the predicted change in MAP with FO-EM was *g* = 0.58 (95% CI: 0.56 – 0.60).

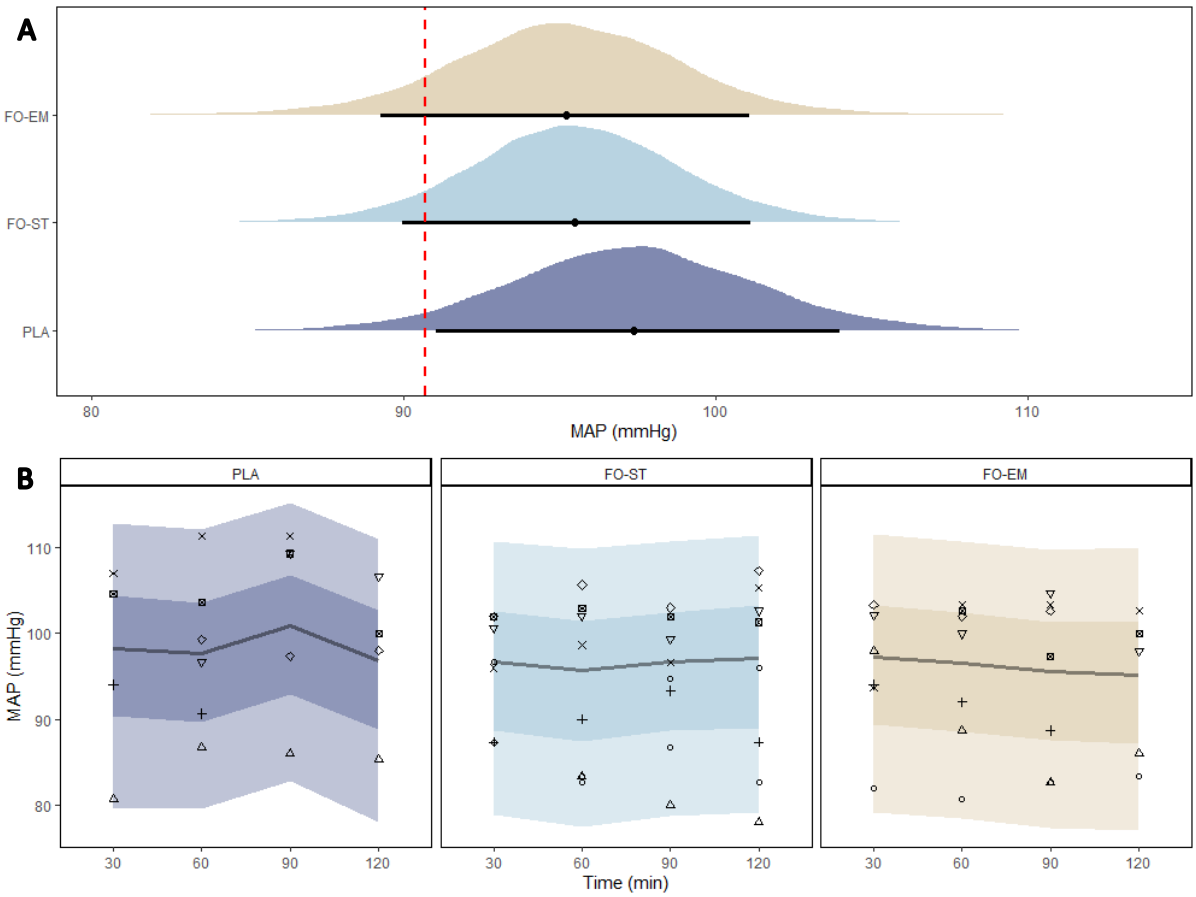

Figure 4.12 – **A)** posterior densities of mean arterial pressure (MAP). **B)** predicted MAP values by time, with original data points shown. Darker shaded regions represent 50% CI, while lighter regions represent 95% CI. Different shapes represent repeat data points from individual participants.

4.3.9 – Rate pressure product

Mean RPP was 20180 ± 1925 during PLA, 20125 ± 2446 during FO-ST and 20024 ± 2172 during FO-EM (figure 4.13A). The SWC for RPP was set at 19353, a 1029 reduction from PLA. The probability that the decrease in RPP exceeded the SWC with FO-ST was 0.176. The effect size for the predicted change in RPP with FO-ST was *g* = -0.19 (-0.21 – 0.17). The probability that the decrease in RPP exceeded the SWC with FO-EM was 0.131. The effect size for the predicted change in RPP with FO-EM was *g* = -0.16 (-0.17 – 0.14).

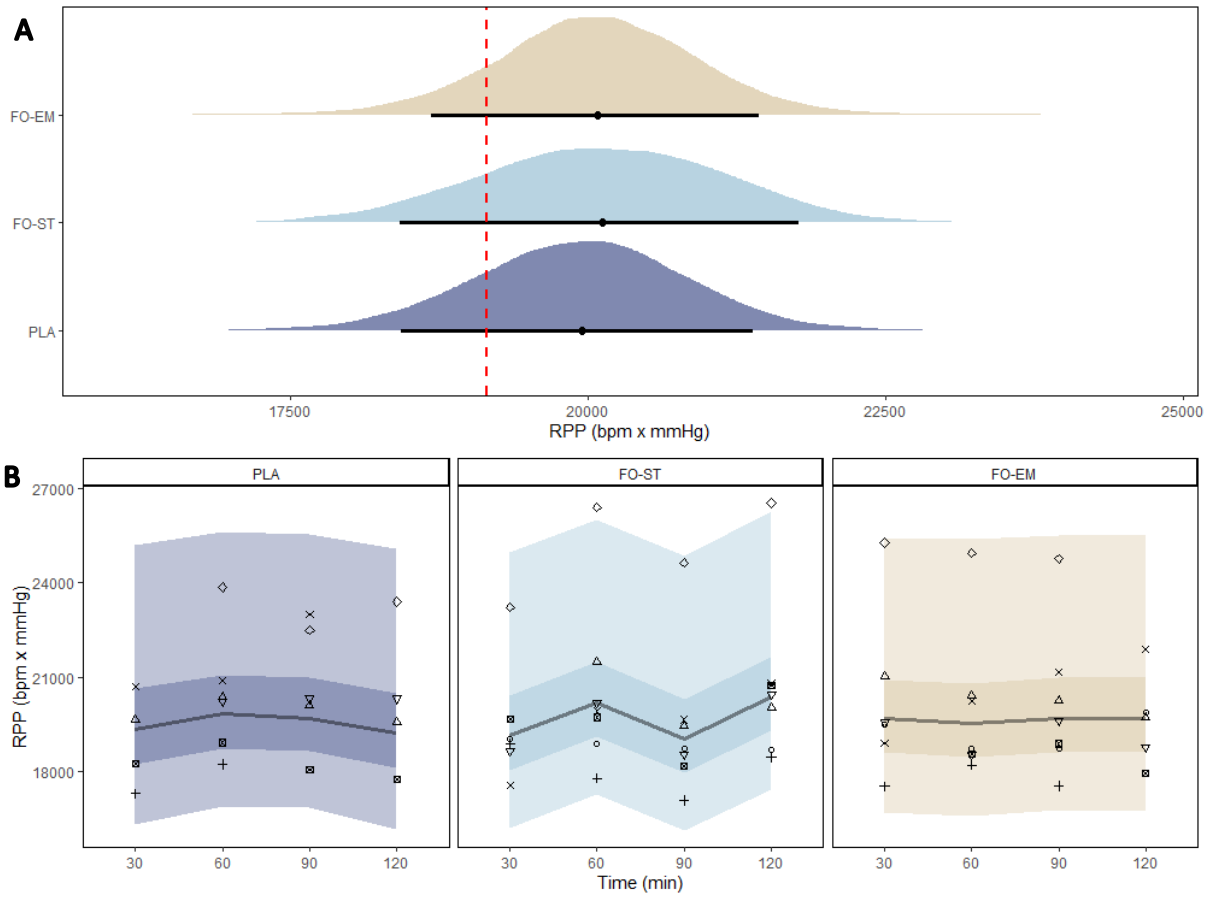

Figure 4.13 – **A)** posterior densities of rate pressure product (RPP). **B)** predicted RPP values by time, with original data points shown. Darker shaded regions represent 50% CI, while lighter regions represent 95% CI. Different shapes represent repeat data points from individual participants.

4.4 – Discussion

The primary aim of this study was to investigate the acute influence of n-3 PUFA supplements on exercise performance in trained cyclists. Baseline n-3 PUFA status was low, as indicated by relative whole-blood concentrations of EPA, DHA and total n-3 PUFA, and a mean ARA:EPA of 17:1 [124]. During the exercise trials, FO-ST and FO-EM increased the relative concentrations of plasma EPA, DHA and total n-3 PUFA, with no change in ARA. Despite higher bioavailability of the emulsified omega-3 supplement (FO-EM), neither FO-EM nor FO-ST elicited improvements in TT performance compared to PLA. Furthermore, n-3 PUFA supplements did not acutely influence HR, VO_2, RPE, MAP or RPP during submaximal cycling at ~60% of VO_{2max} / 48% of W_{max}. Both n-3 PUFA supplements lowered RER, indicating a potential increase in fat oxidation or carbohydrate-sparing. Using a Bayesian statistical approach, we determined there was a very low probability of a positive effect of acute n-3 PUFA supplementation, ingested 1-hour before the start of exercise (3-hours before TT), on exercise function and performance in our cohort of trained male cyclists.

Ingestion of n-3 PUFA supplements shortly before exercise did not improve time trial performance in trained male cyclists with low baseline n-3 PUFA status. Completion of the work target-based time trial was achievable in 30 minutes if participants cycled at 70% of their W_{max}. However, mean TT completion was ~14% higher than this threshold, indicating a substantial level of fatigue in participants following the 2-hour submaximal cycle. The lack of improvement in TT performance with acute n-3 PUFA supplementation agrees with previous research using chronic n-3 PUFA supplementation, which has consistently failed to observe improvements in performance [82]–[85], [111], [114], [115], [120], [122], [302], [329]. Only one previous study investigated the acute influence of n-3 PUFA on exercise performance [126]. During a maximal ramp test, n-3 PUFA reduced maximal HR and power output following a 3-hour infusion of n-3 PUFA emulsion at 0.6 g/kg body weight. An individual weighing 80 kg, the mean baseline weight of participants in our

study, therefore would have received a dose of 48g n-3 PUFA emulsion, of which 20.1% EPA and 18.4% DHA. This is equivalent to 10g EPA and 8.8g DHA, more than double the dose participants received in our study. The study by Delodder et al (2015) did not include a control condition however, and thus it is unclear if the reduction in power output was due to the large amount of fat delivered into the circulation rather than n-3 PUFA per se. Interestingly, the reduction in maximal HR and power output was also observed following oral consumption of the same dose of n-3 PUFA over 3 days. The authors suggest binding of n-3 PUFA to G-protein coupled receptor 120 (GPR120), and influence of n-3 PUFA on lipid rafts and mitochondrial membranes as potential mechanisms for the observed effects on exercise function. Interestingly, the study by Delodder et al (2015) also observed reduced lactate concentrations at exhaustion following both infusion and 3-day oral n-3 PUFA supplementation. Other studies with chronic supplementation have also observed reductions in lactate immediately after exercise [302], [330], although others also report no difference[85]. Reduced lactate concentrations in plasma may indicate reductions in its production or improved clearance. Unfortunately, we did not measure lactate concentrations during the experimental trials. However, the lowering of RER in both n-3 PUFA conditions indicates a shift towards higher fat oxidation and lower carbohydrate oxidation, and therefore may also suggest alterations in lactate production in our study. Nevertheless, the lack of an effect of n-3 PUFA on exercise performance in our study, combined with the observation that a higher dose infusion may be detrimental, suggests the supposed performance-enhancing effects of n-3 PUFA do not manifest acutely following their appearance in circulation.

Previous studies with chronic supplementation of n-3 PUFA have also been unable to detect improvements in exercise performance. The mechanisms responsible for potential alterations in exercise function with chronic n-3 PUFA have been suggested to be primarily related to the incorporation of DHA into working tissue [114], [331]. As such, a diet rich in DHA may reduce

muscle fatigue, potentially through improvements in efficiency of oxygen use or improvements in calcium cycling [112]. However, even studies that have supplemented participants with high-dose DHA have failed to show improvements in exercise performance, both in real-world and lab-based settings. That is, supplementation with 3.04g/d n-3 PUFA for 10 weeks in trained football players did not improve running TTE during an incremental workload test [115]. Trained cyclists, supplemented with 0.8g/d EPA and 2.4g/d DHA for 8 weeks, did not experience an increase in cycling TTE at 55% W_{max} [111]. Australian rules footballers also experienced no improvement in treadmill run TTE with 0.36g/d EPA and 1.56g/d DHA for 5 weeks [117]. In amateur cyclists, 120 mg/d EPA and 975mg/d DHA for 4 weeks did not improve TTE during an incremental workload test [302]. However, as all these trials used TTE protocols, which hold limited validity and reliability [303], [304], the likelihood of these studies reliably detecting potential improvements in endurance performance was low. The effect size of nutritional interventions on exercise performance is typically low (Cohen's $d < 0.5$), meaning the reliability of the test used is crucial if an effect is to rise above the 'noise' of variation in repeat measurements [308]. There are also indications that the effect size of nutritional supplementation during open-ended tests such as TTE may differ from fixed endpoint tests such as a race or TT [332], [333]. Thus, it is important that the tests used to measure the effects of nutritional supplements on performance match a real-world performance setting.

Others who have studied race time or TT, which are more reliable measurements of performance [304], [334], also observe no influence of chronic n-3 PUFA supplementation. In male marathon runners, 3.6 g/d n-3 PUFA for 6 weeks did not improve race time [83]. In trained cyclists, 140 mg/d EPA and 560 mg/d DHA for 8 weeks did not improve 5-min TT performance after a fatiguing protocol [114]. In male athletes, 375 mg/d EPA, 510 mg/d DHA and 230 mg/d DPA for 3 weeks, did not improve 250 kJ TT performance [120]. One study in trained cyclists, supplemented with 2g/d

EPA and 0.4g/d DHA, and using an exercise protocol close to ours in design, also observed no improvement in 10-km TT performance, at the end of a 3-hour submaximal cycle at ~57% W_{max} [82]. Another study in trained male cyclists using a work-target based time trial protocol, like in our study, also observed no improvement with 1.06g/d EPA and 0.75g/d DHA for 3 weeks [85]. Further, 240 mg/d EPA and 120 mg/d DHA for 6 weeks did not improve work target-based TT performance (designed to last ~60 min) in males and females [84]. Finally, 601 mg/d EPA and 253 mg/d DHA for 4 weeks did not affect 15 min TT work output in trained male cyclists[122]. Thus, it appears that our finding, that n-3 PUFA supplements do not improve exercise performance within hours after their consumption, agrees with results from chronic n-3 PUFA supplementation studies varying in dosage and duration.

Contrary to chronic supplementation, we hypothesised n-3 PUFA ingested shortly before exercise would acutely affect exercise function through mechanisms unrelated to their incorporation into muscle phospholipid membranes. N-3 PUFA, and particularly EPA, can be incorporated into phospholipid membranes of leukocytes and platelets within 60 min of their infusion, although the acute time course for other types of tissue such as muscle is unknown [125], [126]. Nevertheless, acute metabolic effects of n-3 PUFA have been suggested to be mediated by direct action of PUFA and eicosanoids [126]. By binding to G-protein coupled receptors (GPCR), which are expressed in macrophages and other immune cells, n-3 PUFA may influence production of (inflammatory) cytokines [27], [28]. These cytokines act as signalling agents to mediate tissue responses during and after metabolically stressful situations such as exercise [89]. Additionally, PUFA-derived eicosanoids contribute to regulation of cardiovascular responses during (exercise-induced) oxidative stress [57], [76], [297]. Production of specific eicosanoids has been shown to increase during exercise, potentially as a mechanism to regulate vascular function [66], [73]. We therefore hypothesised that increasing the availability of n-3 PUFA during exercise would alter eicosanoid

production, which in turn would result in improved vascular function and ultimately performance. The lack of change in MAP and RPP in our study suggests such an effect did not occur. One explanation for the lack of an effect may be the reduction in RER we observed, which indicates fat oxidation was increased. As such, this may have limited the availability of n-3 PUFA for formation of eicosanoids, and any consequent beneficial effects on vascular function these may have had. Future research which combines measurement of eicosanoid concentrations with functional outcomes during exercise may provide further insight.

In our study, both FO-EM and FO-ST successfully increased relative concentrations of plasma EPA, DHA and total n-3 PUFA. As pilot testing and previous literature [313] suggested peak n-3 PUFA levels occurred ~2-3 hours following ingestion, participants consumed the supplements 1 hour prior to the start of exercise. As such, in FO-EM, peak plasma levels of n-3 PUFA were reached during the latter stages of the submaximal pre-load and the performance task (Figure 1). Thus, if n-3 PUFA were able to influence exercise function and performance in this way, we would expect to have had a good chance to observe changes in these parameters. Knowledge on the mechanisms responsible for eicosanoid production, and therefore also the time course of their production, is however limited. Assuming n-3 PUFA influence exercise function through changes in eicosanoids (or other regulators such as nitric oxide), it may be the case that a longer period is needed between elevated plasma levels and purported effects of eicosanoid activity. In studies where (non-emulsified) n-3 PUFA were consumed as part of a high-fat meal, improvements in vascular function were typically observed after ~4-5 hours [283], [285], although others that observed earlier timeframes (0-2 hours) also noted improvements [284]. As a previous study demonstrated increased peak and total concentrations of plasma n-3 PUFA with pre-emulsified oil, we hypothesised FO-EM would have improved uptake and n-3 PUFA would appear in plasma earlier and with a higher peak [313]. This proved successful, with the increases in EPA, DHA and total n-3

PUFA occurring at earlier time points during exercise, and with a greater peak. It is possible that plasma levels of n-3 PUFA continued to rise post-TT in FO-ST, ultimately resulting in a similar bioactivity as FO-EM. However, the study by Garaiova, et al. (2007) suggests this is unlikely, as although they observed peak values in the oil condition after 6 hours, this peak was much lower than the peak of the emulsion condition. Regardless, as peak plasma n-3 PUFA levels were reached during the most intense period of exercise with FO-EM, we would have expected to observe its effects on parameters of exercise function and TT performance, if there were any.

As ingestion of n-3 PUFA influences vascular function acutely [283]–[285], generally indicated by reductions in markers of vasoconstriction, we expected to observe reductions in related parameters. Nevertheless, MAP and RPP were unaffected by n-3 PUFA in our study. One previous study, in which participants consumed EPA or DHA rich meals, observed reductions in systemic vascular resistance (SVR), diastolic blood pressure (DBP) and mean arterial pressure (MAP) during light exercise (25W + 25W/3min for 12 min) at 5h post-DHA meal. One previous study with chronic n-3 PUFA supplementation observed maintenance of increased plasma levels of n-3 PUFA following an overnight fast [82]. As such, it may be that changes in DBP [117] and RPP [111] during submaximal exercise following chronic supplementation, but with an overnight fast or standard (n-3 PUFA free) meal before the exercise trial, were due to effects of n-3 PUFA in plasma. Although we did not observe any effects of increased plasma n-3 PUFA on vascular function, previous observations appear in line with the suggested effects of long-term supplementation on general cardiovascular health. That is, n-3 PUFA is believed to create a less inflammatory, less thrombotic state, which is associated with reductions in plasma triglycerides (TG), atherosclerosis and cardiac events [5], [206], [280], [335]. The TG-lowering effect of n-3 PUFA has been suggested to be mediated by reductions in lipogenesis, but also increased fat uptake and oxidation [167]. This would appear consistent with the observed reduction in RER during exercise in our study. As such,

the direction of substrate metabolism towards higher fat oxidation may indicate consistency with the reductions in TG observed with chronic supplementation. Regardless of changes in the concentration of lipids in circulation, acute improvements in vascular function with n-3 PUFA may improve delivery to and clearance of substrates from working tissue [287]. Therefore, future studies may wish to investigate acute influences of n-3 PUFA on vascular function during exercise further.

Despite increased plasma levels of n-3 PUFA, HR during submaximal cycling was unaffected by acute n-3 PUFA supplementation. This is in contrast with the study by Delodder, et al. (2015), mentioned earlier, which observed a reduction in maximal HR following a 3h infusion of high dose n-3 PUFA. It is also in contrast with some chronic supplementation studies, that have observed reductions in HR at rest [134], during submaximal [111], [117], [336] and maximal exercise [81]. However, there are also studies that observed no effect of n-3 PUFA supplementation on submaximal or maximal HR [113], [122], [337], [338]. Thus, the effects of n-3 PUFA on HR during exercise remain ambiguous and may depend on dose, delivery method and population characteristics. Less ambiguous appears to be the effect of chronic n-3 PUFA on heart rate recovery following exercise, with consistent reporting of improvements [118], [119], [338], [339]. Unfortunately, we did not collect HR data following the time trial, and therefore it remains unclear if HR recovery may be improved with acute n-3 PUFA supplementation.

We did not observe an acute effect of n-3 PUFA on VO_2 during the submaximal cycle, despite previous reports that n-3 PUFA lowers oxygen consumption in this context, albeit with chronic supplementation. The study by Peoples, et al. (2008), reports reductions in whole-body oxygen consumption following 8 weeks of n-3 PUFA supplementation. Another study in trained cyclists supplemented with n-3 PUFA for 8 weeks also observed a reduction in oxygen consumption during

a 5 min TT [114]. In recreationally active males, n-3 PUFA for 8 weeks tended to lower oxygen consumption during submaximal cycling [113]. In this study, the pre-post supplementation change in VO_2 was significantly correlated with the change in erythrocyte EPA incorporation. Incorporation of EPA and DHA into erythrocytes is often used as a proxy for muscle incorporation, as these metrics are thought to be well correlated [15], [340]. The lack of an acute effect of n-3 PUFA in our study suggests lowering of oxygen consumption may be related primarily to incorporation of n-3 PUFA into muscle, as others have suggested [111], [112], [114], [341]. As we did not observe any changes in vascular function (HR, MAP, RPP), it remains to be seen if n-3 PUFA-induced improvements in these parameters may be able to improve oxygen kinetics by improving delivery of O_2 and clearance of CO_2. It also remains to be investigated if acute n-3 PUFA may influence maximal O_2 uptake. Several previous studies have suggested chronic n-3 PUFA consumption may improve maximal aerobic capacity (i.e. VO_{2max}), although reports are inconsistent [81], [85], [113], [115], [342]. Further research is required to determine if n-3 PUFA may alter oxygen consumption, at varying levels of exercise intensity, through acute mechanisms.

As it has been suggested the effects of n-3 PUFA may be more pronounced during oxygen-limited exercise [114], participants in our study completed a 2-hour fatiguing pre-load before completing the performance task. We did not observe differences in VO_2 with FO-EM or FO-ST, even during the latter stages of the pre-load when participants were fatigued. Interestingly, RER values drifted downward over the 2-hour window in all trials, which may indicate diminishing of carbohydrate stores. As such, although RER over the duration of the pre-load was lower in FO-EM and FO-ST, compared to PLA, there was no difference in the change over time. This suggests that the reduction in mean RER with n-3 PUFA was mainly driven by an increase in fat oxidation, rather than a decrease in CHO oxidation. Hingley et al (2017), similarly did not observe reductions in oxygen consumption during fatigue-inducing pre-load exercises, while it was reduced during the

subsequent time trial task. Time trials require exercising at near-maximal effort for prolonged periods, and therefore may be better suited for observing changes in mechanisms that are active during oxygen-limited exercise. As we did not collect data during the time trial other than completion time, it is unclear if n-3 PUFA may have influenced oxygen consumption during TT. Therefore, it also remains unclear if the oxygen consumption-lowering effect of n-3 PUFA is indeed mediated by incorporation of DHA into muscle, as has previously been suggested, or includes acute mechanisms. The observed reduction in RER during exercise in our study is in contrast with some studies, that observed no acute changes with n-3 PUFA as part of a meal [343], or with chronic supplementation [111]. However, others did observe reductions in RER following chronic supplementation, during exercise [343] and at rest [144], [171]. Therefore, the acute effects of n-3 PUFA on substrate metabolism, like its chronic effects, remain unclear and require further investigation.

Although this study employed a relatively strong exercise protocol to determine changes in performance and parameters of efficiency, it is limited by several factors. Firstly, the sample size of 7 was very low. Unfortunately, the lack of data for PLA in one participant limited the ability to account for repeat measurement in that case. Although we used statistical methods to account for small sample size, the results from this study should still be treated with caution. Previous studies, that observed changes in HR and VO_2 with n-3 PUFA, included a larger sample size (~16-30). Although we based our Bayesian prior selection on previous studies that have established coefficients of variation for the same parameters of exercise function, differences in study population, dosage and exercise protocol imposed a limit on the level of certainty in expected values. The strength in Bayesian statistics comes from incorporating prior knowledge, which allows us to update our expectations when new information becomes available. As we did not have much prior knowledge on the expected acute effects of n-3 PUFA on these parameters, we elected for

weakly informative priors. To improve our estimates, and therefore the level of certainty in our predicted probabilities, repetition of the trial would be necessary. In a general sense, this also applies to the wider field of sports science. For each given intervention effect, there are likely to exist conflicting reports, as we noted several times. Nutritional interventions are highly influenced by individual differences, and therefore treating intervention group outcomes as a range of possible values using confidence intervals or Bayesian posterior distributions, rather than relying on mean differences, is desirable [309], [344].

Although differences in other functional outcomes were minor (at most), the increase in RPE with FO-ST suggests there may have been a role of differences in FA structure on the ability to perform the required workloads. That is, FO-EM contained long-chain FA which were pre-emulsified, which aided their uptake and potential ability to be used as a fuel source. PLA contained a mix of palm and soybean oil, and therefore a larger proportion of short and medium chain triglycerides. The increased proportion of these shorter chain FA in PLA may therefore have improved their uptake in the digestive tract compared to FO-ST. The FA in FO-ST were largely non-emulsified n-3 PUFA, and therefore their uptake would have been strained compared to both PLA and FO-EM. Therefore, the uptake of different FA chain structures may have played a role on their ability to influence functional outcomes. Additionally, the droplet size of emulsified oils impacts their uptake in the digestive tract [345]. The increases in plasma n-3 PUFA levels suggest significant improvements in uptake of FO-EM compared to FO-ST, but further improvement may be possible with optimisation of droplet size.

Participants had a relatively high mean training status, as indicated by their pre-testing VO_{2peak} (62.0 ± 5.4). However, this parameter also contained considerable variability (range: 54-71 ml/min/kg). Participants with lower pre-testing VO_{2peak} had longer time trial times. A more

homogenous group of trained cyclists may have prevented the relatively large degree of variability in observations. Furthermore, although participants were requested not to undertake strenuous exercise in the 2 days leading up to each trial, this was only checked verbally as no training diaries were kept. The quality of food diaries was also low, for the most part. As such, we were unable to clearly determine if participants successfully replicated their diets before each trial. Some participants also reported consumption of caffeine on days prior to the trials, although this was consistent for each trial. Nevertheless, recommendations suggest that caffeine consumption should be avoided in the 48h before exercise testing, and it is unclear if caffeine may interact with the purported acute effects of n-3 PUFA. Therefore, differences in the lead-up to trial days may have obscured our ability to observe an intervention effect.

Consumption of 5g EPA and 3.3g DHA, shortly before exercise, did not improve exercise function and performance in trained cyclists with low omega-3 status. Despite higher bioavailability of emulsified fish oil supplements, FO-EM, like FO-ST, did not induce improvements in time trial performance. A Bayesian statistical approach was used to determine the probability of differences in functional outcomes with n-3 PUFA supplementation. As such, we observed that the probability of improvements beyond the smallest worthwhile change for HR, VO_2, RPE, RPP and MAP was low to very low. There was a moderate probability of a decrease in RER with both n-3 PUFA supplements, suggesting potential improvements in fat oxidation. However, the lack of improvement in other parameters of exercise function suggests these effects, which have previously been observed with chronic supplementation, may involve mechanisms related to their incorporation into muscle as others have suggested. Despite robust statistical methodology, the study had a low sample size, and further research is required to validate these findings.

Chapter 5 - General discussion

5.1 – Overview, aims and synthesis of findings

Researchers have been investigating the health modulatory effects of n-3 PUFA for decades. Initial interest stems from a 1971 report by Bang, Dyerberg and Nielsen that Inuit populations with high fish consumption had lower plasma lipid concentrations compared to Danish controls, which was suggested as a potential reason for low observed rates of cardiovascular disease and diabetes [42]. Although the notion that this population experienced lower rates of cardiovascular disease may not have been valid [346], a plethora of research has since attempted to elucidate this relationship and its associated mechanisms. In this time, considerable progress has been made to establish potential mechanisms of health modulatory effects of n-3 PUFA, even beyond the cardiovascular system. We now understand that n-3 PUFA and their oxidised products, eicosanoids, directly and indirectly influence the full scale of cellular functioning, from gene expression to macro-scale cellular structure and interactions [18], [347]. Largely, n-3 PUFA are studied for their potential to ameliorate or prevent aging or disease-induced perturbations such as excessive inflammation, impairments in metabolic control or loss of functional capacity.

However, from these decades of research also come suggestions that n-3 PUFA may be beneficial for 'healthy' people. As such, governing bodies for health around the world recommend consumption of oily fish at least once weekly, or regular supplementation with n-3 PUFA [124], [348]. Typically, these recommendations are based on their supposed protective effects on cardiovascular health, although evidence for this is still equivocal [349]. N-3 PUFA, and their proportion to other types of FA (saturated, monounsaturated and n-6 PUFA) influences cellular function through various pathways, which were set out in detail in **Chapter 1**. In summary, the availability of specific types of PUFA influences: 1) formation of eicosanoids, which act as signalling molecules; 2) intracellular signalling, through direct or indirect binding to G-protein coupled

receptors; 3) gene expression, through direct or indirect binding to nuclear receptors and transcription factors; 4) cellular membrane properties and function, as components of phospholipids and lipid rafts; 5) organelle membrane properties and function (e.g. mitochondria).

Many of the purported effects of n-3 PUFA have been demonstrated in isolated cell models, animal models and clinical human populations. However, there is a limited amount of information on the potential modulatory effects of n-3 PUFA on physiological responses to acute and chronic stressors in healthy human adults. As such, the overall aim of this thesis was to characterise the modulatory effects of increased n-3 PUFA levels, achieved through supplementation, on the response to acute and chronic metabolic stressors in healthy individuals. Building on previously available literature, which was reviewed in the general introduction (Chapter 1), we established the following thesis aims:

1. Investigate the potential for n-3 PUFA supplementation to reduce loss of lean mass during metabolic stress, in this case induced by diet-induced energy restriction, in healthy active young individuals.
2. Investigate the potential for n-3 PUFA supplementation to preserve metabolic and functional health outcomes during ER.
3. Investigate the influence of n-3 PUFA on muscle anabolic signalling and gene expression during ER.
4. Investigate the influence of ex vivo n-3 PUFA-rich human serum on in vitro muscle developmental gene expression and insulin sensitivity.
5. Investigate the time course of appearance in plasma of n-3 PUFA, with standard and emulsified supplements consumed 1 hour before exercise.

6. Investigate the acute influence of n-3 PUFA on whole-body metabolic responses to acute stress, induced by exercise.
7. Investigate the acute influence of n-3 PUFA on time trial performance following a fatiguing submaximal pre-load, in trained cyclists.
8. Investigate the difference in appearance of EPA, DHA and n-3 PUFA in plasma following acute ingestion of standard fish oil or emulsified fish oil supplements, during exercise.

These investigations were discussed in detail in the respective chapters, and their results are summarised here:

1. N-3 PUFA, consumed at 4g/d for 4 weeks prior to, and during a 2-week period of severe energy restriction (40%), did not influence changes in lean, fat and total body mass during ER in healthy, active males. (Chapter 2)
2. N-3 PUFA did not influence changes in whole-body resting metabolism and muscle function during ER. (Chapter 2)
3. N-3 PUFA potentiated muscle anabolic signalling responses to anabolic stimulation through resistance exercise and protein consumption, during ER. N-3 PUFA did not influence the loss of basal anabolic signalling during ER. N-3 PUFA induced small, but non-significant changes in expression of genes related to muscle development, during ER. (Chapter 3)
4. N-3 PUFA appeared to increase insulin-mediated glucose uptake in cultured skeletal muscle cells. In addition, although not statistically analysed due to limited sample size, n-3 PUFA appeared to induce minor changes in expression of genes related to muscle development and autophagy. (Chapter 3)

5. N-3 PUFA, consumed shortly before exercise, did not influence aspects of vascular function and exercise function during submaximal exercise in trained cyclists. (Chapter 4)
6. N-3 PUFA, consumed 3 hours before a cycling time trial at the end of a fatiguing 2-hour pre-load, did not influence exercise performance in trained cyclists. (Chapter 4)
7. The appearance in plasma of EPA, DHA and n-3 PUFA during exercise was quicker and had a higher peak with emulsified n-3 PUFA supplements, compared to standard oil. (Chapter 4)

5.2 – The influence of n-3 PUFA on loss of lean mass during energy restriction

There has been considerable interest from researchers into the potential for n-3 PUFA to modulate total body mass and fat mass, particularly in the context of obesity, which is reflected in the number of studies on these topics [128]–[130]. However, lean mass (or fat free mass), which also has an important metabolic role, is not reported in the majority of these studies. As discussed in **Chapter 2**, preservation of muscle mass is crucial for the maintenance of metabolic and functional capacity during catabolic states, such as anabolic resistance with aging, energy restriction, muscle disuse, disease, and weightlessness. Further, maintenance and optimisation of functional capacity is important for physical performance in athletes and military personnel. Therefore, we investigated the influence of n-3 PUFA supplementation on lean mass changes during a period of metabolic stress, in this case induced by negative energy balance, in healthy adult males. Participants achieved negative energy balance through maintenance of habitual training, and consumption of a 2-week, 60% energy diet relative to energy balance. This study was the first of its kind, as no others had investigated the influence of n-3 PUFA supplementation on lean mass changes during ER in healthy adults. Despite previous research which suggests n-3 PUFA may reduce muscle atrophy in different catabolic situations, we did not detect an influence of 4g/d n-3

PUFA supplementation on whole-body or site-specific changes in lean mass during ER. As such, the loss of lean mass (~1.1 kg) during ER in both groups mirrored the typical loss of lean mass in ER studies of this magnitude [147], [151]. Thus, in healthy individuals, who regularly exercise, and consume normal (recommended) amounts of protein, n-3 PUFA does not appear to provide any benefit to preservation of lean mass during ER. In our study, exercise during ER was not monitored. Future studies may wish to investigate interactions between n-3 PUFA supplementation and controlled exercise programmes and/or high-protein diets during ER. In older adults, provision of n-3 PUFA in addition to exercise training leads to bigger improvements in muscle mass and function [153]. In addition, the modulatory effects of n-3 PUFA on muscle mass and function appear to be greater in females compared to males [47], [106]. As we only studied males, the interaction between n-3 PUFA and lean mass loss during ER in females warrants investigation.

Nevertheless, other researchers have observed considerable ambiguity in the response of lean mass, fat mass and energy metabolism to increased n-3 PUFA intake. As such, although the changes in body composition and metabolism may be clinically relevant, their (limited) magnitude may prevent reliable detection using current study designs. Certainly, the lack of measures to control exercise training (other than verbal instruction) and diet (other than provision of all food and drink and verbal instructions) may have limited our ability to detect the (likely small) effects of n-3 PUFA, if there were any. The ambiguity of results regarding the influence of n-3 PUFA on whole-body energy metabolism and body composition reflects a complex system of metabolic regulation. That is, n-3 PUFA and n-3 PUFA-derived eicosanoids influence gene expression and cellular signalling and thereby may alter regulation of a wide variety of processes, likely in varying ways depending on cell type and location. Whole-body composition and energy metabolism reflect the total sum of all these processes. Therefore, the lack of conformity with molecular studies showing specific directional effects of n-3 PUFA may reflect this divergent nature.

Finally, we used a relatively severe weight loss model, which does not reflect current recommendations for healthy weight loss. In a general sense, the rate of weight loss has been found to be correlated with later weight re-gain [350]. Recent recommendations for weight loss in athletes suggest gradual weight loss, combined with resistance training, and high protein intakes of 1.6-2.4g/kg/day, are optimal for the preservation of lean mass and functional capacity[351]. Therefore, future studies may wish to investigate the influence of n-3 PUFA during ER on lean mass and exercise function using a study design that more closely resembles recent recommendations for healthy weight loss.

5.3 – *The influence of n-3 PUFA on molecular pathways regulating muscle development*

As changes in lean mass during diet-induced ER are believed to be largely determined by changes in muscle protein synthesis (MPS), in **Chapter 3**, we investigated the influence of n-3 PUFA on muscle anabolic signalling during ER. Here, the phosphorylation status of anabolic signalling proteins was intended to be read as a proxy for MPS. As such, we measured the phosphorylation status of anabolic signalling proteins mTOR and rpS6 in muscle collected during EB (pre-weight loss, but following 4 weeks of supplementation) and ER (post-weight loss, following 6 weeks of supplementation). We observed an augmentation of anabolic signalling (as phosphorylated:total protein of mTOR and rpS6) in response to anabolic stimulation with n-3 PUFA compared to PLA. This was in line with some (but not all) previous research, that also found amplification of anabolic signalling and MPS in response to anabolic stimulation with n-3 PUFA supplementation (Chapter 1 – Table 1.1). Certainly, an amplification of mTOR and p70s6k signalling, during ER, would seem beneficial during ER, when anabolic stimulation is sparse. Nevertheless, despite a potential improvement in anabolic signalling observed in **Chapter 3**, n-3 PUFA supplementation, presumably enough to achieve incorporation into muscle, did not affect the overall rate of muscle loss, as observed in **Chapter 2**.

Phosphorylation versus activity

Our initial aim was to build on knowledge from a previous study by McGlory et al. (2016), which had found reductions in activity of anabolic signalling proteins with minor but insignificant increases in MPS. The authors therefore suggested they may have observed an improved efficiency of signalling so that less activity was required for the same rates of MPS. However, MPS rates may have been maximally stimulated by resistance exercise and consumption of 30g high-quality protein. In our study, we therefore wished to investigate the interaction between n-3 PUFA and a

‘suboptimal’ dose of anabolic stimulation, in the form of resistance exercise and 10g protein (without ER). However, as we encountered methodological difficulties with Western Blotting, further limiting our already small sample size, we opted not to pursue this question. We therefore limited ourselves to answering only two key questions: 1) does n-3 PUFA preserve basal anabolic signalling during ER; and 2) does n-3 PUFA potentiate anabolic signalling in response to anabolic stimulation, during ER?

In addition, we were unable to make direct comparisons with the study by McGlory et al., as they had measured activity of anabolic signalling proteins directly, using the ATP Kinase assay [251]. We measured mTOR phosphorylation at Ser2448, which was intended as a proxy for mTOR activity. However, it has been suggested Ser2448 phosphorylation does not actually reflect mTOR activity, but rather is the target of p70S6k in a negative feedback loop [352]. In addition, we quantified rpS6 phosphorylation at Ser235/236, which was intended as a readout of upstream p70S6k activity. We had also aimed to quantify p70s6k phosphorylation at Thr389, and 4EBP1 phosphorylation at Thr37/46, but were unable to do so due to methodological issues. Nevertheless, quantitative analysis of mTOR and rpS6 signalling suggested an ameliorating effect of n-3 PUFA. However, treating phosphorylation status (relative to total protein), acquired via Western Blotting, as a quantitative measure of protein activity, may not have been wholly appropriate. As highlighted by McGlory, et al. (2014), Western Blots inherently possess a limited dynamic range, and may therefore be unable to detect the large fold changes in phosphorylation status. Additionally, p70s6k at Thr389 is known to have low baseline phosphorylation, and the large fold change following stimulation does not necessarily reflect a large change in activity. Others have suggested p70s6k only becomes phosphorylated at Thr389 once other parts of the protein are already highly phosphorylated [99], [252]. It has further been suggested that the phosphorylation of these residues operates under a ‘threshold’ concept, such that only a certain level of phosphorylation is

required for activation. From a mechanistic perspective, in which protein activity is dependent on location and association with other proteins, this certainly would make sense. For example, phosphorylation of 4EBP1 leads to its disassociation from eIF4E, which allows formation of the eIF4F complex and subsequent translation initiation [353]. In this context, it seems plausible that dissociation is triggered upon reaching a certain phosphorylation threshold. Any further phosphorylation beyond this threshold may only be relevant if it were to influence the duration of action. Thus, although measurement of phosphorylation of specific serine and threonine residues may give us some insight of upstream activity, quantitative interpretation may not be appropriate. This was further demonstrated by Greenhaff, et al. (2008), who observed a disconnect between signalling activity and MPS [249]. The mTOR signalling pathway reflects a complex cascade with several dynamic points of regulation, and it has been suggested that optimisation steps (e.g. determination of dynamic range, optimisation of protein loading) are required for reliable quantification by Western blotting [282].

Measurement

It should also be considered that measurements of protein status represent 'snapshots-in-time', and therefore may not fully reflect the total sum of activity. In our study (**Chapter 3**), we sampled muscle immediately after ("0 min", but was actually a few minutes after as they had to walk between laboratories), and 90 minutes after resistance exercise and 10g protein. As such, we aimed to determine the influence of n-3 PUFA on the change in anabolic signalling, from a basal to an anabolically stimulated state. The 90-minute time point was based on previous observations of peak insulin, EAA and MPS rates during the 60–90-minute timeframe following oral ingestion of protein [354], [355]. We did not have a true baseline of signalling activity, as we did not sample muscle before the resistance exercise bout. Therefore, some activity may have occurred acutely

following exercise, which may have had an influence on the phosphorylation status of proteins measured at the 0-minute time point.

Other models of assessing protein metabolism exist, which may be more suitable for assessing the effects of n-3 PUFA on muscle anabolism. Researchers have recently used deuterium oxide (D_2O) to measure rates of MPS over a duration of days-weeks [106], [356], [357]. Both MPS and MPB are dynamic processes, that fluctuate throughout the day in response to various stimuli. Different signalling pathways determine the overall muscle protein synthetic and breakdown responses, which may include feedback loops (e.g. myostatin) to regulate sustainment of these processes [358]. As such, a method which captures the total sum of these processes, and not just a snapshot at one time point, may provide better understanding of the mechanistic effects of n-3 PUFA supplementation. One study, that used the D_2O technique, was able to demonstrate preservation of MPS with n-3 PUFA, during a situation of muscle disuse [106]. Therefore, further research may wish to investigate the influence of n-3 PUFA on the overall muscle protein synthetic response over time during free-living ER conditions.

5.4 – Modulating periods of acute metabolic stress

The majority of research, particularly in exercise science, on potential health modulatory effects of n-3 PUFA, has focused on mechanisms related to the long-term incorporation of EPA and DHA into phospholipid membranes of working tissue (i.e. muscle). However, as was established in **Chapters 1 & 4**, n-3 PUFA may also have modulatory actions on aspects of physiological functioning in the immediate timeframe following their uptake. N-3 PUFA and n-3 PUFA-derived eicosanoids bind to G-protein coupled receptors (GPCR), transmembrane domains that bind extracellular molecules and regulate intracellular signalling pathways [27], [359]. Through binding to GPCR on leukocytes (of which macrophages have been studied in particular), n-3 PUFA may influence the acute

inflammatory response to oxidative stress [24], [27]. The ability for n-3 PUFA to acutely (within hours) influence local function was previously shown in haemodynamic responses to acute stressors [283], [284], [292]. These acute effects have been linked to endothelium-dependent (i.e. nitric oxide and prostacyclin (PGI_2), which act as vasodilators) and endothelium-independent mechanisms [283], [284], [360]. Eicosanoids are further suggested to influence respiratory function, potentially through changes in bronchial tone [18], [301], [359]. Through acute changes in the eicosanoid profile, it was therefore hypothesised that n-3 PUFA consumption shortly before exercise may improve exercise function. In **Chapter 4**, we attempted to investigate whether this acute response to n-3 PUFA ingestion may result in detectable changes in whole-body parameters of exercise function. Using a Bayesian statistical approach, we determined the probability of physiologically relevant improvements in parameters of exercise function was low-to-very low. By consuming n-3 PUFA supplements 1 hour before starting the cycling trial, EPA, DHA and total n-3 PUFA concentrations rose steeply and appeared to reach peak values during the TT task (3 hours following ingestion), particularly with the emulsified fish oil supplements. However, despite these large increases in plasma n-3 PUFA status, performance was not improved by n-3 PUFA. We did not measure eicosanoids or inflammatory markers. However, assuming our acute dose of n-3 PUFA was able to induce changes in the eicosanoid profile, this was not enough to provide an acute benefit to exercise function and performance on a whole-body scale. Future studies may wish to combine direct measurement of specific eicosanoids during exercise following acute ingestion of n-3 PUFA, with measurement of vascular function, to determine whether acute alterations in the profile of eicosanoids may improve exercise function.

Recovery, inflammation and oxidative stress

Several studies suggest n-3 PUFA may improve recovery from intense or damaging exercise, with reductions in subjective muscle soreness, muscle volume, and maintenance of range of motion [361]. Some studies have combined these observations of muscle damage with measurement of inflammatory (i.e. cytokines) and muscle damage (i.e. C-reactive protein, creatine kinase) markers [53], [362], [363]. As such, some have shown chronic n-3 PUFA supplementation inhibits the increase in, for example, creatine kinase, TNF-α and IL-6 concentrations following eccentric, damaging exercise [363]–[365]. However, others have also failed to show effects on these, and other markers [337], [365]–[367]. The discrepancies in these observations may stem from issues with the design of studies to assess muscle health after eccentric exercise, which are suggested to include a high risk of bias [53]. In addition, differences in the timing of measurement in relation to exercise, dose of supplementation, and heterogeneity in study populations, may also obscure our understanding around the effects of n-3 PUFA on recovery from exercise. Furthermore, some studies have attempted to measure changes in oxidative stress with n-3 PUFA supplementation using the thiobarbituric acid-reactive substance (TBARS) assay to assess lipid peroxidation. As such, some showed no change [84], or a reduction [367] in TBARS with n-3 PUFA. However, the TBARS assay as a method to quantify lipid peroxidation has been criticised due to its non-specificity [368]. Thus, generally, methodological issues may underpin the observed ambiguity of changes in markers of oxidative stress and inflammation with n-3 PUFA supplementation.

No pain, no gain?

While there are suggestions that n-3 PUFA may improve recovery from (damaging) exercise, the associated changes in inflammation may not necessarily be desirable at all times. As noted in **Chapter 1**, the production of reactive (oxygen/nitrogen) species contributes to force production

during exercise. In addition, the state of oxidative stress produced by exercise initiates an inflammatory response which mediates recovery from potential damage, but also adaptation for improved efficiency and function in the future [62], [65]. It remains to be determined whether supplementation with n-3 PUFA may inhibit training adaptation. That is, as n-3 PUFA and their derived eicosanoids are suggested to reduce the overall state of inflammation, could it be that they also impair training adaptation? In the context of NSAIDs, it has been suggested that their use may indeed do so [77], [78]. As NSAIDs inhibit the action of COX enzymes, which facilitate oxidation of PUFA, alterations in the n-3:n-6 ratio also may play an important role in balancing inflammation, resolution, and improvements in functional capacity of muscle [66]. Interestingly, studies investigating the effects of n-3 PUFA on various physiological parameters often include vitamin E (α-tocopherol) in the supplementation. As vitamin E is an antioxidant, it therefore may limit the oxidation of n-3 PUFA while it is being transported in the circulation, and thereby increase their chances to reach tissues. However, the increase in antioxidants may also limit their oxidation into eicosanoids during periods of oxidative stress. Therefore, it also remains to be determined if co-ingestion of antioxidants may impair the potential actions of n-3 PUFA by limiting eicosanoid formation.

Improvement?

The idea that n-3 PUFA improves exercise function, and therefore may also improve performance, in part comes from observations of reductions in oxygen consumption and heart rate during submaximal workloads. As such, it can be posited that a lower demand placed on cardiovascular and respiratory systems to perform the same amount of work is beneficial for fatigue resistance. Nevertheless, most studies that did find improvements in parameters of exercise function such as HR, VO_2 and RPE either did not measure exercise performance [111], [113], observed no

improvement [114], [117] or observed a detriment [126]. Thus, it appears unlikely that reductions in oxygen consumption or heart rate by n-3 PUFA supplementation can translate into performance improvements, and in fact none have been observed. N-3 PUFA are often suggested to be anti-inflammatory, likely because they compete with n-6 PUFA for production of eicosanoids, which act as inflammatory mediators [18]. However, at this point, it remains relatively unclear how alterations in eicosanoids and other inflammation-related signalling molecules influence smooth and skeletal muscle function. Although disease states are associated with increased levels of inflammatory cytokines, reductions in inflammation may not necessarily be desired in healthy individuals or in the context of exercise. Inflammation plays a crucial role in mediating responses to metabolic stress, thereby influencing gene expression and signalling networks related to repair of and adaptation to damage. Individuals who are unaccustomed to exercise, or those who undergo strenuous, damaging exercise, typically experience higher levels of muscle damage, and therefore soreness [369]. There is evidence that n-3 PUFA supplementation may reduce inflammation and muscle soreness, and it has therefore been suggested that n-3 PUFA may improve recovery from exercise [53]. However, at the same time, it has been suggested that maximising metabolic stress is beneficial for adaptation to endurance training, such as mitochondrial biogenesis and aerobic capacity [62]. Similarly, changes in type and size of muscle fibres may be influenced by the effects of n-3 PUFA on inflammatory processes [78], [370]. In rats, a n-3 PUFA-rich diet was demonstrated to inhibit recovery from muscle disuse atrophy, which was associated with reductions in Akt-p70s6k (muscle anabolic) signalling [371]. Similarly, improvements in adaptation associated with changes in the ratio of n-3:n-6 PUFA may be secondary to overall increases in PUFA available to cells, as ARA (20:4n-6) has been observed to increase eicosanoid-dependent muscle cell growth [372], just as EPA and DHA have [91], [92]. Thus, while n-3 PUFA may be advantageous for exercise recovery, and thereby performance during periods of intensified training or competition, the associated reductions in inflammation may actually limit positive aspects of muscle functioning.

5.5 – Studies in exercise science

Within exercise science, there naturally is a lot of interest in strategies to improve physiological functioning for performance. As discussed in **Chapter 4**, the results of scientific studies have traditionally been assessed using Null Hypothesis Significance Testing (NHST). However, NHST, and associated p-values were not meant to be interpreted the way they often are [310]. That is, although incorrect, researchers often interpret significant p-values as there being an effect of the intervention. Over the last two decades, within exercise science, efforts have been made to improve statistical methods to better evaluate intervention effects. As such, magnitude-based inference (MBI) was created and adopted by some, even though it was never published in a statistical journal, and its workings not shown [210], [373]. MBI is only available on the creators' own website, and is not accepted by many journals. After being challenged on their methodology [211], the authors also claimed that MBI is a Bayesian approach, although it is not [210]. Nevertheless, although MBI is not the answer, the intent to improve thinking around statistical approaches in exercise science was justified. Instead, researchers in exercise science are now advocating for the use of Bayesian statistics [311]. As we showed in **Chapter 4**, Bayesian analysis can effectively be applied to statistical evaluation of different types of data (e.g. linear models and ordinal models). As such, we evaluated the probability of an effect of n-3 PUFA compared to the smallest worthwhile change (SWC) for different functional outcomes during exercise. Several other researcher groups in physiology and exercise science have also recently published papers using Bayesian statistical approaches to assess, for example, the effects of peer pressure on ratings of perceived exertion, the effect of sleep deprivation on physical and cognitive performance, or the effect of acute glutamine supplementation on cycling performance [374]–[376].

In addition to changes in statistical approaches, improvements in study design and methodology are also required. Meta-analyses regarding the effects of n-3 PUFA on different physiological

parameters often conclude there is insufficient, or not enough high-quality evidence to support an effect (as discussed in **Chapter 1.10**). In addition, it was suggested by different authors that studies that did observe significant effects of n-3 PUFA were at higher risk of bias. As has been discussed throughout, there are many contexts where specific directional effects of n-3 PUFA failed to translate from in vitro or animal studies to human contexts. As Cunningham (2002) aptly stated: "a mouse is not a rat is not a human."[377] That is, while many metabolic mechanisms may act in similar ways in different organisms, there are many confounders to translating animal studies to human contexts. The causes of discrepancies between in vitro and animal, and human studies, and between different human studies, were elegantly laid out by Lalia and Lanza (2016) [21]. As table 5.1 shows, there are many possible factors that have introduced heterogeneity between different studies on the effects of n-3 PUFA, and they all contribute to our ability to make recommendations in a wide context. Therefore, it is important to consider the context in which findings were made.

Table 5.1 – Confounding factors in n-3 PUFA research. Figure adapted from Lalia and Lanza (2016) [21].

	Causes for discrepancies in studies of n-3 PUFA action
1	Dosage of n-3 PUFA
2	Ratio of EPA:DHA
3	Source of fish oil
4	Absorption and Bioavailability
5	Duration of intervention
6	Type of placebo
7	n-6:n-3 ratio
8	Type of cohort (age, weight, race, inflammatory status, metabolic status, comorbidities, usual dietary habits, lifestyle, physical activity levels)
9	Method of assessment
10	Preventive study or therapeutic
11	Size and power of study

The same is true for this thesis. Here, we evaluated the effects of n-3 PUFA on physiological outcomes exclusively in male participants. The aim of this was to reduce the heterogeneity of the population sampled in our studies. However, within exercise science, and science in general, women are historically under-represented [378], [379]. As was discussed in **Chapters 2 and 3**,

previous research on n-3 PUFA suggests their effects may differ depending on sex, with indications that they may be more pronounced in females. Therefore, it is entirely possible that we would have made different observations if we had included females in our studies.

Furthermore, to accurately assess the independent effects of n-3 PUFA, control of external conditions and participant behaviour in the lead up to measurement is crucial. Therefore, participants are typically asked to come to testing sessions in an overnight fasted state, having not done any exercise in the 1-2 days before, and refrain from consuming foods and drinks that are understood to interfere. In addition, the testing laboratory is set up in a standardized way, with controlled temperature and humidity. However, other factors, such as sleep duration and quality, psychological well-being, and hydration status also impact performance [374], [380], [381]. It is impossible to control for all of these, and other perhaps unknown factors. Controlling as many as possible improves the reliability of observations. However, it may never be possible to observe purely the effects of our intervention. Therefore, repeating of studies, and thereby constantly refining our expectations based on newly available knowledge, is crucial. This is yet another reason why Bayesian statistics is optimally suited for exercise science. It allows us to do exactly this, by constantly improving our models with newly generated data.

5.6 – Future directions and conclusions

Taken together, the work detailed in this thesis does not appear to support the use of n-3 PUFA as a supplemental aid for whole-body improvements in body composition and function in healthy young males. However, we did observe indications that n-3 PUFA may benefit metabolic processes on a smaller scale. It remains to be seen whether these potential minor changes may provide worthwhile benefits. In a general sense, individuals with the available knowledge and resources to increase their consumption of fish or n-3 PUFA, are more likely to already possess satisfactory

management of metabolic stress due to other lifestyle factors (e.g. diet and physical activity). At the same time, it is possible that n-3 PUFA does elicit worthwhile improvements in whole-body physiology, despite our failure to observe them. In future research, it is likely that optimisation of study design is required, with particular emphasis on a longer duration of supplementation and number of participants.

Finally, we are left with several key questions, that future research may wish to address:

i) Can n-3 PUFA preserve muscle mass in situations of weight loss which follow recent recommendations, i.e. gradual, controlled and longer-term, with increased protein intake?

ii) Can n-3 PUFA improve/maintain muscle metabolic health during catabolic situations? That is, does n-3 PUFA promote healthy turnover of proteins and maintenance of function, besides overall changes in mass?

iii) Does n-3 PUFA influence muscle protein turnover (MPS and MPB) during ER, on a worthwhile, whole-body scale?

iv) Does n-3 PUFA supplementation acutely influence formation of eicosanoids during exercise, and does this influence exercise function?

References

[1] A. J. Hulbert and P. L. Else, "Membranes as possible pacemakers of metabolism," *J Theor Biol*, vol. 199, no. 3, pp. 257–274, Aug. 1999, doi: 10.1006/jtbi.1999.0955.

[2] G. C. Burdge and P. C. Calder, "Conversion of **α** -linolenic acid to longer-chain polyunsaturated fatty acids in human adults," *Reprod. Nutr. Dev.*, vol. 45, no. 5, pp. 581–597, Sep. 2005, doi: 10.1051/rnd:2005047.

[3] H. Sprecher, "The roles of anabolic and catabolic reactions in the synthesis and recycling of polyunsaturated fatty acids," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 67, no. 2, pp. 79–83, Aug. 2002, doi: 10.1054/plef.2002.0402.

[4] P. P. Toth, "Triglyceride-rich lipoproteins as a causal factor for cardiovascular disease," *Vasc Health Risk Manag*, vol. 12, pp. 171–183, May 2016, doi: 10.2147/VHRM.S104369.

[5] A. C. Skulas-Ray *et al.*, "Omega-3 Fatty Acids for the Management of Hypertriglyceridemia: A Science Advisory From the American Heart Association," *Circulation*, Sep. 2019, doi: 10.1161/CIR.0000000000000709.

[6] J. Ukropec *et al.*, "The hypotriglyceridemic effect of dietary n−3 FA is associated with increased β-oxidation and reduced leptin expression," *Lipids*, vol. 38, no. 10, pp. 1023–1029, 2003, doi: 10.1007/s11745-006-1156-z.

[7] W. S. Harris and D. Bulchandani, "Why do omega-3 fatty acids lower serum triglycerides?," *Current Opinion in Lipidology*, vol. 17, no. 4, pp. 387–393, Aug. 2006, doi: 10.1097/01.mol.0000236363.63840.16.

[8] I. IKEDA *et al.*, "Effects of Dietary α-Linolenic, Eicosapentaenoic and Docosahexaenoic Acids on Hepatic Lipogenesis and β-Oxidation in Rats," *Bioscience, Biotechnology, and Biochemistry*, vol. 62, no. 4, pp. 675–680, Jan. 1998, doi: 10.1271/bbb.62.675.

[9] M. H. Davidson, "Mechanisms for the Hypotriglyceridemic Effect of Marine Omega-3 Fatty Acids," *The American Journal of Cardiology*, vol. 98, no. 4, Supplement 1, pp. 27–33, Aug. 2006, doi: 10.1016/j.amjcard.2005.12.024.

[10] S. Kotwal, M. Jun, D. Sullivan, V. Perkovic, and B. Neal, "Omega 3 Fatty Acids and Cardiovascular Outcomes," *Circulation: Cardiovascular Quality and Outcomes*, vol. 5, no. 6, pp. 808–818, Nov. 2012, doi: 10.1161/CIRCOUTCOMES.112.966168.

[11] A. S. Abdelhamid *et al.*, "Omega-3 fatty acids for the primary and secondary prevention of cardiovascular disease," *Cochrane Database of Systematic Reviews*, no. 3, 2020, doi: 10.1002/14651858.CD003177.pub5.

[12] Q. Li *et al.*, "Docosahexaenoic acid changes lipid composition and interleukin-2 receptor signaling in membrane rafts," *Journal of Lipid Research*, vol. 46, no. 9, pp. 1904–1913, Sep. 2005, doi: 10.1194/jlr.M500033-JLR200.

[13] T. Y. Hou, D. N. McMurray, and R. S. Chapkin, "Omega-3 fatty acids, lipid rafts, and T cell signaling," *European Journal of Pharmacology*, vol. 785, pp. 2–9, Aug. 2016, doi: 10.1016/j.ejphar.2015.03.091.

[14] W. E. Lands *et al.*, "Maintenance of lower proportions of (n - 6) eicosanoid precursors in phospholipids of human plasma in response to added dietary (n - 3) fatty acids," *Biochim Biophys Acta*, vol. 1180, no. 2, pp. 147–162, Dec. 1992, doi: 10.1016/0925-4439(92)90063-s.

[15] C. McGlory *et al.*, "Temporal changes in human skeletal muscle and blood lipid composition with fish oil supplementation," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 90, no. 6, pp. 199–206, Jun. 2014, doi: 10.1016/j.plefa.2014.03.001.

[16] D. B. Jump, "The Biochemistry of n-3 Polyunsaturated Fatty Acids * 210," *Journal of Biological Chemistry*, vol. 277, no. 11, pp. 8755–8758, Mar. 2002, doi: 10.1074/jbc.R100062200.

[17] D. A. Pratt, K. A. Tallman, and N. A. Porter, "Free radical oxidation of polyunsaturated lipids: New mechanistic insights and the development of peroxyl radical clocks," *Acc Chem Res*, vol. 44, no. 6, pp. 458–467, Jun. 2011, doi: 10.1021/ar200024c.

[18] P. C. Calder, “Eicosanoids,” *Essays in Biochemistry*, vol. 64, no. 3, pp. 423–441, Aug. 2020, doi: 10.1042/EBC20190083.
[19] T. C. L. Bargut, E. D. C. Frantz, C. A. Mandarim-de-Lacerda, and M. B. Aguila, “Effects of a Diet Rich in n-3 Polyunsaturated Fatty Acids on Hepatic Lipogenesis and Beta-Oxidation in Mice,” *Lipids*, vol. 49, no. 5, pp. 431–444, May 2014, doi: 10.1007/s11745-014-3892-9.
[20] P. Flachs *et al.*, “Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce β-oxidation in white fat,” *Diabetologia*, vol. 48, no. 11, pp. 2365–2375, Nov. 2005, doi: 10.1007/s00125-005-1944-7.
[21] A. Z. Lalia and I. R. Lanza, “Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation?,” *Nutrients*, vol. 8, no. 6, Art. no. 6, Jun. 2016, doi: 10.3390/nu8060329.
[22] P. C. Calder, “Mechanisms of Action of (n-3) Fatty Acids,” *The Journal of Nutrition*, vol. 142, no. 3, pp. 592S-599S, Mar. 2012, doi: 10.3945/jn.111.155259.
[23] C. von Schacky, R. Kiefl, A. J. Marcus, M. J. Broekman, and W. E. Kaminski, “Dietary n-3 fatty acids accelerate catabolism of leukotriene B4 in human granulocytes,” *Biochim Biophys Acta*, vol. 1166, no. 1, pp. 20–24, Feb. 1993, doi: 10.1016/0005-2760(93)90278-h.
[24] P. Maderna and C. Godson, “Lipoxins: resolutionary road,” *British Journal of Pharmacology*, vol. 158, no. 4, pp. 947–959, 2009, doi: 10.1111/j.1476-5381.2009.00386.x.
[25] C. N. Serhan, N. Chiang, and T. E. Van Dyke, “Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators,” *Nat Rev Immunol*, vol. 8, no. 5, Art. no. 5, May 2008, doi: 10.1038/nri2294.
[26] J. I. Nesman, J. E. Tungen, A. Vik, and T. V. Hansen, “Stereoselective synthesis of the specialized pro-resolving and anti-inflammatory mediator resolvin E1,” *Tetrahedron*, vol. 76, no. 3, p. 130821, Jan. 2020, doi: 10.1016/j.tet.2019.130821.
[27] D. Y. Oh *et al.*, “GPR120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-inflammatory and Insulin-Sensitizing Effects,” *Cell*, vol. 142, no. 5, pp. 687–698, Sep. 2010, doi: 10.1016/j.cell.2010.07.041.
[28] S. Talukdar, J. M. Olefsky, and O. Osborn, “Targeting GPR120 and other fatty acid-sensing GPCRs ameliorates insulin resistance and inflammatory diseases,” *Trends in Pharmacological Sciences*, vol. 32, no. 9, pp. 543–550, Sep. 2011, doi: 10.1016/j.tips.2011.04.004.
[29] Y. Williams-Bey *et al.*, “Omega-3 Free Fatty Acids Suppress Macrophage Inflammasome Activation by Inhibiting NF-κB Activation and Enhancing Autophagy,” *PLOS ONE*, vol. 9, no. 6, p. e97957, Jun. 2014, doi: 10.1371/journal.pone.0097957.
[30] F. Huang, H. Wei, H. Luo, S. Jiang, and J. Peng, “EPA inhibits the inhibitor of κBα (IκBα)/NF-κB/muscle RING finger 1 pathway in C2C12 myotubes in a PPARγ-dependent manner,” *British Journal of Nutrition*, vol. 105, no. 3, pp. 348–356, Feb. 2011, doi: 10.1017/S0007114510003703.
[31] A. Polus *et al.*, “Omega-3 fatty acid supplementation influences the whole blood transcriptome in women with obesity, associated with pro-resolving lipid mediator production,” *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, vol. 1861, no. 11, pp. 1746–1755, Nov. 2016, doi: 10.1016/j.bbalip.2016.08.005.
[32] I. Rudkowska *et al.*, “Transcriptomic and metabolomic signatures of an n-3 polyunsaturated fatty acids supplementation in a normolipidemic/normocholesterolemic Caucasian population,” *The Journal of Nutritional Biochemistry*, vol. 24, no. 1, pp. 54–61, Jan. 2013, doi: 10.1016/j.jnutbio.2012.01.016.
[33] L. Cordain *et al.*, “Origins and evolution of the Western diet: health implications for the 21st century,” *The American Journal of Clinical Nutrition*, vol. 81, no. 2, pp. 341–354, Feb. 2005, doi: 10.1093/ajcn.81.2.341.
[34] F. K. Ho *et al.*, “Associations of fat and carbohydrate intake with cardiovascular disease and mortality: prospective cohort study of UK Biobank participants,” *BMJ*, vol. 368, p. m688, Mar. 2020, doi: 10.1136/bmj.m688.
[35] W. S. Yancy, C.-C. Wang, and M. L. Maciejewski, “Trends in energy and macronutrient

intakes by weight status over four decades," *Public Health Nutrition*, vol. 17, no. 2, pp. 256–265, Feb. 2014, doi: 10.1017/S1368980012005423.
[36] W. Kopp, "How Western Diet And Lifestyle Drive The Pandemic Of Obesity And Civilization Diseases," *Diabetes Metab Syndr Obes*, vol. 12, pp. 2221–2236, Oct. 2019, doi: 10.2147/DMSO.S216791.
[37] D. Grigg, "The pattern of world protein consumption," *Geoforum*, vol. 26, no. 1, pp. 1–17, Feb. 1995, doi: 10.1016/0016-7185(94)00020-8.
[38] S. van Vliet, N. A. Burd, and L. J. van Loon, "The Skeletal Muscle Anabolic Response to Plant- versus Animal-Based Protein Consumption," *The Journal of Nutrition*, vol. 145, no. 9, pp. 1981–1991, Sep. 2015, doi: 10.3945/jn.114.204305.
[39] D. Cuthbertson *et al.*, "Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle," *The FASEB Journal*, vol. 19, no. 3, pp. 1–22, 2005, doi: 10.1096/fj.04-2640fje.
[40] N. A. Burd, S. H. Gorissen, and L. J. C. van Loon, "Anabolic Resistance of Muscle Protein Synthesis with Aging," *Exercise and Sport Sciences Reviews*, vol. 41, no. 3, p. 169, Jul. 2013, doi: 10.1097/JES.0b013e318292f3d5.
[41] B. E. Phillips, D. S. Hill, and P. J. Atherton, "Regulation of muscle protein synthesis in humans," *Current Opinion in Clinical Nutrition & Metabolic Care*, vol. 15, no. 1, pp. 58–63, Jan. 2012, doi: 10.1097/MCO.0b013e32834d19bc.
[42] H. O. Bang, J. Dyerberg, and A. B. Nielsen, "Plasma lipid and lipoprotein pattern in Greenlandic West-coast Eskimos," *Lancet*, vol. 1, no. 7710, pp. 1143–1145, Jun. 1971, doi: 10.1016/s0140-6736(71)91658-8.
[43] S. Jeromson, I. J. Gallagher, S. D. R. Galloway, and D. L. Hamilton, "Omega-3 Fatty Acids and Skeletal Muscle Health," *Marine Drugs*, vol. 13, no. 11, Art. no. 11, Nov. 2015, doi: 10.3390/md13116977.
[44] C. McGlory, P. C. Calder, and E. A. Nunes, "The Influence of Omega-3 Fatty Acids on Skeletal Muscle Protein Turnover in Health, Disuse, and Disease," *Front. Nutr.*, vol. 6, 2019, doi: 10.3389/fnut.2019.00144.
[45] C. L. Rodacki *et al.*, "Fish-oil supplementation enhances the effects of strength training in elderly women," *The American Journal of Clinical Nutrition*, vol. 95, no. 2, pp. 428–436, Feb. 2012, doi: 10.3945/ajcn.111.021915.
[46] G. I. Smith, S. Julliand, D. N. Reeds, D. R. Sinacore, S. Klein, and B. Mittendorfer, "Fish oil–derived n−3 PUFA therapy increases muscle mass and function in healthy older adults1," *The American Journal of Clinical Nutrition*, vol. 102, no. 1, pp. 115–122, Jul. 2015, doi: 10.3945/ajcn.114.105833.
[47] M. Da Boit *et al.*, "Sex differences in the effect of fish-oil supplementation on the adaptive response to resistance exercise training in older people: a randomized controlled trial," *The American Journal of Clinical Nutrition*, vol. 105, no. 1, pp. 151–158, Jan. 2017, doi: 10.3945/ajcn.116.140780.
[48] A. Z. Lalia *et al.*, "Influence of omega-3 fatty acids on skeletal muscle protein metabolism and mitochondrial bioenergetics in older adults," *Aging (Albany NY)*, vol. 9, no. 4, pp. 1096–1115, Apr. 2017, doi: 10.18632/aging.101210.
[49] C. McGlory *et al.*, "Fish oil supplementation suppresses resistance exercise and feeding-induced increases in anabolic signaling without affecting myofibrillar protein synthesis in young men," *Physiological Reports*, vol. 4, no. 6, p. e12715, 2016, doi: 10.14814/phy2.12715.
[50] A. O. Akinkuolie, J. S. Ngwa, J. B. Meigs, and L. Djoussé, "Omega-3 polyunsaturated fatty acid and insulin sensitivity: A meta-analysis of randomized controlled trials," *Clinical Nutrition*, vol. 30, no. 6, pp. 702–707, Dec. 2011, doi: 10.1016/j.clnu.2011.08.013.
[51] R.-J. Shei, M. R. Lindley, and T. D. Mickleborough, "Omega-3 Polyunsaturated Fatty Acids in the Optimization of Physical Performance," *Military Medicine*, vol. 179, no. suppl_11, pp. 144–156, Nov. 2014, doi: 10.7205/MILMED-D-14-00160.

[52] J. D. Philpott, O. C. Witard, and S. D. R. Galloway, “Applications of omega-3 polyunsaturated fatty acid supplementation for sport performance,” *Research in Sports Medicine*, vol. 27, no. 2, pp. 219–237, Apr. 2019, doi: 10.1080/15438627.2018.1550401.
[53] R. Anthony, M. J. Macartney, and G. E. Peoples, “The Influence of Long-Chain Omega-3 Fatty Acids on Eccentric Exercise-Induced Delayed Muscle Soreness: Reported Outcomes Are Compromised by Study Design Issues,” *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 31, no. 2, pp. 143–153, Jan. 2021, doi: 10.1123/ijsnem.2020-0238.
[54] Z. Lv, J. Zhang, and W. Zhu, “Omega-3 Polyunsaturated Fatty Acid Supplementation for Reducing Muscle Soreness after Eccentric Exercise: A Systematic Review and Meta-Analysis of Randomized Controlled Trials,” *BioMed Research International*, vol. 2020, p. e8062017, Apr. 2020, doi: 10.1155/2020/8062017.
[55] N. A. Lewis, D. Daniels, P. C. Calder, L. M. Castell, and C. R. Pedlar, “Are There Benefits from the Use of Fish Oil Supplements in Athletes? A Systematic Review,” *Advances in Nutrition*, vol. 11, no. 5, pp. 1300–1314, Sep. 2020, doi: 10.1093/advances/nmaa050.
[56] C. Gómez Candela, L. M. Bermejo López, and V. Loria Kohen, “Importance of a balanced omega 6/omega 3 ratio for the maintenance of health: nutritional recommendations,” *Nutr Hosp*, vol. 26, no. 2, pp. 323–329, Apr. 2011, doi: 10.1590/S0212-16112011000200013.
[57] P. C. Norris and E. A. Dennis, “A lipidomic perspective on inflammatory macrophage eicosanoid signaling,” *Adv Biol Regul*, vol. 54, pp. 99–110, Jan. 2014, doi: 10.1016/j.jbior.2013.09.009.
[58] A. Kumar, Y. Takada, A. Boriek, and B. Aggarwal, “Nuclear factor-kappaB: Its role in health and disease,” *Journal of molecular medicine (Berlin, Germany)*, vol. 82, pp. 434–48, Aug. 2004, doi: 10.1007/s00109-004-0555-y.
[59] M. K. Crow, M. Olferiev, and K. A. Kirou, “Type I Interferons in Autoimmune Disease,” *Annual Review of Pathology: Mechanisms of Disease*, vol. 14, no. 1, pp. 369–393, 2019, doi: 10.1146/annurev-pathol-020117-043952.
[60] H. Wang, T.-L. Li, S. Hsia, I.-L. Su, Y.-L. Chan, and C.-J. Wu, “Skeletal muscle atrophy is attenuated in tumor-bearing mice under chemotherapy by treatment with fish oil and selenium,” *Oncotarget*, vol. 6, no. 10, pp. 7758–7773, Mar. 2015.
[61] P. Magee, S. Pearson, and J. Allen, “The omega-3 fatty acid, eicosapentaenoic acid (EPA), prevents the damaging effects of tumour necrosis factor (TNF)-alpha during murine skeletal muscle cell differentiation,” *Lipids in Health and Disease*, vol. 7, no. 1, p. 24, Jul. 2008, doi: 10.1186/1476-511X-7-24.
[62] K. Baar, “Nutrition and the Adaptation to Endurance Training,” *Sports Med*, vol. 44, no. 1, pp. 5–12, May 2014, doi: 10.1007/s40279-014-0146-1.
[63] C. Malm, “Exercise-induced muscle damage and inflammation: fact or fiction?,” *Acta Physiologica Scandinavica*, vol. 171, no. 3, pp. 233–239, 2001, doi: 10.1046/j.1365-201x.2001.00825.x.
[64] J. E. Belizário, C. C. Fontes-Oliveira, J. P. Borges, J. A. Kashiabara, and E. Vannier, “Skeletal muscle wasting and renewal: a pivotal role of myokine IL-6,” *SpringerPlus*, vol. 5, no. 1, p. 619, May 2016, doi: 10.1186/s40064-016-2197-2.
[65] A. Yavari, M. Javadi, P. Mirmiran, and Z. Bahadoran, “Exercise-Induced Oxidative Stress and Dietary Antioxidants,” *Asian J Sports Med*, vol. 6, no. 1, p. e24898, Mar. 2015, doi: 10.5812/asjsm.24898.
[66] J. F. Markworth *et al.*, “Human inflammatory and resolving lipid mediator responses to resistance exercise and ibuprofen treatment,” *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 305, no. 11, pp. R1281–R1296, Dec. 2013, doi: 10.1152/ajpregu.00128.2013.
[67] R. Brigelius-Flohé, “Commentary: oxidative stress reconsidered,” *Genes Nutr*, vol. 4, no. 3, pp. 161–163, Sep. 2009, doi: 10.1007/s12263-009-0131-8.
[68] D. R. Gough and T. G. Cotter, “Hydrogen peroxide: a Jekyll and Hyde signalling molecule,” *Cell Death Dis*, vol. 2, p. e213, Oct. 2011, doi: 10.1038/cddis.2011.96.

[69] M. B. Reid and W. J. Durham, “Generation of reactive oxygen and nitrogen species in contracting skeletal muscle: potential impact on aging,” *Ann N Y Acad Sci*, vol. 959, pp. 108–116, Apr. 2002, doi: 10.1111/j.1749-6632.2002.tb02087.x.
[70] E. J. Anderson *et al.*, “Do Fish Oil Omega-3 Fatty Acids Enhance Antioxidant Capacity and Mitochondrial Fatty Acid Oxidation in Human Atrial Myocardium via PPARγ Activation?,” *Antioxidants & Redox Signaling*, vol. 21, no. 8, pp. 1156–1163, Sep. 2014, doi: 10.1089/ars.2014.5888.
[71] C. Sakai *et al.*, “Fish oil omega-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage in vascular endothelial cells,” *PLOS ONE*, vol. 12, no. 11, p. e0187934, Nov. 2017, doi: 10.1371/journal.pone.0187934.
[72] S. Williamson, D. Varma, M. Brown, and S. Jansen, “Eicosanoid Production following One Bout of Exercise in Middle-Aged African American Pre- and Stage 1 Hypertensives,” *Journal of Aging Research*, vol. 2011, p. e302802, May 2011, doi: 10.4061/2011/302802.
[73] K. Laustiola, E. Seppälä, T. Nikkari, and H. Vapaatalo, “Exercise-induced increase in plasma arachidonic acid and thromboxane B2 in healthy men: effect of beta-adrenergic blockade,” *J Cardiovasc Pharmacol*, vol. 6, no. 3, pp. 449–454, May 1984, doi: 10.1097/00005344-198405000-00012.
[74] A. Daci, Z. Celik, G. Ozen, M. Dashwood, B. S. U. Dogan, and G. Topal, “Effect of omega-3 polyunsaturated fatty acids in modulation of vascular tone under physiological and pathological conditions,” *European Journal of Pharmaceutical Sciences*, vol. 153, p. 105499, Oct. 2020, doi: 10.1016/j.ejps.2020.105499.
[75] V. B. O’Donnell, B. Maskrey, and G. W. Taylor, “Eicosanoids: Generation and Detection in Mammalian Cells,” in *Lipid Signaling Protocols*, B. Larijani, R. Woscholski, and C. A. Rosser, Eds. Totowa, NJ: Humana Press, 2009, pp. 1–19. doi: 10.1007/978-1-60327-115-8_1.
[76] J. D. Imig, “Eicosanoid blood vessel regulation in physiological and pathological states,” *Clinical Science*, vol. 134, no. 20, pp. 2707–2727, Oct. 2020, doi: 10.1042/CS20191209.
[77] S. J. Warden, “Prophylactic Use of NSAIDs by Athletes: A Risk/Benefit Assessment,” *The Physician and Sportsmedicine*, vol. 38, no. 1, pp. 132–138, Apr. 2010, doi: 10.3810/psm.2010.04.1770.
[78] M. Lilja *et al.*, “High doses of anti-inflammatory drugs compromise muscle strength and hypertrophic adaptations to resistance training in young adults,” *Acta Physiol (Oxf)*, vol. 222, no. 2, Feb. 2018, doi: 10.1111/apha.12948.
[79] M. P. Wymann and R. Schneiter, “Lipid signalling in disease,” *Nat Rev Mol Cell Biol*, vol. 9, no. 2, pp. 162–176, Feb. 2008, doi: 10.1038/nrm2335.
[80] A. C. Skulas-Ray, M. R. Flock, C. K. Richter, W. S. Harris, S. G. West, and P. M. Kris-Etherton, “Red Blood Cell Docosapentaenoic Acid (DPA n-3) is Inversely Associated with Triglycerides and C-reactive Protein (CRP) in Healthy Adults and Dose-Dependently Increases Following n-3 Fatty Acid Supplementation,” *Nutrients*, vol. 7, no. 8, pp. 6390–6404, Aug. 2015, doi: 10.3390/nu7085291.
[81] A. Żebrowska, K. Mizia-Stec, M. Mizia, Z. Gąsior, and S. Poprzęcki, “Omega-3 fatty acids supplementation improves endothelial function and maximal oxygen uptake in endurance-trained athletes,” *Eur J Sport Sci*, vol. 15, no. 4, Art. no. 4, 2015, doi: 10.1080/17461391.2014.949310.
[82] D. C. Nieman, D. A. Henson, S. R. McAnulty, F. Jin, and K. R. Maxwell, “n-3 Polyunsaturated Fatty Acids Do Not Alter Immune and Inflammation Measures in Endurance Athletes,” *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 19, no. 5, pp. 536–546, Oct. 2009, doi: 10.1123/ijsnem.19.5.536.
[83] A. D. Toft *et al.*, “N-3 polyunsaturated fatty acids do not affect cytokine response to strenuous exercise,” *Journal of Applied Physiology*, vol. 89, no. 6, pp. 2401–2406, Dec. 2000, doi: 10.1152/jappl.2000.89.6.2401.
[84] M. Da Boit, I. Mastalurova, G. Brazaite, N. McGovern, K. Thompson, and S. R. Gray, “The Effect of Krill Oil Supplementation on Exercise Performance and Markers of Immune

Function," *PLOS ONE*, vol. 10, no. 9, p. e0139174, Sep. 2015, doi: 10.1371/journal.pone.0139174.
[85] G. S. Oostenbrug, R. P. Mensink, M. R. Hardeman, T. De Vries, F. Brouns, and G. Hornstra, "Exercise performance, red blood cell deformability, and lipid peroxidation: effects of fish oil and vitamin E," *Journal of Applied Physiology*, vol. 83, no. 3, pp. 746–752, Sep. 1997, doi: 10.1152/jappl.1997.83.3.746.
[86] G. I. Smith *et al.*, "Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia–hyperaminoacidaemia in healthy young and middle-aged men and women," *Clinical Science*, vol. 121, no. 6, pp. 267–278, May 2011, doi: 10.1042/CS20100597.
[87] G. I. Smith *et al.*, "Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older adults: a randomized controlled trial," *The American Journal of Clinical Nutrition*, vol. 93, no. 2, pp. 402–412, Feb. 2011, doi: 10.3945/ajcn.110.005611.
[88] J. H. Lee and H.-S. Jun, "Role of Myokines in Regulating Skeletal Muscle Mass and Function," *Frontiers in Physiology*, vol. 10, 2019, Accessed: Mar. 09, 2022. [Online]. Available: https://www.frontiersin.org/article/10.3389/fphys.2019.00042
[89] B. K. Pedersen, "Exercise and cytokines," *Immunology & Cell Biology*, vol. 78, no. 5, pp. 532–535, 2000, doi: 10.1111/j.1440-1711.2000.t01-11-.x.
[90] R. R. Wolfe, "The underappreciated role of muscle in health and disease," *Am J Clin Nutr*, vol. 84, no. 3, pp. 475–482, Sep. 2006, doi: 10.1093/ajcn/84.3.475.
[91] T. Kamolrat and S. R. Gray, "The effect of eicosapentaenoic and docosahexaenoic acid on protein synthesis and breakdown in murine C2C12 myotubes," *Biochemical and Biophysical Research Communications*, vol. 432, no. 4, pp. 593–598, Mar. 2013, doi: 10.1016/j.bbrc.2013.02.041.
[92] A. S. Whitehouse and M. J. Tisdale, "Downregulation of Ubiquitin-Dependent Proteolysis by Eicosapentaenoic Acid in Acute Starvation," *Biochemical and Biophysical Research Communications*, vol. 285, no. 3, pp. 598–602, Jul. 2001, doi: 10.1006/bbrc.2001.5209.
[93] H. J. Smith, N. A. Greenberg, and M. J. Tisdale, "Effect of eicosapentaenoic acid, protein and amino acids on protein synthesis and degradation in skeletal muscle of cachectic mice," *Br J Cancer*, vol. 91, no. 2, pp. 408–412, Jul. 2004, doi: 10.1038/sj.bjc.6601981.
[94] E. Castillero, A. I. Martín, M. López-Menduiña, M. A. Villanúa, and A. López-Calderón, "Eicosapentaenoic acid attenuates arthritis-induced muscle wasting acting on atrogin-1 and on myogenic regulatory factors," *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, vol. 297, no. 5, pp. R1322–R1331, Nov. 2009, doi: 10.1152/ajpregu.00388.2009.
[95] Y. Liu *et al.*, "Fish Oil Increases Muscle Protein Mass and Modulates Akt/FOXO, TLR4, and NOD Signaling in Weanling Piglets After Lipopolysaccharide Challenge," *The Journal of Nutrition*, vol. 143, no. 8, pp. 1331–1339, Aug. 2013, doi: 10.3945/jn.113.176255.
[96] M. Laplante and D. M. Sabatini, "mTOR signaling at a glance," *Journal of Cell Science*, vol. 122, no. 20, pp. 3589–3594, Oct. 2009, doi: 10.1242/jcs.051011.
[97] Y. Sancak, L. Bar-Peled, R. Zoncu, A. L. Markhard, S. Nada, and D. M. Sabatini, "Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids," *Cell*, vol. 141, no. 2, pp. 290–303, Apr. 2010, doi: 10.1016/j.cell.2010.02.024.
[98] A. Philp, D. L. Hamilton, and K. Baar, "Signals mediating skeletal muscle remodeling by resistance exercise: PI3-kinase independent activation of mTORC1," *Journal of Applied Physiology*, vol. 110, no. 2, Art. no. 2, Feb. 2011, doi: 10.1152/japplphysiol.00941.2010.
[99] N. Pullen and G. Thomas, "The modular phosphorylation and activation of p70s6k," *FEBS Letters*, vol. 410, no. 1, pp. 78–82, Jun. 1997, doi: 10.1016/S0014-5793(97)00323-2.
[100] J. Kim, M. Kundu, B. Viollet, and K.-L. Guan, "AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1," *Nat Cell Biol*, vol. 13, no. 2, pp. 132–141, Feb. 2011, doi: 10.1038/ncb2152.

[101] N. Mizushima and M. Komatsu, “Autophagy: Renovation of Cells and Tissues,” *Cell*, vol. 147, no. 4, pp. 728–741, Nov. 2011, doi: 10.1016/j.cell.2011.10.026.
[102] J. M. Dickinson *et al.*, “Rapamycin does not affect post-absorptive protein metabolism in human skeletal muscle,” *Metabolism*, vol. 62, no. 1, pp. 144–151, Jan. 2013, doi: 10.1016/j.metabol.2012.07.003.
[103] A.-A. Gingras *et al.*, “Long-chain omega-3 fatty acids regulate bovine whole-body protein metabolism by promoting muscle insulin signalling to the Akt–mTOR–S6K1 pathway and insulin sensitivity,” *The Journal of Physiology*, vol. 579, no. 1, pp. 269–284, 2007, doi: 10.1113/jphysiol.2006.121079.
[104] S. Tesseraud *et al.*, “Modulation of the insulin anabolic signalling cascade in growing chickens by n-3 PUFA,” *British Journal of Nutrition*, vol. 111, no. 5, pp. 761–772, Mar. 2014, doi: 10.1017/S0007114513003085.
[105] J.-S. Y.-S. You, M.-N. P.-N. Park, W. S. Song, and Y.-S. L.-S. Lee, “Dietary fish oil alleviates soleus atrophy during immobilization in association with Akt signaling to p70s6k and E3 ubiquitin ligases in rats,” *Applied Physiology, Nutrition, and Metabolism*, May 2010, doi: 10.1139/H10-022.
[106] C. McGlory *et al.*, “Omega-3 fatty acid supplementation attenuates skeletal muscle disuse atrophy during two weeks of unilateral leg immobilization in healthy young women,” *The FASEB Journal*, vol. 33, no. 3, pp. 4586–4597, 2019, doi: 10.1096/fj.201801857RRR.
[107] S. Schmidt, F. Stahl, K.-O. Mutz, T. Scheper, A. Hahn, and J. P. Schuchardt, “Transcriptome-based identification of antioxidative gene expression after fish oil supplementation in normo- and dyslipidemic men,” *Nutrition & Metabolism*, vol. 9, no. 1, p. 45, May 2012, doi: 10.1186/1743-7075-9-45.
[108] T. Kamolrat, S. R. Gray, and M. Carole Thivierge, “Fish oil positively regulates anabolic signalling alongside an increase in whole-body gluconeogenesis in ageing skeletal muscle,” *Eur J Nutr*, vol. 52, no. 2, pp. 647–657, Mar. 2013, doi: 10.1007/s00394-012-0368-7.
[109] S. Pepe and P. L. McLennan, “Cardiac membrane fatty acid composition modulates myocardial oxygen consumption and postischemic recovery of contractile function,” *Circulation*, vol. 105, no. 19, pp. 2303–2308, May 2002, doi: 10.1161/01.cir.0000015604.88808.74.
[110] S. Pepe and P. L. McLennan, “(n-3) Long chain PUFA dose-dependently increase oxygen utilization efficiency and inhibit arrhythmias after saturated fat feeding in rats,” *J Nutr*, vol. 137, no. 11, pp. 2377–2383, Nov. 2007, doi: 10.1093/jn/137.11.2377.
[111] G. E. Peoples, P. L. McLennan, P. R. C. Howe, and H. Groeller, “Fish Oil Reduces Heart Rate and Oxygen Consumption During Exercise,” *Journal of Cardiovascular Pharmacology*, vol. 52, no. 6, pp. 540–547, Dec. 2008, doi: 10.1097/FJC.0b013e3181911913.
[112] G. E. Peoples and P. L. McLennan, “Long-chain n-3 DHA reduces the extent of skeletal muscle fatigue in the rat in vivo hindlimb model,” *British Journal of Nutrition*, vol. 111, no. 6, pp. 996–1003, Mar. 2014, doi: 10.1017/S0007114513003449.
[113] F. Kawabata, M. Neya, K. Hamazaki, Y. Watanabe, S. Kobayashi, and T. Tsuji, “Supplementation with eicosapentaenoic acid-rich fish oil improves exercise economy and reduces perceived exertion during submaximal steady-state exercise in normal healthy untrained men,” *Bioscience, Biotechnology, and Biochemistry*, vol. 78, no. 12, pp. 2081–2088, Dec. 2014, doi: 10.1080/09168451.2014.946392.
[114] L. Hingley, M. J. Macartney, M. A. Brown, P. L. McLennan, and G. E. Peoples, “DHA-rich Fish Oil Increases the Omega-3 Index and Lowers the Oxygen Cost of Physiologically Stressful Cycling in Trained Individuals,” *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 27, no. 4, pp. 335–343, Aug. 2017, doi: 10.1123/ijsnem.2016-0150.
[115] T. Raastad, A. T. Høstmark, and S. B. Strømme, “Omega-3 fatty acid supplementation does not improve maximal aerobic power, anaerobic threshold and running performance in well-trained soccer players,” *Scand J Med Sci Sports*, vol. 7, no. 1, pp. 25–31, Feb. 1997, doi:

10.1111/j.1600-0838.1997.tb00113.x.
[116] L. Gravina *et al.*, "n-3 Fatty Acid Supplementation During 4 Weeks of Training Leads to Improved Anaerobic Endurance Capacity, but not Maximal Strength, Speed, or Power in Soccer Players," *Int J Sport Nutr Exerc Metab*, vol. 27, no. 4, pp. 305–313, Aug. 2017, doi: 10.1123/ijsnem.2016-0325.
[117] J. D. Buckley, S. Burgess, K. J. Murphy, and P. R. C. Howe, "DHA-rich fish oil lowers heart rate during submaximal exercise in elite Australian Rules footballers," *Journal of Science and Medicine in Sport*, vol. 12, no. 4, Art. no. 4, Jul. 2009, doi: 10.1016/j.jsams.2008.01.011.
[118] S. W. Farrell *et al.*, "Higher omega-3 index is associated with more rapid heart rate recovery in healthy men and women," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 163, p. 102206, Dec. 2020, doi: 10.1016/j.plefa.2020.102206.
[119] M. J. Macartney, L. Hingley, M. A. Brown, G. E. Peoples, and P. L. McLennan, "Intrinsic heart rate recovery after dynamic exercise is improved with an increased omega-3 index in healthy males," *British Journal of Nutrition*, vol. 112, no. 12, pp. 1984–1992, Dec. 2014, doi: 10.1017/S0007114514003146.
[120] E. J. H. Lewis, P. W. Radonic, T. M. S. Wolever, and G. D. Wells, "21 days of mammalian omega-3 fatty acid supplementation improves aspects of neuromuscular function and performance in male athletes compared to olive oil placebo," *Journal of the International Society of Sports Nutrition*, vol. 12, no. 1, p. 28, Jun. 2015, doi: 10.1186/s12970-015-0089-4.
[121] K. E. Black *et al.*, "Adding omega-3 fatty acids to a protein-based supplement during pre-season training results in reduced muscle soreness and the better maintenance of explosive power in professional Rugby Union players," *European Journal of Sport Science*, vol. 18, no. 10, pp. 1357–1367, Nov. 2018, doi: 10.1080/17461391.2018.1491626.
[122] L. James *et al.*, "Four Weeks of Omega-3 Supplementation does not Improve Cycling Time Trial Performance in Trained Cyclists," *Archives of Sports Medicine*, vol. 4, no. 2, Art. no. 2, Nov. 2020, Accessed: Nov. 25, 2021. [Online]. Available: https://www.scholars.direct/Articles/sports-medicine/aspm-4-037.php?jid=sports-medicine
[123] W. S. Harris, "The Omega-3 Index: Clinical Utility for Therapeutic Intervention," *Curr Cardiol Rep*, vol. 12, no. 6, pp. 503–508, Nov. 2010, doi: 10.1007/s11886-010-0141-6.
[124] P. C. Calder, "Chapter 2 - Health benefits of omega-3 fatty acids," in *Omega-3 Delivery Systems*, P. J. García-Moreno, C. Jacobsen, A.-D. Moltke Sørensen, and B. Yesiltas, Eds. Academic Press, 2021, pp. 25–53. doi: 10.1016/B978-0-12-821391-9.00006-5.
[125] Y. A. Carpentier, M. Hacquebard, L. Portois, I. E. Dupont, R. J. Deckelbaum, and W. J. Malaisse, "Rapid cellular enrichment of eicosapentaenoate after a single intravenous injection of a novel medium-chain triacylglycerol:fish-oil emulsion in humans," *The American Journal of Clinical Nutrition*, vol. 91, no. 4, pp. 875–882, Apr. 2010, doi: 10.3945/ajcn.2009.27951.
[126] F. Delodder, L. Tappy, L. Liaudet, P. Schneiter, C. Perrudet, and M. M. Berger, "Incorporation and washout of n-3 PUFA after high dose intravenous and oral supplementation in healthy volunteers," *Clinical Nutrition*, vol. 34, no. 3, pp. 400–408, Jun. 2015, doi: 10.1016/j.clnu.2014.07.005.
[127] A. Dewey, C. Baughan, T. Dean, B. Higgins, and I. Johnson, "Eicosapentaenoic acid (EPA, an omega-3 fatty acid from fish oils) for the treatment of cancer cachexia," *Cochrane Database Syst Rev*, no. 1, p. CD004597, Jan. 2007, doi: 10.1002/14651858.CD004597.pub2.
[128] N. Bender, M. Portmann, Z. Heg, K. Hofmann, M. Zwahlen, and M. Egger, "Fish or n3-PUFA intake and body composition: a systematic review and meta-analysis," *Obesity Reviews*, vol. 15, no. 8, pp. 657–665, 2014, doi: 10.1111/obr.12189.
[129] Y. Y. Zhang, W. Liu, T. Y. Zhao, and H. M. Tian, "EFFICACY OF OMEGA-3 POLYUNSATURATED FATTY ACIDS SUPPLEMENTATION IN MANAGING OVERWEIGHT AND OBESITY: A META-ANALYSIS OF RANDOMIZED CLINICAL TRIALS," *J Nutr Health Aging*, vol. 21, no. 2, p. 6, 2017.
[130] S. Du, J. Jin, W. Fang, and Q. Su, "Does Fish Oil Have an Anti-Obesity Effect in

Overweight/Obese Adults? A Meta-Analysis of Randomized Controlled Trials," *PLOS ONE*, vol. 10, no. 11, p. e0142652, Nov. 2015, doi: 10.1371/journal.pone.0142652.
[131] J. H. Y. Wu *et al.*, "Omega-3 fatty acids and incident type 2 diabetes: a systematic review and meta-analysis," *British Journal of Nutrition*, vol. 107, no. S2, pp. S214–S227, Jun. 2012, doi: 10.1017/S0007114512001602.
[132] H. Gao, T. Geng, T. Huang, and Q. Zhao, "Fish oil supplementation and insulin sensitivity: a systematic review and meta-analysis," *Lipids Health Dis*, vol. 16, no. 1, p. 131, Jul. 2017, doi: 10.1186/s12944-017-0528-0.
[133] Y.-H. Huang, W.-C. Chiu, Y.-P. Hsu, Y.-L. Lo, and Y.-H. Wang, "Effects of Omega-3 Fatty Acids on Muscle Mass, Muscle Strength and Muscle Performance among the Elderly: A Meta-Analysis," *Nutrients*, vol. 12, no. 12, Art. no. 12, Dec. 2020, doi: 10.3390/nu12123739.
[134] D. Mozaffarian, A. Geelen, I. A. Brouwer, J. M. Geleijnse, P. L. Zock, and M. B. Katan, "Effect of fish oil on heart rate in humans: a meta-analysis of randomized controlled trials," *Circulation*, vol. 112, no. 13, pp. 1945–1952, Sep. 2005, doi: 10.1161/CIRCULATIONAHA.105.556886.
[135] Q. Wang *et al.*, "Effect of omega-3 fatty acids supplementation on endothelial function: A meta-analysis of randomized controlled trials," *Atherosclerosis*, vol. 221, no. 2, pp. 536–543, Apr. 2012, doi: 10.1016/j.atherosclerosis.2012.01.006.
[136] J. H. Y. Wu, L. E. Cahill, and D. Mozaffarian, "Effect of Fish Oil on Circulating Adiponectin: A Systematic Review and Meta-Analysis of Randomized Controlled Trials," *The Journal of Clinical Endocrinology & Metabolism*, vol. 98, no. 6, pp. 2451–2459, Jun. 2013, doi: 10.1210/jc.2012-3899.
[137] J. Rausch, S. Gillespie, T. Orchard, A. Tan, and J. C. McDaniel, "Systematic review of marine-derived omega-3 fatty acid supplementation effects on leptin, adiponectin, and the leptin-to-adiponectin ratio," *Nutrition Research*, vol. 85, pp. 135–152, Jan. 2021, doi: 10.1016/j.nutres.2020.11.002.
[138] Y. Liao *et al.*, "Efficacy of omega-3 PUFAs in depression: A meta-analysis," *Transl Psychiatry*, vol. 9, no. 1, Art. no. 1, Aug. 2019, doi: 10.1038/s41398-019-0515-5.
[139] M. H. Bloch and J. Hannestad, "Omega-3 fatty acids for the treatment of depression: systematic review and meta-analysis," *Mol Psychiatry*, vol. 17, no. 12, Art. no. 12, Dec. 2012, doi: 10.1038/mp.2011.100.
[140] J. P. A. Ioannidis, "Why Most Published Research Findings Are False," *PLOS Medicine*, vol. 2, no. 8, p. e124, Aug. 2005, doi: 10.1371/journal.pmed.0020124.
[141] T. P. Wycherley, L. J. Moran, P. M. Clifton, M. Noakes, and G. D. Brinkworth, "Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: a meta-analysis of randomized controlled trials," *The American Journal of Clinical Nutrition*, vol. 96, no. 6, pp. 1281–1298, Dec. 2012, doi: 10.3945/ajcn.112.044321.
[142] Biobank UK, "Omega-3 Fatty Acids to Total Fatty Acids percentage," *Biobank UK*. https://biobank.ndph.ox.ac.uk/showcase/field.cgi?id=23451 (accessed Apr. 26, 2022).
[143] E. E. Noreen, M. J. Sass, M. L. Crowe, V. A. Pabon, J. Brandauer, and L. K. Averill, "Effects of supplemental fish oil on resting metabolic rate, body composition, and salivary cortisol in healthy adults," *Journal of the International Society of Sports Nutrition*, vol. 7, no. 1, p. 31, Oct. 2010, doi: 10.1186/1550-2783-7-31.
[144] S. L. Logan and L. L. Spriet, "Omega-3 Fatty Acid Supplementation for 12 Weeks Increases Resting and Exercise Metabolic Rate in Healthy Community-Dwelling Older Females," *PLOS ONE*, vol. 10, no. 12, p. e0144828, Dec. 2015, doi: 10.1371/journal.pone.0144828.
[145] S. Jannas-Vela, S. L. Klingel, D. M. Mutch, and L. L. Spriet, "DHA supplementation decreases resting metabolic rate in healthy young females," *Appl Physiol Nutr Metab*, vol. 45, no. 2, pp. 221–225, Feb. 2020, doi: 10.1139/apnm-2019-0581.
[146] S. B. Heymsfield *et al.*, "Voluntary weight loss: systematic review of early phase body composition changes," *Obesity Reviews*, vol. 12, no. 5, pp. e348–e361, 2011, doi: 10.1111/j.1467-789X.2010.00767.x.

[147] S. Mettler, N. Mitchell, and K. D. Tipton, “Increased Protein Intake Reduces Lean Body Mass Loss during Weight Loss in Athletes,” *Medicine & Science in Sports & Exercise*, vol. 42, no. 2, pp. 326–337, Feb. 2010, doi: 10.1249/MSS.0b013e3181b2ef8e.
[148] J. S. Garrow and C. D. Summerbell, “Meta-analysis: effect of exercise, with or without dieting, on the body composition of overweight subjects,” *Eur J Clin Nutr*, vol. 49, no. 1, pp. 1–10, Jan. 1995.
[149] E. Cava, N. C. Yeat, and B. Mittendorfer, “Preserving Healthy Muscle during Weight Loss,” *Advances in Nutrition*, vol. 8, no. 3, pp. 511–519, May 2017, doi: 10.3945/an.116.014506.
[150] S. Morris *et al.*, “Inadequacy of Protein Intake in Older UK Adults,” *Geriatrics*, vol. 5, no. 1, Art. no. 1, Mar. 2020, doi: 10.3390/geriatrics5010006.
[151] S. M. Pasiakos *et al.*, “Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: a randomized controlled trial,” *The FASEB Journal*, vol. 27, no. 9, pp. 3837–3847, 2013, doi: 10.1096/fj.13-230227.
[152] A. A. Sneddon *et al.*, “Effect of a Conjugated Linoleic Acid and ω-3 Fatty Acid Mixture on Body Composition and Adiponectin,” *Obesity*, vol. 16, no. 5, pp. 1019–1024, 2008, doi: 10.1038/oby.2008.41.
[153] Y.-H. Huang, W.-C. Chiu, Y.-P. Hsu, Y.-L. Lo, and Y.-H. Wang, “Effects of Omega-3 Fatty Acids on Muscle Mass, Muscle Strength and Muscle Performance among the Elderly: A Meta-Analysis,” *Nutrients*, vol. 12, no. 12, Art. no. 12, Dec. 2020, doi: 10.3390/nu12123739.
[154] I. Thorsdottir *et al.*, “Randomized trial of weight-loss-diets for young adults varying in fish and fish oil content,” *Int J Obes*, vol. 31, no. 10, pp. 1560–1566, Oct. 2007, doi: 10.1038/sj.ijo.0803643.
[155] I. A. Munro and M. L. Garg, “Prior supplementation with long chain omega-3 polyunsaturated fatty acids promotes weight loss in obese adults: a double-blinded randomised controlled trial,” *Food Funct.*, vol. 4, no. 4, pp. 650–658, Mar. 2013, doi: 10.1039/C3FO60038F.
[156] I. A. Munro and M. L. Garg, “Dietary supplementation with long chain omega-3 polyunsaturated fatty acids and weight loss in obese adults,” *Obesity Research & Clinical Practice*, vol. 7, no. 3, pp. e173–e181, May 2013, doi: 10.1016/j.orcp.2011.11.001.
[157] I. A. Munro and M. L. Garg, “Dietary supplementation with n-3 PUFA does not promote weight loss when combined with a very-low-energy diet,” *British Journal of Nutrition*, vol. 108, no. 8, pp. 1466–1474, Oct. 2012, doi: 10.1017/S0007114511006817.
[158] J. W. Beals *et al.*, “Anabolic sensitivity of postprandial muscle protein synthesis to the ingestion of a protein-dense food is reduced in overweight and obese young adults,” *The American Journal of Clinical Nutrition*, vol. 104, no. 4, pp. 1014–1022, Oct. 2016, doi: 10.3945/ajcn.116.130385.
[159] H. L. Hutchins-Wiese *et al.*, “The impact of supplemental N-3 long chain polyunsaturated fatty acids and dietary antioxidants on physical performance in postmenopausal women,” *J Nutr Health Aging*, vol. 17, no. 1, pp. 76–80, Jan. 2013, doi: 10.1007/s12603-012-0415-3.
[160] Y. Rolland *et al.*, “Effect of Long-Term Omega 3 Polyunsaturated Fatty Acid Supplementation with or without Multidomain Lifestyle Intervention on Muscle Strength in Older Adults: Secondary Analysis of the Multidomain Alzheimer Preventive Trial (MAPT),” *Nutrients*, vol. 11, no. 8, Art. no. 8, Aug. 2019, doi: 10.3390/nu11081931.
[161] R. Krzymińska-Siemaszko *et al.*, “The Effect of a 12-Week Omega-3 Supplementation on Body Composition, Muscle Strength and Physical Performance in Elderly Individuals with Decreased Muscle Mass,” *International Journal of Environmental Research and Public Health*, vol. 12, no. 9, Art. no. 9, Sep. 2015, doi: 10.3390/ijerph120910558.
[162] S. M. Cornish, S. B. Myrie, E. M. Bugera, J. E. Chase, D. Turczyn, and M. Pinder, “Omega-3 supplementation with resistance training does not improve body composition or lower biomarkers of inflammation more so than resistance training alone in older men,” *Nutrition Research*, vol. 60, pp. 87–95, Dec. 2018, doi: 10.1016/j.nutres.2018.09.005.
[163] W. Ma *et al.*, “Effect of n-3 polyunsaturated fatty acid supplementation on muscle mass and

function with aging: A meta-analysis of randomized controlled trials☆," *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 165, p. 102249, Feb. 2021, doi: 10.1016/j.plefa.2021.102249.
[164] S. M. Pasiakos *et al.*, "Acute Energy Deprivation Affects Skeletal Muscle Protein Synthesis and Associated Intracellular Signaling Proteins in Physically Active Adults," *The Journal of Nutrition*, vol. 140, no. 4, pp. 745–751, Apr. 2010, doi: 10.3945/jn.109.118372.
[165] J. L. Areta *et al.*, "Reduced resting skeletal muscle protein synthesis is rescued by resistance exercise and protein ingestion following short-term energy deficit," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 306, no. 8, pp. E989–E997, Apr. 2014, doi: 10.1152/ajpendo.00590.2013.
[166] J. W. Carbone, S. M. Pasiakos, L. M. Vislocky, J. M. Anderson, and N. R. Rodriguez, "Effects of short-term energy deficit on muscle protein breakdown and intramuscular proteolysis in normal-weight young adults," *Appl. Physiol. Nutr. Metab.*, vol. 39, no. 8, pp. 960–968, Aug. 2014, doi: 10.1139/apnm-2013-0433.
[167] N. Willumsen, J. Skorve, S. Hexeberg, A. C. Rustan, and R. K. Berge, "The hypotriglyceridemic effect of eicosapentaenoic acid in rats is reflected in increased mitochondrial fatty acid oxidation followed by diminished lipogenesis," *Lipids*, vol. 28, no. 8, pp. 683–690, Aug. 1993, doi: 10.1007/BF02535987.
[168] D. B. Jump, S. D. Clarke, A. Thelen, and M. Liimatta, "Coordinate regulation of glycolytic and lipogenic gene expression by polyunsaturated fatty acids.," *Journal of Lipid Research*, vol. 35, no. 6, Art. no. 6, Jun. 1994, doi: 10.1016/S0022-2275(20)40103-8.
[169] C. Couet, J. Delarue, P. Ritz, J.-M. Antoine, and F. Lamisse, "Effect of dietary fish oil on body fat mass and basal fat oxidation in healthy adults," *Int J Obes*, vol. 21, no. 8, pp. 637–643, Aug. 1997, doi: 10.1038/sj.ijo.0800451.
[170] M. Da Boit, A. M. Hunter, and S. R. Gray, "Fit with good fat? The role of n-3 polyunsaturated fatty acids on exercise performance," *Metabolism*, vol. 66, pp. 45–54, Jan. 2017, doi: 10.1016/j.metabol.2016.10.007.
[171] S. Jannas-Vela, K. Roke, S. Boville, D. M. Mutch, and L. L. Spriet, "Lack of effects of fish oil supplementation for 12 weeks on resting metabolic rate and substrate oxidation in healthy young men: A randomized controlled trial," *PLOS ONE*, vol. 12, no. 2, p. e0172576, Feb. 2017, doi: 10.1371/journal.pone.0172576.
[172] C. J. Gerling, J. Whitfield, K. Mukai, and L. L. Spriet, "Variable effects of 12 weeks of omega-3 supplementation on resting skeletal muscle metabolism," *Appl. Physiol. Nutr. Metab.*, vol. 39, no. 9, Art. no. 9, Sep. 2014, doi: 10.1139/apnm-2014-0049.
[173] M. Fogelholm, "Effects of Bodyweight Reduction on Sports Performance," *Sports Med*, vol. 18, no. 4, pp. 249–267, Oct. 1994, doi: 10.2165/00007256-199418040-00004.
[174] I. Garthe, T. Raastad, P. E. Refsnes, A. Koivisto, and J. Sundgot-Borgen, "Effect of Two Different Weight-Loss Rates on Body Composition and Strength and Power-Related Performance in Elite Athletes," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 21, no. 2, pp. 97–104, Apr. 2011, doi: 10.1123/ijsnem.21.2.97.
[175] C. Bouchard, A. Tremblay, C. Leblanc, G. Lortie, R. Savard, and G. Thériault, "A method to assess energy expenditure in children and adults," *Am J Clin Nutr*, vol. 37, no. 3, pp. 461–467, Mar. 1983, doi: 10.1093/ajcn/37.3.461.
[176] N. Rodriguez-Sanchez and S. D. R. Galloway, "Errors in Dual Energy X-Ray Absorptiometry Estimation of Body Composition Induced by Hypohydration," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 25, no. 1, pp. 60–68, Feb. 2015, doi: 10.1123/ijsnem.2014-0067.
[177] J. D. Philpott *et al.*, "Influence of Fish Oil-Derived n-3 Fatty Acid Supplementation on Changes in Body Composition and Muscle Strength During Short-Term Weight Loss in Resistance-Trained Men," *Front. Nutr.*, vol. 0, 2019, doi: 10.3389/fnut.2019.00102.
[178] J. B. de V. Weir, "New methods for calculating metabolic rate with special reference to protein metabolism," *J Physiol*, vol. 109, no. 1–2, pp. 1–9, Aug. 1949.

[179] M. J. Marfell-Jones, A. D. Stewart, and J. H. de Ridder, *International standards for anthropometric assessment.* 2012. Accessed: Apr. 07, 2022. [Online]. Available: https://repository.openpolytechnic.ac.nz/handle/11072/1510
[180] R. T. Withers, N. P. Craig, P. C. Bourdon, and K. I. Norton, "Relative body fat and anthropometric prediction of body density of male athletes," *Eur J Appl Physiol Occup Physiol*, vol. 56, no. 2, pp. 191–200, 1987, doi: 10.1007/BF00640643.
[181] T. Reilly, K. George, M. Marfell-Jones, M. Scott, L. Sutton, and J. A. Wallace, "How well do skinfold equations predict percent body fat in elite soccer players?," *Int J Sports Med*, vol. 30, no. 8, pp. 607–613, Aug. 2009, doi: 10.1055/s-0029-1202353.
[182] S. Pillen and N. van Alfen, "Skeletal muscle ultrasound," *Neurol Res*, vol. 33, no. 10, pp. 1016–1024, Dec. 2011, doi: 10.1179/1743132811Y.0000000010.
[183] J. G. Bell, E. E. Mackinlay, J. R. Dick, I. Younger, B. Lands, and T. Gilhooly, "Using a fingertip whole blood sample for rapid fatty acid measurement: method validation and correlation with erythrocyte polar lipid compositions in UK subjects," *British Journal of Nutrition*, vol. 106, no. 9, pp. 1408–1415, Nov. 2011, doi: 10.1017/S0007114511001978.
[184] D. McNeish, "Small Sample Methods for Multilevel Modeling: A Colloquial Elucidation of REML and the Kenward-Roger Correction," *Multivariate Behavioral Research*, vol. 52, no. 5, pp. 661–670, Sep. 2017, doi: 10.1080/00273171.2017.1344538.
[185] M. G. Kenward and J. H. Roger, "Small Sample Inference for Fixed Effects from Restricted Maximum Likelihood," *Biometrics*, vol. 53, no. 3, pp. 983–997, 1997, doi: 10.2307/2533558.
[186] J. W. Tukey, "Comparing Individual Means in the Analysis of Variance," *Biometrics*, vol. 5, no. 2, pp. 99–114, 1949, doi: 10.2307/3001913.
[187] J. Cohen, *Statistical Power Analysis for the Behavioral Sciences*, 2nd ed. New York: Routledge, 1988. doi: 10.4324/9780203771587.
[188] L. V. Hedges, "Estimation of effect size from a series of independent experiments," *Psychological Bulletin*, vol. 92, no. 2, pp. 490–499, 1982, doi: 10.1037/0033-2909.92.2.490.
[189] C. O. Fritz, P. E. Morris, and J. J. Richler, "Effect size estimates: current use, calculations, and interpretation," *J Exp Psychol Gen*, vol. 141, no. 1, pp. 2–18, Feb. 2012, doi: 10.1037/a0024338.
[190] J. W. Beals, N. A. Burd, D. R. Moore, and S. van Vliet, "Obesity Alters the Muscle Protein Synthetic Response to Nutrition and Exercise," *Frontiers in Nutrition*, vol. 6, 2019, Accessed: Jan. 29, 2023. [Online]. Available: https://www.frontiersin.org/articles/10.3389/fnut.2019.00087
[191] D. Dardevet, D. Rémond, M.-A. Peyron, I. Papet, I. Savary-Auzeloux, and L. Mosoni, "Muscle Wasting and Resistance of Muscle Anabolism: The 'Anabolic Threshold Concept' for Adapted Nutritional Strategies during Sarcopenia," *The Scientific World Journal*, vol. 2012, p. e269531, Dec. 2012, doi: 10.1100/2012/269531.
[192] A. Nana, G. J. Slater, A. D. Stewart, and L. M. Burke, "Methodology Review: Using Dual-Energy X-Ray Absorptiometry (DXA) for the Assessment of Body Composition in Athletes and Active People," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 25, no. 2, pp. 198–215, Apr. 2015, doi: 10.1123/ijsnem.2013-0228.
[193] D. L. Ballor and Eric. T. Poehlman, "A meta-analysis of the effects of exercise and/or dietary restriction on resting metabolic rate," *Eur J Appl Physiol*, vol. 71, no. 6, pp. 535–542, Nov. 1995, doi: 10.1007/BF00238557.
[194] J. L. Thompson, M. M. Manore, and J. R. Thomas, "Effects of Diet and Diet-Plus-Exercise Programs on Resting Metabolic Rate: A Meta-Analysis," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 6, no. 1, pp. 41–61, Mar. 1996, doi: 10.1123/ijsn.6.1.41.
[195] L. F. DeFina, L. G. Marcoux, S. M. Devers, J. P. Cleaver, and B. L. Willis, "Effects of omega-3 supplementation in combination with diet and exercise on weight loss and body composition," *The American Journal of Clinical Nutrition*, vol. 93, no. 2, pp. 455–462, Feb. 2011, doi: 10.3945/ajcn.110.002741.

[196] C. Compher, D. Frankenfield, N. Keim, and L. Roth-Yousey, “Best Practice Methods to Apply to Measurement of Resting Metabolic Rate in Adults: A Systematic Review,” *Journal of the American Dietetic Association*, vol. 106, no. 6, pp. 881–903, Jun. 2006, doi: 10.1016/j.jada.2006.02.009.
[197] D. F. Rolfe and G. C. Brown, “Cellular energy utilization and molecular origin of standard metabolic rate in mammals,” *Physiological Reviews*, vol. 77, no. 3, pp. 731–758, Jul. 1997, doi: 10.1152/physrev.1997.77.3.731.
[198] A. J. Hulbert, “Membrane Fatty Acids as Pacemakers of Animal Metabolism,” *Lipids*, vol. 42, no. 9, pp. 811–819, 2007, doi: 10.1007/s11745-007-3058-0.
[199] E. A. F. Herbst *et al.*, “Omega-3 supplementation alters mitochondrial membrane composition and respiration kinetics in human skeletal muscle,” *J Physiol*, vol. 592, no. Pt 6, pp. 1341–1352, Mar. 2014, doi: 10.1113/jphysiol.2013.267336.
[200] R. A. Vaughan, R. Garcia-Smith, M. Bisoffi, C. A. Conn, and K. A. Trujillo, “Conjugated linoleic acid or omega 3 fatty acids increase mitochondrial biosynthesis and metabolism in skeletal muscle cells,” *Lipids Health Dis*, vol. 11, no. 1, p. 142, Oct. 2012, doi: 10.1186/1476-511X-11-142.
[201] I. R. Lanza *et al.*, “Influence of fish oil on skeletal muscle mitochondrial energetics and lipid metabolites during high-fat diet,” *Am J Physiol Endocrinol Metab*, vol. 304, no. 12, pp. E1391-1403, Jun. 2013, doi: 10.1152/ajpendo.00584.2012.
[202] K. Salin *et al.*, “The relationship between membrane fatty acid content and mitochondrial efficiency differs within- and between- omega-3 dietary treatments,” *Marine Environmental Research*, vol. 163, p. 105205, Jan. 2021, doi: 10.1016/j.marenvres.2020.105205.
[203] S. C. Parkes, A. N, elcastro, L. J. Mccargar, and D. Mckenzie, “Effect of Energy Restriction on Muscle Function and Calcium Stimulated Protease Activity in Recreationally Active Women,” *Can. J. Appl. Physiol.*, vol. 23, no. 3, pp. 279–292, Jun. 1998, doi: 10.1139/h98-016.
[204] P. A. Molé, “Daily exercise enhances fat utilization and maintains metabolic rate during severe energy restriction in humans,” *Sports Medicine, Training and Rehabilitation*, vol. 7, no. 1, pp. 39–48, Jul. 1996, doi: 10.1080/15438629609512069.
[205] N. Willumsen, H. Vaagenes, Ø. Lie, A. C. Rustan, and R. K. Berge, “Eicosapentaenoic acid, but not docosahexaenoic acid, increases mitochondrial fatty acid oxidation and upregulates 2,4-dienoyl-CoA reductase gene expression in rats,” *Lipids*, vol. 31, no. 6, Art. no. 6, 1996, doi: 10.1007/BF02523828.
[206] P. Weber and D. Raederstorff, “Triglyceride-lowering effect of omega-3 LC-polyunsaturated fatty acids--a review,” *Nutr Metab Cardiovasc Dis*, vol. 10, no. 1, pp. 28–37, Feb. 2000.
[207] J. Lopes, D. M. Russell, J. Whitwell, and K. N. Jeejeebhoy, “Skeletal muscle function in malnutrition,” *The American Journal of Clinical Nutrition*, vol. 36, no. 4, pp. 602–610, Oct. 1982, doi: 10.1093/ajcn/36.4.602.
[208] J. L. Nuzzo, J. M. McBride, P. Cormie, and G. O. McCaulley, “Relationship Between Countermovement Jump Performance and Multijoint Isometric and Dynamic Tests of Strength,” *The Journal of Strength & Conditioning Research*, vol. 22, no. 3, pp. 699–707, May 2008, doi: 10.1519/JSC.0b013e31816d5eda.
[209] G. Markovic, D. Dizdar, I. Jukic, and M. Cardinale, “Reliability and Factorial Validity of Squat and Countermovement Jump Tests,” *The Journal of Strength & Conditioning Research*, vol. 18, no. 3, pp. 551–555, Aug. 2004.
[210] K. L. Sainani, K. R. Lohse, P. R. Jones, and A. Vickers, “Magnitude-based Inference is not Bayesian and is not a valid method of inference,” *Scand J Med Sci Sports*, vol. 29, no. 9, pp. 1428–1436, Sep. 2019, doi: 10.1111/sms.13491.
[211] A. H. Welsh and E. J. Knight, “‘Magnitude-based inference’: a statistical review,” *Med Sci Sports Exerc*, vol. 47, no. 4, pp. 874–884, Apr. 2015, doi: 10.1249/MSS.0000000000000451.
[212] G. E. Peoples and P. L. McLennan, “Dietary fish oil reduces skeletal muscle oxygen consumption, provides fatigue resistance and improves contractile recovery in the rat in vivo

hindlimb," *British Journal of Nutrition*, vol. 104, no. 12, pp. 1771–1779, Dec. 2010, doi: 10.1017/S0007114510002928.
[213] J. F. da Silva Santos, M. Y. Takito, G. G. Artioli, and E. Franchini, "Weight loss practices in Taekwondo athletes of different competitive levels," *J Exerc Rehabil*, vol. 12, no. 3, pp. 202–208, Jun. 2016, doi: 10.12965/jer.1632610.305.
[214] D. R. Moore *et al.*, "Daytime pattern of post-exercise protein intake affects whole-body protein turnover in resistance-trained males," *Nutrition & Metabolism*, vol. 9, no. 1, p. 91, Oct. 2012, doi: 10.1186/1743-7075-9-91.
[215] J. L. Areta *et al.*, "Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis," *The Journal of Physiology*, vol. 591, no. 9, pp. 2319–2331, 2013, doi: 10.1113/jphysiol.2012.244897.
[216] P. T. Price, C. M. Nelson, and S. D. Clarke, "Omega-3 polyunsaturated fatty acid regulation of gene expression," *Current Opinion in Lipidology*, vol. 11, no. 1, pp. 3–7, Feb. 2000.
[217] A. J. Hector, C. McGlory, F. Damas, N. Mazara, S. K. Baker, and S. M. Phillips, "Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise," *The FASEB Journal*, vol. 32, no. 1, pp. 265–275, 2018, doi: https://doi.org/10.1096/fj.201700158RR.
[218] S. M. Phillips and C. McGlory, "CrossTalk proposal: The dominant mechanism causing disuse muscle atrophy is decreased protein synthesis," *The Journal of Physiology*, vol. 592, no. 24, pp. 5341–5343, 2014, doi: 10.1113/jphysiol.2014.273615.
[219] M. B. Reid, A. R. Judge, and S. C. Bodine, "CrossTalk opposing view: The dominant mechanism causing disuse muscle atrophy is proteolysis," *The Journal of Physiology*, vol. 592, no. 24, pp. 5345–5347, 2014, doi: 10.1113/jphysiol.2014.279406.
[220] G. I. Smith *et al.*, "Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia–hyperaminoacidaemia in healthy young and middle-aged men and women," *Clinical Science*, vol. 121, no. 6, Art. no. 6, May 2011, doi: 10.1042/CS20100597.
[221] P. J. Atherton and K. Smith, "Muscle protein synthesis in response to nutrition and exercise," *The Journal of Physiology*, vol. 590, no. 5, pp. 1049–1057, 2012, doi: 10.1113/jphysiol.2011.225003.
[222] S. C. Bodine *et al.*, "Identification of ubiquitin ligases required for skeletal muscle atrophy," *Science*, vol. 294, no. 5547, pp. 1704–1708, Nov. 2001, doi: 10.1126/science.1065874.
[223] S. C. Bodine, "Edward F. Adolph Distinguished Lecture. Skeletal muscle atrophy: Multiple pathways leading to a common outcome," *Journal of Applied Physiology*, vol. 129, no. 2, pp. 272–282, Aug. 2020, doi: 10.1152/japplphysiol.00381.2020.
[224] M. Sandri, "Protein breakdown in muscle wasting: Role of autophagy-lysosome and ubiquitin-proteasome," *The International Journal of Biochemistry & Cell Biology*, vol. 45, no. 10, pp. 2121–2129, Oct. 2013, doi: 10.1016/j.biocel.2013.04.023.
[225] S. H. Lecker, V. Solomon, W. E. Mitch, and A. L. Goldberg, "Muscle Protein Breakdown and the Critical Role of the Ubiquitin-Proteasome Pathway in Normal and Disease States," *The Journal of Nutrition*, vol. 129, no. 1, pp. 227S-237S, Jan. 1999, doi: 10.1093/jn/129.1.227S.
[226] K. Jing *et al.*, "Docosahexaenoic acid induces autophagy through p53/AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53," *Autophagy*, vol. 7, no. 11, pp. 1348–1358, Nov. 2011, doi: 10.4161/auto.7.11.16658.
[227] S. Shin *et al.*, "The Omega-3 Polyunsaturated Fatty Acid DHA Induces Simultaneous Apoptosis and Autophagy via Mitochondrial ROS-Mediated Akt-mTOR Signaling in Prostate Cancer Cells Expressing Mutant p53," *BioMed Research International*, vol. 2013, p. e568671, Jun. 2013, doi: 10.1155/2013/568671.
[228] J. Yoshino, G. I. Smith, S. C. Kelly, S. Julliand, D. N. Reeds, and B. Mittendorfer, "Effect of dietary n-3 PUFA supplementation on the muscle transcriptome in older adults," *Physiological Reports*, vol. 4, no. 11, p. e12785, 2016, doi: 10.14814/phy2.12785.

[229] A. Ramel, J. A. Martinez, M. Kiely, N. M. Bandarra, and I. Thorsdottir, “Effects of weight loss and seafood consumption on inflammation parameters in young, overweight and obese European men and women during 8 weeks of energy restriction,” *Eur J Clin Nutr*, vol. 64, no. 9, pp. 987–993, Sep. 2010, doi: 10.1038/ejcn.2010.99.
[230] J. Lefils-Lacourtablaise *et al.*, “The eicosapentaenoic acid metabolite 15-deoxy-δ(12,14)-prostaglandin J3 increases adiponectin secretion by adipocytes partly via a PPARγ-dependent mechanism,” *PLoS One*, vol. 8, no. 5, p. e63997, 2013, doi: 10.1371/journal.pone.0063997.
[231] B. Gray, F. Steyn, P. S. W. Davies, and L. Vitetta, “Omega-3 fatty acids: a review of the effects on adiponectin and leptin and potential implications for obesity management,” *Eur J Clin Nutr*, vol. 67, no. 12, pp. 1234–1242, Dec. 2013, doi: 10.1038/ejcn.2013.197.
[232] M. Itoh *et al.*, “Increased Adiponectin Secretion by Highly Purified Eicosapentaenoic Acid in Rodent Models of Obesity and Human Obese Subjects,” *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 27, no. 9, pp. 1918–1925, Sep. 2007, doi: 10.1161/ATVBAHA.106.136853.
[233] N. Guelzim *et al.*, “N-3 fatty acids improve body composition and insulin sensitivity during energy restriction in the rat,” *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 91, no. 5, pp. 203–211, Nov. 2014, doi: 10.1016/j.plefa.2014.07.007.
[234] B. Cohen, D. Novick, and M. Rubinstein, “Modulation of Insulin Activities by Leptin,” *Science*, vol. 274, no. 5290, pp. 1185–1188, Nov. 1996, doi: 10.1126/science.274.5290.1185.
[235] M. Hariri, R. Ghiasvand, A. Shiranian, G. Askari, B. Iraj, and A. Salehi-Abargouei, “Does omega-3 fatty acids supplementation affect circulating leptin levels? A systematic review and meta-analysis on randomized controlled clinical trials,” *Clinical Endocrinology*, vol. 82, no. 2, pp. 221–228, 2015, doi: 10.1111/cen.12508.
[236] D. M. Muoio, G. L. Dohm, F. T. Fiedorek, E. B. Tapscott, R. A. Coleman, and G. L. Dohn, “Leptin directly alters lipid partitioning in skeletal muscle,” *Diabetes*, vol. 46, no. 8, pp. 1360–1363, Aug. 1997, doi: 10.2337/diab.46.8.1360.
[237] G. R. Steinberg, A. Bonen, and D. J. Dyck, “Fatty acid oxidation and triacylglycerol hydrolysis are enhanced after chronic leptin treatment in rats,” *American Journal of Physiology-Endocrinology and Metabolism*, vol. 282, no. 3, Art. no. 3, Mar. 2002, doi: 10.1152/ajpendo.00303.2001.
[238] G. R. Steinberg *et al.*, “Chronic Leptin Administration Decreases Fatty Acid Uptake and Fatty Acid Transporters in Rat Skeletal Muscle *,” *Journal of Biological Chemistry*, vol. 277, no. 11, pp. 8854–8860, Mar. 2002, doi: 10.1074/jbc.M107683200.
[239] T. R. Baechle and R. W. Earle, *Essentials of Strength Training and Conditioning*. Human Kinetics, 2008.
[240] T. Nagasawa, F. Yoshizawa, and N. Nishizawa, “Plasma N tau-methylhistidine concentration is a sensitive index of myofibrillar protein degradation during starvation in rats,” *Biosci Biotechnol Biochem*, vol. 60, no. 3, pp. 501–502, Mar. 1996, doi: 10.1271/bbb.60.501.
[241] J. A. Rathmacher, G. A. Link, P. J. Flakoll, and S. L. Nissen, “Gas chromatographic/mass spectrometric analysis of stable isotopes of 3-methylhistidine in biological fluids: Application to plasma kineticsin vivo,” *Biol. Mass Spectrom.*, vol. 21, no. 11, pp. 560–566, Nov. 1992, doi: 10.1002/bms.1200211107.
[242] J. Bergström, “Percutaneous Needle Biopsy of Skeletal Muscle in Physiological and Clinical Research,” *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 35, no. 7, pp. 609–616, Jan. 1975, doi: 10.1080/00365517509095787.
[243] M. A. Tarnopolsky, E. Pearce, K. Smith, and B. Lach, “Suction-modified Bergström muscle biopsy technique: Experience with 13,500 procedures,” *Muscle & Nerve*, vol. 43, no. 5, pp. 716–725, 2011, doi: 10.1002/mus.21945.
[244] R. Van Thienen, G. D’Hulst, L. Deldicque, and P. Hespel, “Biochemical artifacts in experiments involving repeated biopsies in the same muscle,” *Physiological Reports*, vol. 2, no. 5, p. e00286, 2014, doi: 10.14814/phy2.286.
[245] J. Vandesompele *et al.*, “Accurate normalization of real-time quantitative RT-PCR data by

geometric averaging of multiple internal control genes," *Genome Biology*, vol. 3, no. 7, p. research0034.1, Jun. 2002, doi: 10.1186/gb-2002-3-7-research0034.
[246] B. P. Carson *et al.*, "Regulation of muscle protein synthesis in an in vitro cell model using ex vivo human serum," *Experimental Physiology*, vol. 103, no. 6, pp. 783–789, 2018, doi: 10.1113/EP086860.
[247] M. Delacre, D. Lakens, and C. Leys, "Why Psychologists Should by Default Use Welch's *t*-test Instead of Student's *t*-test," *International Review of Social Psychology*, vol. 30, no. 1, Art. no. 1, Apr. 2017, doi: 10.5334/irsp.82.
[248] T. D. Schmittgen and K. J. Livak, "Analyzing real-time PCR data by the comparative CT method," *Nat Protoc*, vol. 3, no. 6, Art. no. 6, Jun. 2008, doi: 10.1038/nprot.2008.73.
[249] P. L. Greenhaff *et al.*, "Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle," *Am J Physiol Endocrinol Metab*, vol. 295, no. 3, pp. E595-604, Sep. 2008, doi: 10.1152/ajpendo.90411.2008.
[250] K. D. Tipton, E. Borsheim, S. E. Wolf, A. P. Sanford, and R. R. Wolfe, "Acute response of net muscle protein balance reflects 24-h balance after exercise and amino acid ingestion," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 284, no. 1, pp. E76–E89, Jan. 2003, doi: 10.1152/ajpendo.00234.2002.
[251] C. McGlory *et al.*, "Application of the [γ-32P] ATP kinase assay to study anabolic signaling in human skeletal muscle," *Journal of Applied Physiology*, vol. 116, no. 5, pp. 504–513, Mar. 2014, doi: 10.1152/japplphysiol.01072.2013.
[252] S. J. Crozier, S. R. Kimball, S. W. Emmert, J. C. Anthony, and L. S. Jefferson, "Oral Leucine Administration Stimulates Protein Synthesis in Rat Skeletal Muscle," *The Journal of Nutrition*, vol. 135, no. 3, pp. 376–382, Mar. 2005, doi: 10.1093/jn/135.3.376.
[253] M. Gassmann, B. Grenacher, B. Rohde, and J. Vogel, "Quantifying Western blots: pitfalls of densitometry," *Electrophoresis*, vol. 30, no. 11, pp. 1845–1855, Jun. 2009, doi: 10.1002/elps.200800720.
[254] J. D. MacDougall, M. J. Gibala, M. A. Tarnopolsky, J. R. MacDonald, S. A. Interisano, and K. E. Yarasheski, "The Time Course for Elevated Muscle Protein Synthesis Following Heavy Resistance Exercise," *Can. J. Appl. Physiol.*, vol. 20, no. 4, pp. 480–486, Dec. 1995, doi: 10.1139/h95-038.
[255] O. C. Witard, "Defining the anabolic window of opportunity," *Sport Nutrition - Agro FOOD Industry Hi-Tech*, vol. 25, p. 4, 2014.
[256] T. A. Babcock, A. Kurland, W. S. Helton, A. Rahman, K. N. Anwar, and N. J. Espat, "Inhibition of activator protein-1 transcription factor activation by omega-3 fatty acid modulation of mitogen-activated protein kinase signaling kinases," *JPEN J Parenter Enteral Nutr*, vol. 27, no. 3, pp. 176–180; discussion 181, Jun. 2003, doi: 10.1177/0148607103027003176.
[257] P. Magee, S. Pearson, J. Whittingham-Dowd, and J. Allen, "PPARγ as a molecular target of EPA anti-inflammatory activity during TNF-α-impaired skeletal muscle cell differentiation," *J Nutr Biochem*, vol. 23, no. 11, pp. 1440–1448, Nov. 2012, doi: 10.1016/j.jnutbio.2011.09.005.
[258] L. M. Margolis, D. A. Rivas, S. M. Pasiakos, J. P. McClung, L. Ceglia, and R. A. Fielding, "Upregulation of circulating myomiR following short-term energy restriction is inversely associated with whole body protein synthesis," *Am J Physiol Regul Integr Comp Physiol*, vol. 313, no. 3, pp. R298–R304, Sep. 2017, doi: 10.1152/ajpregu.00054.2017.
[259] H. J. Smith, M. J. Lorite, and M. J. Tisdale, "Effect of a Cancer Cachectic Factor on Protein Synthesis/Degradation in Murine C2C12 Myoblasts: Modulation by Eicosapentaenoic Acid," *Cancer Res*, vol. 59, no. 21, pp. 5507–5513, Nov. 1999.
[260] Y. Wang, Q. Lin, P. Zheng, J. Zhang, and F. Huang, "DHA inhibits protein degradation more efficiently than EPA by regulating the PPARγ/NFκB pathway in C2C12 myotubes," *Biomed Res Int*, vol. 2013, p. 318981, 2013, doi: 10.1155/2013/318981.
[261] S. C. Bodine and L. M. Baehr, "Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1

and MAFbx/atrogin-1," *American Journal of Physiology-Endocrinology and Metabolism*, vol. 307, no. 6, pp. E469–E484, Sep. 2014, doi: 10.1152/ajpendo.00204.2014.
[262] H. Li, S. Malhotra, and A. Kumar, "Nuclear factor-kappa B signaling in skeletal muscle atrophy," *J Mol Med*, vol. 86, no. 10, pp. 1113–1126, Oct. 2008, doi: 10.1007/s00109-008-0373-8.
[263] M. Figueras, M. Olivan, S. Busquets, F. J. López-Soriano, and J. M. Argilés, "Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status," *Obesity (Silver Spring)*, vol. 19, no. 2, Art. no. 2, Feb. 2011, doi: 10.1038/oby.2010.194.
[264] A. S. Whitehouse and M. J. Tisdale, "Increased expression of the ubiquitin – proteasome pathway in murine myotubes by proteolysis-inducing factor (PIF) is associated with activation of the transcription factor NF-κB," *Br J Cancer*, vol. 89, no. 6, pp. 1116–1122, Sep. 2003, doi: 10.1038/sj.bjc.6601132.
[265] K. Lim, C. Han, Y. Dai, M. Shen, and T. Wu, "OMEGA-3 POLYUNSATURATED FATTY ACIDS INHIBIT HEPATOCELLULAR CARCINOMA CELL GROWTH THROUGH BLOCKING β-CATENIN AND COX-2," *Mol Cancer Ther*, vol. 8, no. 11, pp. 3046–3055, Nov. 2009, doi: 10.1158/1535-7163.MCT-09-0551.
[266] D. Rovito *et al.*, "Omega-3 DHA- and EPA–dopamine conjugates induce PPARγ-dependent breast cancer cell death through autophagy and apoptosis," *Biochimica et Biophysica Acta (BBA) - General Subjects*, vol. 1850, no. 11, pp. 2185–2195, Nov. 2015, doi: 10.1016/j.bbagen.2015.08.004.
[267] L. Li, Y. Chen, and S. B. Gibson, "Starvation-induced autophagy is regulated by mitochondrial reactive oxygen species leading to AMPK activation," *Cellular Signalling*, vol. 25, no. 1, pp. 50–65, Jan. 2013, doi: 10.1016/j.cellsig.2012.09.020.
[268] M. B. Menon and S. Dhamija, "Beclin 1 Phosphorylation – at the Center of Autophagy Regulation," *Frontiers in Cell and Developmental Biology*, vol. 6, p. 137, 2018, doi: 10.3389/fcell.2018.00137.
[269] M. G. MacKenzie, D. L. Hamilton, J. T. Murray, and K. Baar, "mVps34 is activated by an acute bout of resistance exercise," *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1314–1316, Oct. 2007, doi: 10.1042/BST0351314.
[270] M. G. MacKenzie, D. L. Hamilton, J. T. Murray, P. M. Taylor, and K. Baar, "mVps34 is activated following high-resistance contractions," *The Journal of Physiology*, vol. 587, no. 1, pp. 253–260, 2009, doi: 10.1113/jphysiol.2008.159830.
[271] M.-S. Yoon, K. Son, E. Arauz, J. M. Han, S. Kim, and J. Chen, "Leucyl-tRNA Synthetase Activates Vps34 in Amino Acid-Sensing mTORC1 Signaling," *Cell Reports*, vol. 16, no. 6, pp. 1510–1517, Aug. 2016, doi: 10.1016/j.celrep.2016.07.008.
[272] J. Zhang and P. A. Ney, "Role of BNIP3 and NIX in cell death, autophagy, and mitophagy," *Cell Death Differ*, vol. 16, no. 7, pp. 939–946, Jul. 2009, doi: 10.1038/cdd.2009.16.
[273] M. R. de Oliveira, S. F. Nabavi, S. M. Nabavi, and F. R. Jardim, "Omega-3 polyunsaturated fatty acids and mitochondria, back to the future," *Trends in Food Science & Technology*, vol. 67, pp. 76–92, Sep. 2017, doi: 10.1016/j.tifs.2017.06.019.
[274] J. H. Hurley and L. N. Young, "Mechanisms of Autophagy Initiation," *Annu Rev Biochem*, vol. 86, pp. 225–244, Jun. 2017, doi: 10.1146/annurev-biochem-061516-044820.
[275] C. Mammucari *et al.*, "FoxO3 controls autophagy in skeletal muscle in vivo," *Cell Metab*, vol. 6, no. 6, pp. 458–471, Dec. 2007, doi: 10.1016/j.cmet.2007.11.001.
[276] D. J. Klionsky *et al.*, "Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition)," *Autophagy*, vol. 12, no. 1, pp. 1–222, Jan. 2016, doi: 10.1080/15548627.2015.1100356.
[277] F. Haidari, M. Tavakoli, H. Heybar, B. Helli, and M. Mohamadshahi, "The Effect of Omega-3 on the Level of Serum Lipid Profile, Adipocytokines, and Indicator of Vascular Inflammation in Patients Diagnosed with Myocardial Infarction," *Journal of Babol University of Medical Sciences*, vol. 17, no. 4, pp. 7–17, Apr. 2015, doi:

10.22088/jbums.17.4.7.
[278] J. D. Krebs *et al.*, “Additive benefits of long-chain n -3 polyunsaturated fatty acids and weight-loss in the management of cardiovascular disease risk in overweight hyperinsulinaemic women,” *Int J Obes*, vol. 30, no. 10, pp. 1535–1544, Oct. 2006, doi: 10.1038/sj.ijo.0803309.
[279] T. Thomas *et al.*, “Relationship of serum leptin levels with body composition and sex steroid and insulin levels in men and women,” *Metabolism*, vol. 49, no. 10, pp. 1278–1284, Oct. 2000, doi: 10.1053/meta.2000.9519.
[280] P. C. Calder, “The role of marine omega-3 (n-3) fatty acids in inflammatory processes, atherosclerosis and plaque stability,” *Molecular Nutrition & Food Research*, vol. 56, no. 7, pp. 1073–1080, 2012, doi: 10.1002/mnfr.201100710.
[281] R. Ghosh, J. E. Gilda, and A. V. Gomes, “The necessity of and strategies for improving confidence in the accuracy of western blots,” *Expert Rev Proteomics*, vol. 11, no. 5, pp. 549–560, Oct. 2014, doi: 10.1586/14789450.2014.939635.
[282] S. C. Taylor, T. Berkelman, G. Yadav, and M. Hammond, “A Defined Methodology for Reliable Quantification of Western Blot Data,” *Mol Biotechnol*, vol. 55, no. 3, pp. 217–226, Nov. 2013, doi: 10.1007/s12033-013-9672-6.
[283] C. K. Armah, K. G. Jackson, I. Doman, L. James, F. Cheghani, and A. M. Minihane, “Fish oil fatty acids improve postprandial vascular reactivity in healthy men,” *Clinical Science*, vol. 114, no. 11, pp. 679–686, Jun. 2008, doi: 10.1042/CS20070277.
[284] M. F.-F. Chong, S. Lockyer, C. J. Saunders, and J. A. Lovegrove, “Long chain n-3 PUFA-rich meal reduced postprandial measures of arterial stiffness,” *Clin Nutr*, vol. 29, no. 5, pp. 678–681, Oct. 2010, doi: 10.1016/j.clnu.2010.02.001.
[285] V. G. Rontoyanni, W. L. Hall, S. Pombo-Rodrigues, A. Appleton, R. Chung, and T. A. B. Sanders, “A comparison of the changes in cardiac output and systemic vascular resistance during exercise following high-fat meals containing DHA or EPA,” *British Journal of Nutrition*, vol. 108, no. 3, pp. 492–499, Aug. 2012, doi: 10.1017/S0007114511005721.
[286] R. A. Vogel, M. C. Corretti, and G. D. Plotnick, “The postprandial effect of components of the mediterranean diet on endothelial function,” *Journal of the American College of Cardiology*, vol. 36, no. 5, pp. 1455–1460, Nov. 2000, doi: 10.1016/S0735-1097(00)00896-2.
[287] D. C. Poole, B. J. Behnke, and T. I. Musch, “The role of vascular function on exercise capacity in health and disease,” *The Journal of Physiology*, vol. 599, no. 3, pp. 889–910, 2021, doi: 10.1113/JP278931.
[288] A. M. Hill, J. D. Buckley, K. J. Murphy, and P. R. C. Howe, “Combining fish-oil supplements with regular aerobic exercise improves body composition and cardiovascular disease risk factors,” *Am J Clin Nutr*, vol. 85, no. 5, pp. 1267–1274, May 2007, doi: 10.1093/ajcn/85.5.1267.
[289] B. Walser and C. L. Stebbins, “Omega-3 fatty acid supplementation enhances stroke volume and cardiac output during dynamic exercise,” *Eur J Appl Physiol*, vol. 104, no. 3, Art. no. 3, Oct. 2008, doi: 10.1007/s00421-008-0791-x.
[290] F. Dangardt *et al.*, “Omega-3 fatty acid supplementation improves vascular function and reduces inflammation in obese adolescents,” *Atherosclerosis*, vol. 212, no. 2, pp. 580–585, Oct. 2010, doi: 10.1016/j.atherosclerosis.2010.06.046.
[291] G. Colussi, C. Catena, M. Novello, N. Bertin, and L. A. Sechi, “Impact of omega-3 polyunsaturated fatty acids on vascular function and blood pressure: Relevance for cardiovascular outcomes,” *Nutrition, Metabolism and Cardiovascular Diseases*, vol. 27, no. 3, pp. 191–200, Mar. 2017, doi: 10.1016/j.numecd.2016.07.011.
[292] W. L. Hall, K. A. Sanders, T. A. B. Sanders, and P. J. Chowienczyk, “A High-Fat Meal Enriched with Eicosapentaenoic Acid Reduces Postprandial Arterial Stiffness Measured by Digital Volume Pulse Analysis in Healthy Men,” *The Journal of Nutrition*, vol. 138, no. 2, pp. 287–291, Feb. 2008, doi: 10.1093/jn/138.2.287.
[293] J. W. Senefeld, C. C. Wiggins, R. J. Regimbal, P. B. Dominelli, S. E. Baker, and M. J. Joyner,

"Ergogenic Effect of Nitrate Supplementation: A Systematic Review and Meta-analysis," *Medicine & Science in Sports & Exercise*, vol. 52, no. 10, pp. 2250–2261, Oct. 2020, doi: 10.1249/MSS.0000000000002363.
[294] K. E. Lansley *et al.*, "Acute Dietary Nitrate Supplementation Improves Cycling Time Trial Performance," *Medicine & Science in Sports & Exercise*, vol. 43, no. 6, pp. 1125–1131, Jun. 2011, doi: 10.1249/MSS.0b013e31821597b4.
[295] M. P. Murphy, "Antioxidants as therapies: can we improve on nature?," *Free Radical Biology and Medicine*, vol. 66, pp. 20–23, Jan. 2014, doi: 10.1016/j.freeradbiomed.2013.04.010.
[296] D. Richard, K. Kefi, U. Barbe, P. Bausero, and F. Visioli, "Polyunsaturated fatty acids as antioxidants," *Pharmacological Research*, vol. 57, no. 6, pp. 451–455, Jun. 2008, doi: 10.1016/j.phrs.2008.05.002.
[297] M. Y. Abeywardena and R. J. Head, "Longchain n−3 polyunsaturated fatty acids and blood vessel function," *Cardiovascular Research*, vol. 52, no. 3, pp. 361–371, Dec. 2001, doi: 10.1016/S0008-6363(01)00406-0.
[298] R. Purcell, S. H. Latham, K. M. Botham, W. L. Hall, and C. P. Wheeler-Jones, "High-fat meals rich in EPA plus DHA compared with DHA only have differential effects on postprandial lipemia and plasma 8-isoprostane F2α concentrations relative to a control high–oleic acid meal: a randomized controlled trial," *The American Journal of Clinical Nutrition*, vol. 100, no. 4, pp. 1019–1028, Oct. 2014, doi: 10.3945/ajcn.114.091223.
[299] M. Zanetti *et al.*, "Omega-3 Polyunsaturated Fatty Acids: Structural and Functional Effects on the Vascular Wall," *BioMed Research International*, vol. 2015, p. e791978, Aug. 2015, doi: 10.1155/2015/791978.
[300] M. Yuan *et al.*, "Omega-3 polyunsaturated fatty acid supplementation improves lipid metabolism and endothelial function by providing a beneficial eicosanoid-pattern in patients with acute myocardial infarction: A randomized, controlled trial," *Clinical Nutrition*, vol. 40, no. 2, pp. 445–459, Feb. 2021, doi: 10.1016/j.clnu.2020.05.034.
[301] J. Sabater, J. R. Masclans, J. Sacanell, P. Chacon, P. Sabin, and M. Planas, "Effects of an omega-3 fatty acid-enriched lipid emulsion on eicosanoid synthesis in acute respiratory distress syndrome (ARDS): A prospective, randomized, double-blind, parallel group study," *Nutr Metab (Lond)*, vol. 8, no. 1, p. 22, Apr. 2011, doi: 10.1186/1743-7075-8-22.
[302] V. Ávila-Gandía, A. Torregrosa-García, A. J. Luque-Rubia, M. S. Abellán-Ruiz, D. Victoria-Montesinos, and F. J. López-Román, "Re-esterified DHA improves ventilatory threshold 2 in competitive amateur cyclists," *J Int Soc Sports Nutr*, vol. 17, no. 1, p. 51, Oct. 2020, doi: 10.1186/s12970-020-00379-0.
[303] K. Currell and A. E. Jeukendrup, "Validity, Reliability and Sensitivity of Measures of Sporting Performance," *Sports Med*, vol. 38, no. 4, Art. no. 4, Apr. 2008, doi: 10.2165/00007256-200838040-00003.
[304] A. Jeukendrup, W. H. M. Saris, F. Brouns, and A. D. M. Kester, "A new validated endurance performance test:," *Medicine &amp Science in Sports &amp Exercise*, vol. 28, no. 2, pp. 266–270, Feb. 1996, doi: 10.1097/00005768-199602000-00017.
[305] R. M. Malcata and W. G. Hopkins, "Variability of competitive performance of elite athletes: a systematic review," *Sports Med*, vol. 44, no. 12, pp. 1763–1774, Dec. 2014, doi: 10.1007/s40279-014-0239-x.
[306] R. M. Hobson, B. Saunders, G. Ball, R. C. Harris, and C. Sale, "Effects of β-alanine supplementation on exercise performance: a meta-analysis," *Amino Acids*, vol. 43, no. 1, pp. 25–37, Jul. 2012, doi: 10.1007/s00726-011-1200-z.
[307] M. W. Hoon, N. A. Johnson, P. G. Chapman, and L. M. Burke, "The Effect of Nitrate Supplementation on Exercise Performance in Healthy Individuals: A Systematic Review and Meta-Analysis," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 23, no. 5, pp. 522–532, Oct. 2013, doi: 10.1123/ijsnem.23.5.522.
[308] L. M. Burke, "Practical Issues in Evidence-Based Use of Performance Supplements: Supplement Interactions, Repeated Use and Individual Responses," *Sports Med*, vol. 47, no.

Suppl 1, pp. 79–100, 2017, doi: 10.1007/s40279-017-0687-1.
[309] P. A. Swinton, B. S. Hemingway, B. Saunders, B. Gualano, and E. Dolan, “A Statistical Framework to Interpret Individual Response to Intervention: Paving the Way for Personalized Nutrition and Exercise Prescription,” *Frontiers in Nutrition*, vol. 5, 2018, Accessed: Feb. 04, 2022. [Online]. Available: https://www.frontiersin.org/article/10.3389/fnut.2018.00041
[310] R. L. Wasserstein and N. A. Lazar, “The ASA Statement on p-Values: Context, Process, and Purpose,” *The American Statistician*, vol. 70, no. 2, pp. 129–133, Apr. 2016, doi: 10.1080/00031305.2016.1154108.
[311] K. L. Mengersen, C. C. Drovandi, C. P. Robert, D. B. Pyne, and C. J. Gore, “Bayesian Estimation of Small Effects in Exercise and Sports Science,” *PLOS ONE*, vol. 11, no. 4, p. e0147311, Apr. 2016, doi: 10.1371/journal.pone.0147311.
[312] J. K. Kruschke and T. M. Liddell, “Bayesian data analysis for newcomers,” *Psychon Bull Rev*, vol. 25, no. 1, pp. 155–177, Feb. 2018, doi: 10.3758/s13423-017-1272-1.
[313] I. Garaiova, I. A. Guschina, S. F. Plummer, J. Tang, D. Wang, and N. T. Plummer, “A randomised cross-over trial in healthy adults indicating improved absorption of omega-3 fatty acids by pre-emulsification,” *Nutr J*, vol. 6, no. 1, Art. no. 1, Jan. 2007, doi: 10.1186/1475-2891-6-4.
[314] M. E. Sublette *et al.*, “Validation of a food frequency questionnaire to assess intake of n-3 polyunsaturated fatty acids in subjects with and without major depressive disorder,” *J Am Diet Assoc*, vol. 111, no. 1, pp. 117-123.e1–2, Jan. 2011, doi: 10.1016/j.jada.2010.10.007.
[315] A. M. Minihane *et al.*, “Consumption of Fish Oil Providing Amounts of Eicosapentaenoic Acid and Docosahexaenoic Acid That Can Be Obtained from the Diet Reduces Blood Pressure in Adults with Systolic Hypertension: A Retrospective Analysis,” *The Journal of Nutrition*, vol. 146, no. 3, pp. 516–523, Mar. 2016, doi: 10.3945/jn.115.220475.
[316] B. Carpenter *et al.*, “Stan: A Probabilistic Programming Language,” *Journal of Statistical Software*, vol. 76, pp. 1–32, Jan. 2017, doi: 10.18637/jss.v076.i01.
[317] “Applied Regression Modeling via RStan — rstanarm-package.” http://mc-stan.org/rstanarm/reference/rstanarm-package.html (accessed Apr. 21, 2022).
[318] J. Kruschke, *Doing Bayesian Data Analysis: A Tutorial with R, JAGS, and Stan*. Academic Press, 2014.
[319] G. A. Borg, “Psychophysical bases of perceived exertion,” *Med Sci Sports Exerc*, vol. 14, no. 5, pp. 377–381, 1982.
[320] B. Zinoubi, S. Zbidi, H. Vandewalle, K. Chamari, and T. Driss, “Relationships between rating of perceived exertion, heart rate and blood lactate during continuous and alternated-intensity cycling exercises,” *Biol Sport*, vol. 35, no. 1, pp. 29–37, Mar. 2018, doi: 10.5114/biolsport.2018.70749.
[321] T. M. Liddell and J. K. Kruschke, “Analyzing ordinal data with metric models: What could possibly go wrong?,” *Journal of Experimental Social Psychology*, vol. 79, pp. 328–348, Nov. 2018, doi: 10.1016/j.jesp.2018.08.009.
[322] C. Muth, Z. Oravecz, and J. Gabry, “User-friendly Bayesian regression modeling: A tutorial with rstanarm and shinystan,” *TQMP*, vol. 14, no. 2, pp. 99–119, Apr. 2018, doi: 10.20982/tqmp.14.2.p099.
[323] H. Robbins, “AN EMPIRICAL BAYES APPROACH TO STATISTICS,” *Proc. 3rd Berkeley Symp. Math. Statit. Probab.*, no. 1, pp. 157–163, 1956.
[324] L. D. Brown, “In-season prediction of batting averages: A field test of empirical Bayes and Bayes methodologies,” *arXiv:0803.3697 [stat]*, Mar. 2008, doi: 10.1214/07-AOAS138.
[325] U. Wergel-Kolmert, A. Ågehäll, N. Rosenberg, and B. Wohlfart, “Day-to-day variation in oxygen consumption at submaximal loads during ergometer cycling by adolescents,” *Clinical Physiology*, vol. 21, no. 2, pp. 135–140, 2001, doi: 10.1046/j.1365-2281.2001.00313.x.
[326] J. H. Goedecke, A. S. C. Gibson, L. Grobler, M. Collins, T. D. Noakes, and E. V. Lambert, “Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes,” *American Journal of Physiology-Endocrinology and Metabolism*, vol. 279,

no. 6, pp. E1325–E1334, Dec. 2000, doi: 10.1152/ajpendo.2000.279.6.E1325.
[327] R. P. Lamberts and M. I. Lambert, “Day-to-Day Variation in Heart Rate at Different Levels of Submaximal Exertion: Implications for Monitoring Training,” *The Journal of Strength & Conditioning Research*, vol. 23, no. 3, pp. 1005–1010, May 2009, doi: 10.1519/JSC.0b013e3181a2dcdc.
[328] J. H. Wilmore *et al.*, “Reproducibility of cardiovascular, respiratory, and metabolic responses to submaximal exercise: The HERITAGE Family Study,” *Medicine & Science in Sports & Exercise*, vol. 30, no. 2, pp. 259–265, Feb. 1998.
[329] A. Boss, V. Lecoultre, C. Ruffieux, L. Tappy, and P. Schneiter, “Combined effects of endurance training and dietary unsaturated fatty acids on physical performance, fat oxidation and insulin sensitivity,” *British Journal of Nutrition*, vol. 103, no. 8, pp. 1151–1159, Apr. 2010, doi: 10.1017/S000711450999287X.
[330] P. Lembke, J. Capodice, K. Hebert, and T. Swenson, “Influence of Omega-3 (N3) Index on Performance and Wellbeing in Young Adults after Heavy Eccentric Exercise,” *J Sports Sci Med*, vol. 13, no. 1, pp. 151–156, Jan. 2014.
[331] M. J. Macartney, G. E. Peoples, T. M. Treweek, and P. L. McLennan, “Docosahexaenoic acid varies in rat skeletal muscle membranes according to fibre type and provision of dietary fish oil,” *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 151, pp. 37–44, Dec. 2019, doi: 10.1016/j.plefa.2019.08.006.
[332] B. Saunders *et al.*, “β-alanine supplementation to improve exercise capacity and performance: a systematic review and meta-analysis,” *Br J Sports Med*, vol. 51, no. 8, pp. 658–669, Apr. 2017, doi: 10.1136/bjsports-2016-096396.
[333] P. M. Christensen, Y. Shirai, C. Ritz, and N. B. Nordsborg, “Caffeine and Bicarbonate for Speed. A Meta-Analysis of Legal Supplements Potential for Improving Intense Endurance Exercise Performance,” *Frontiers in Physiology*, vol. 8, 2017, Accessed: Feb. 17, 2022. [Online]. Available: https://www.frontiersin.org/article/10.3389/fphys.2017.00240
[334] W. G. Hopkins, J. A. Hawley, and L. M. Burke, “Design and analysis of research on sport performance enhancement,” *Med Sci Sports Exerc*, vol. 31, no. 3, pp. 472–485, Mar. 1999, doi: 10.1097/00005768-199903000-00018.
[335] C. Von Schacky, “Omega-3 Index and Cardiovascular Health,” *Nutrients*, vol. 6, no. 2, Art. no. 2, Feb. 2014, doi: 10.3390/nu6020799.
[336] D. M. Ninio, A. M. Hill, P. R. Howe, J. D. Buckley, and D. A. Saint, “Docosahexaenoic acid-rich fish oil improves heart rate variability and heart rate responses to exercise in overweight adults,” *British Journal of Nutrition*, vol. 100, no. 5, pp. 1097–1103, Nov. 2008, doi: 10.1017/S0007114508959225.
[337] R. J. Bloomer, D. E. Larson, K. H. Fisher-Wellman, A. J. Galpin, and B. K. Schilling, “Effect of eicosapentaenoic and docosahexaenoic acid on resting and exercise-induced inflammatory and oxidative stress biomarkers: a randomized, placebo controlled, cross-over study,” *Lipids Health Dis*, vol. 8, no. 1, p. 36, Aug. 2009, doi: 10.1186/1476-511X-8-36.
[338] L. Hingley, M. Macartney, M. Brown, P. McLennan, and G. Peoples, “Low dose fish oil increases the omega-3 index improving cycling efficiency and heart rate recovery,” *Journal of Science and Cycling*, vol. 3, no. 2, Art. no. 2, Aug. 2014.
[339] J. H. O’Keefe, H. Abuissa, A. Sastre, D. M. Steinhaus, and W. S. Harris, “Effects of Omega-3 Fatty Acids on Resting Heart Rate, Heart Rate Recovery After Exercise, and Heart Rate Variability in Men With Healed Myocardial Infarctions and Depressed Ejection Fractions,” *The American Journal of Cardiology*, vol. 97, no. 8, pp. 1127–1130, Apr. 2006, doi: 10.1016/j.amjcard.2005.11.025.
[340] W. C. Tu, B. S. Mühlhäusler, L. N. Yelland, and R. A. Gibson, “Correlations between blood and tissue omega-3 LCPUFA status following dietary ALA intervention in rats,” *Prostaglandins, Leukotrienes and Essential Fatty Acids*, vol. 88, no. 1, pp. 53–60, Jan. 2013, doi: 10.1016/j.plefa.2012.04.005.
[341] G. E. Peoples and P. L. McLennan, “Dietary fish oil delays hypoxic skeletal muscle fatigue

and enhances caffeine-stimulated contractile recovery in the rat in vivo hindlimb," *Applied Physiology, Nutrition, and Metabolism*, Jan. 2017, doi: 10.1139/apnm-2016-0501.
[342] M. Bortolotti, L. Tappy, and P. Schneiter, "Fish oil supplementation does not alter energy efficiency in healthy males," *Clin Nutr*, vol. 26, no. 2, pp. 225–230, Apr. 2007, doi: 10.1016/j.clnu.2006.11.006.
[343] D. M. Huffman, J. L. Michaelson, and T. R. Thomas, "Chronic Supplementation with Fish Oil Increases Fat Oxidation During Exercise in Young Men," *Professionalization of Exercise Physiology*, vol. 7, no. 1, pp. 48–56, Feb. 2004.
[344] B. de Roos and L. Brennan, "Personalised Interventions—A Precision Approach for the Next Generation of Dietary Intervention Studies," *Nutrients*, vol. 9, no. 8, p. 847, Aug. 2017, doi: 10.3390/nu9080847.
[345] M. Armand *et al.*, "Digestion and absorption of 2 fat emulsions with different droplet sizes in the human digestive tract," *The American Journal of Clinical Nutrition*, vol. 70, no. 6, pp. 1096–1106, Dec. 1999, doi: 10.1093/ajcn/70.6.1096.
[346] J. G. Fodor, E. Helis, N. Yazdekhasti, and B. Vohnout, "'Fishing' for the Origins of the 'Eskimos and Heart Disease' Story: Facts or Wishful Thinking?," *Canadian Journal of Cardiology*, vol. 30, no. 8, pp. 864–868, Aug. 2014, doi: 10.1016/j.cjca.2014.04.007.
[347] P. C. Calder, "Very long chain omega-3 (n-3) fatty acids and human health," *European Journal of Lipid Science and Technology*, vol. 116, no. 10, pp. 1280–1300, 2014, doi: 10.1002/ejlt.201400025.
[348] Food and Agriculture Organization of the United Nations, Ed., *Fats and fatty acids in human nutrition: report of an expert consultation: 10-14 November 2008, Geneva*. Rome: Food and Agriculture Organization of the United Nations, 2010.
[349] A. Abdelhamid *et al.*, "The Relationship Between Omega-3, Omega-6 and Total Polyunsaturated Fat and Musculoskeletal Health and Functional Status in Adults: A Systematic Review and Meta-analysis of RCTs," *Calcif Tissue Int*, vol. 105, no. 4, pp. 353–372, Oct. 2019, doi: 10.1007/s00223-019-00584-3.
[350] J. Turicchi, R. O'Driscoll, G. Finlayson, K. Beaulieu, K. Deighton, and R. J. Stubbs, "Associations between the rate, amount, and composition of weight loss as predictors of spontaneous weight regain in adults achieving clinically significant weight loss: A systematic review and meta-regression," *Obesity Reviews*, vol. 20, no. 7, pp. 935–946, 2019, doi: 10.1111/obr.12849.
[351] A. J. Hector and S. M. Phillips, "Protein Recommendations for Weight Loss in Elite Athletes: A Focus on Body Composition and Performance," *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 28, no. 2, pp. 170–177, Mar. 2018, doi: 10.1123/ijsnem.2017-0273.
[352] V. C. Figueiredo, J. F. Markworth, and D. Cameron-Smith, "Considerations on mTOR regulation at serine 2448: implications for muscle metabolism studies," *Cell Mol Life Sci*, vol. 74, no. 14, pp. 2537–2545, Jul. 2017, doi: 10.1007/s00018-017-2481-5.
[353] A.-C. Gingras *et al.*, "Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism," *Genes Dev.*, vol. 13, no. 11, pp. 1422–1437, Jan. 1999.
[354] P. J. Atherton *et al.*, "Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling," *The American Journal of Clinical Nutrition*, vol. 92, no. 5, pp. 1080–1088, Nov. 2010, doi: 10.3945/ajcn.2010.29819.
[355] L. S. Macnaughton *et al.*, "The response of muscle protein synthesis following whole-body resistance exercise is greater following 40 g than 20 g of ingested whey protein," *Physiological Reports*, vol. 4, no. 15, p. e12893, 2016, doi: 10.14814/phy2.12893.
[356] A. J. Hector, C. McGlory, F. Damas, N. Mazara, S. K. Baker, and S. M. Phillips, "Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise," *The FASEB Journal*, vol. 32, no. 1, Art. no. 1, 2018, doi:

https://doi.org/10.1096/fj.201700158RR.
[357] A. J. MacDonald *et al.*, “A novel oral tracer procedure for measurement of habitual myofibrillar protein synthesis,” *Rapid Communications in Mass Spectrometry*, vol. 27, no. 15, pp. 1769–1777, 2013, doi: 10.1002/rcm.6622.
[358] A. Philp, D. L. Hamilton, and K. Baar, “Signals mediating skeletal muscle remodeling by resistance exercise: PI3-kinase independent activation of mTORC1,” *Journal of Applied Physiology*, vol. 110, no. 2, pp. 561–568, Feb. 2011, doi: 10.1152/japplphysiol.00941.2010.
[359] M. Sanak, “Eicosanoid Mediators in the Airway Inflammation of Asthmatic Patients: What is New?,” *Allergy Asthma Immunol Res*, vol. 8, no. 6, pp. 481–490, Aug. 2016, doi: 10.4168/aair.2016.8.6.481.
[360] I. Fleming, “The factor in EDHF: Cytochrome P450 derived lipid mediators and vascular signaling,” *Vascular Pharmacology*, vol. 86, pp. 31–40, Nov. 2016, doi: 10.1016/j.vph.2016.03.001.
[361] J. L. Heileson and L. K. Funderburk, “The effect of fish oil supplementation on the promotion and preservation of lean body mass, strength, and recovery from physiological stress in young, healthy adults: a systematic review,” *Nutrition Reviews*, vol. 78, no. 12, pp. 1001–1014, Dec. 2020, doi: 10.1093/nutrit/nuaa034.
[362] J. D. Philpott, O. C. Witard, and S. D. R. Galloway, “Applications of omega-3 polyunsaturated fatty acid supplementation for sport performance,” *Research in Sports Medicine*, vol. 27, no. 2, pp. 219–237, Apr. 2019, doi: 10.1080/15438627.2018.1550401.
[363] Y. Tsuchiya, K. Yanagimoto, K. Nakazato, K. Hayamizu, and E. Ochi, “Eicosapentaenoic and docosahexaenoic acids-rich fish oil supplementation attenuates strength loss and limited joint range of motion after eccentric contractions: a randomized, double-blind, placebo-controlled, parallel-group trial,” *Eur J Appl Physiol*, vol. 116, no. 6, pp. 1179–1188, Jun. 2016, doi: 10.1007/s00421-016-3373-3.
[364] B. Tartibian, B. H. Maleki, and A. Abbasi, “The Effects of Ingestion of Omega-3 Fatty Acids on Perceived Pain and External Symptoms of Delayed Onset Muscle Soreness in Untrained Men,” *Clinical Journal of Sport Medicine*, vol. 19, no. 2, pp. 115–119, Mar. 2009, doi: 10.1097/JSM.0b013e31819b51b3.
[365] E. Ochi and Y. Tsuchiya, “Eicosapentaenoic Acid (EPA) and Docosahexaneoic Acid (DHA) in Muscle Damage and Function,” *Nutrients*, vol. 10, no. 5, Art. no. 5, May 2018, doi: 10.3390/nu10050552.
[366] J. R. Jakeman, D. M. Lambrick, B. Wooley, J. A. Babraj, and J. A. Faulkner, “Effect of an acute dose of omega-3 fish oil following exercise-induced muscle damage,” *Eur J Appl Physiol*, vol. 117, no. 3, pp. 575–582, Mar. 2017, doi: 10.1007/s00421-017-3543-y.
[367] P. Gray, A. Chappell, A. M. Jenkinson, F. Thies, and S. R. Gray, “Fish Oil Supplementation Reduces Markers of Oxidative Stress But Not Muscle Soreness After Eccentric Exercise,” *International Journal of Sport Nutrition and Exercise Metabolism*, vol. 24, no. 2, pp. 206–214, Apr. 2014, doi: 10.1123/ijsnem.2013-0081.
[368] J. N. Cobley, G. L. Close, D. M. Bailey, and G. W. Davison, “Exercise redox biochemistry: Conceptual, methodological and technical recommendations,” *Redox Biology*, vol. 12, pp. 540–548, Aug. 2017, doi: 10.1016/j.redox.2017.03.022.
[369] F. Damas, C. A. Libardi, and C. Ugrinowitsch, “The development of skeletal muscle hypertrophy through resistance training: the role of muscle damage and muscle protein synthesis,” *Eur J Appl Physiol*, vol. 118, no. 3, pp. 485–500, Mar. 2018, doi: 10.1007/s00421-017-3792-9.
[370] T. J. Koh and F. X. Pizza, “Do inflammatory cells influence skeletal muscle hypertrophy?,” *Front Biosci (Elite Ed)*, vol. 1, no. 1, pp. 60–71, Jun. 2009, doi: 10.2741/E7.
[371] J.-S. You, M.-N. Park, and Y.-S. Lee, “Dietary fish oil inhibits the early stage of recovery of atrophied soleus muscle in rats via Akt–p70s6k signaling and PGF2α,” *The Journal of Nutritional Biochemistry*, vol. 21, no. 10, pp. 929–934, Oct. 2010, doi: 10.1016/j.jnutbio.2009.07.007.

[372] J. F. Markworth and D. Cameron-Smith, “Arachidonic acid supplementation enhances in vitro skeletal muscle cell growth via a COX-2-dependent pathway,” *American Journal of Physiology-Cell Physiology*, vol. 304, no. 1, pp. C56–C67, Jan. 2013, doi: 10.1152/ajpcell.00038.2012.
[373] A. M. Batterham and W. G. Hopkins, “Making Meaningful Inferences About Magnitudes,” *International Journal of Sports Physiology and Performance*, vol. 1, no. 1, pp. 50–57, Mar. 2006, doi: 10.1123/ijspp.1.1.50.
[374] T. Cullen, G. Thomas, A. J. Wadley, and T. Myers, “The effects of a single night of complete and partial sleep deprivation on physical and cognitive performance: A Bayesian analysis,” *Journal of Sports Sciences*, vol. 37, no. 23, pp. 2726–2734, Dec. 2019, doi: 10.1080/02640414.2019.1662539.
[375] J. O. Osborne, I. B. Stewart, K. W. Beagley, D. N. Borg, and G. M. Minett, “Acute glutamine supplementation does not improve 20-km self-paced cycling performance in the heat,” *Eur J Appl Physiol*, vol. 119, no. 11, pp. 2567–2578, Dec. 2019, doi: 10.1007/s00421-019-04234-2.
[376] G. M. Minett, V. Fels-Camilleri, J. J. Bon, F. M. Impellizzeri, and D. N. Borg, “Peer Presence Increases Session Ratings of Perceived Exertion,” *Int J Sports Physiol Perform*, pp. 1–5, Sep. 2021, doi: 10.1123/ijspp.2021-0080.
[377] M. L. Cunningham, “A Mouse Is Not a Rat Is Not a Human: Species Differences Exist,” *Toxicological Sciences*, vol. 70, no. 2, pp. 157–158, Dec. 2002, doi: 10.1093/toxsci/70.2.157.
[378] I. Mujika and R. S. Taipale, “Sport Science on Women, Women in Sport Science,” *International Journal of Sports Physiology and Performance*, vol. 14, no. 8, pp. 1013–1014, Sep. 2019, doi: 10.1123/ijspp.2019-0514.
[379] E. S. Cowley, A. A. Olenick, K. L. McNulty, and E. Z. Ross, “‘Invisible Sportswomen’: The Sex Data Gap in Sport and Exercise Science Research,” *Women in Sport and Physical Activity Journal*, vol. 29, no. 2, pp. 146–151, Sep. 2021, doi: 10.1123/wspaj.2021-0028.
[380] A. McCormick, C. Meijen, and S. Marcora, “Psychological Determinants of Whole-Body Endurance Performance,” *Sports Med*, vol. 45, no. 7, pp. 997–1015, Jul. 2015, doi: 10.1007/s40279-015-0319-6.
[381] R. A. Jacobs *et al.*, “Determinants of time trial performance and maximal incremental exercise in highly trained endurance athletes,” *Journal of Applied Physiology*, vol. 111, no. 5, pp. 1422–1430, Nov. 2011, doi: 10.1152/japplphysiol.00625.2011.

Appendices

A3.1 – Gene expression in skeletal muscle cells

Table A3.1 – Gene expression in skeletal muscle cells conditioned with ex vivo human serum (Chapter 3.3.3). Cells were conditioned with serum collected during fasting, resting conditions, and 90 minutes following resistance exercise and 10g protein consumption, at pre- and post-ER. Lower and upper confidence limits of 95% confidence interval are displayed. Mean differences are between FO and PLA.
* Indicates no standard deviation / standard error calculation possible due to only 1 observation.

Gene	Time	State	Mean Diff	SEM	LCI	UCI
FBXO32	Pre	Rested	0.0026	0.0073	-0.0118	0.0170
	Pre	Exercised	-0.0173	0.0070	-0.0310	-0.0036
	Post	Rested	-0.0179	0.0053	-0.0283	-0.0075
	Post	Exercised	0.0102	0.0075	-0.0045	0.0249
NFKB	Pre	Rested	-0.0084	0.0034	-0.0150	-0.0018
	Pre	Exercised	-0.0144	0.0037	-0.0217	-0.0071
	Post	Rested	-0.0281	0.0105	-0.0487	-0.0075
	Post	Exercised	-0.0027	0.0020	-0.0066	0.0011
PIK3C3	Pre	Rested	-0.0011	0.0025	-0.0061	0.0038
	Pre	Exercised	-0.0172	0.0018	-0.0208	-0.0136
	Post	Rested	-0.0198	0.0138	-0.0469	0.0072
	Post	Exercised	0.0034	0.0053	-0.0070	0.0137
MYOG	Pre	Rested	-0.1241	0.0160	-0.1555	-0.0928
	Pre	Exercised	-0.0943	0.0124	-0.1186	-0.0699
	Post	Rested	0.1806	0.0243	0.1330	0.2282
	Post	Exercised	0.0235	0.0223	-0.0202	0.0672
MYOD	Pre	Rested	-0.0013	0.0006	-0.0025	-0.0001
	Pre	Exercised	0.0002	0.0003	-0.0005	0.0009
	Post	Rested	0.0099	0.0050	0.0001	0.0197
	Post	Exercised	0.0004	0.0002	0.0000	0.0008
PTGS2	Pre	Rested	0.0119	0.0032	0.0056	0.0181
	Pre	Exercised	0.0004	0.0007	-0.0009	0.0017
	Post	Rested	0.0001	0.0019	-0.0036	0.0038
	Post	Exercised	0.0084	0.0053	-0.0020	0.0189
BECN1	Pre	Rested	0.0194	0.0086	0.0026	0.0362
	Pre	Exercised	-0.0579	0.0072	-0.0720	-0.0439
	Post	Rested	-0.0298	0.0156	-0.0604	0.0008
	Post	Exercised	-0.0055	0.0160	-0.0369	0.0259
BNIP3	Pre	Rested	0.0360	0.0249	-0.0128	0.0849
	Pre	Exercised	-0.0603	0.0052	-0.0704	-0.0502
	Post	Rested	-0.0677	*	*	*
	Post	Exercised	0.0233	0.0285	-0.0325	0.0791
BNIP3L	Pre	Rested	0.4393	*	*	*
	Pre	Exercised	-0.0990	0.0049	-0.1087	-0.0893
	Post	Rested	-0.0708	0.0088	-0.0881	-0.0535
	Post	Exercised	-0.0615	0.0173	-0.0955	-0.0276
GABARAPL1	Pre	Rested	0.0115	0.0023	0.0070	0.0159
	Pre	Exercised	-0.0032	0.0026	-0.0082	0.0018
	Post	Rested	0.0112	0.0030	0.0054	0.0170

	Post	Exercised	0.0081	0.0020	0.0042	0.0121
MAP1LC3B	Pre	Rested	0.0174	0.0397	-0.0604	0.0952
	Pre	Exercised	-0.1018	0.0277	-0.1560	-0.0475
	Post	Rested	-0.0529	0.0130	-0.0783	-0.0275
	Post	Exercised	0.0055	0.0196	-0.0328	0.0439

A4.1 – MCMC trace plots for each model in Chapter 4

For each model, Markov Chain Monte Carlo sampling was used. Each of the 4 chains consisted of 2000 iterations, of which the first 1000 were discarded as warmup (default settings).

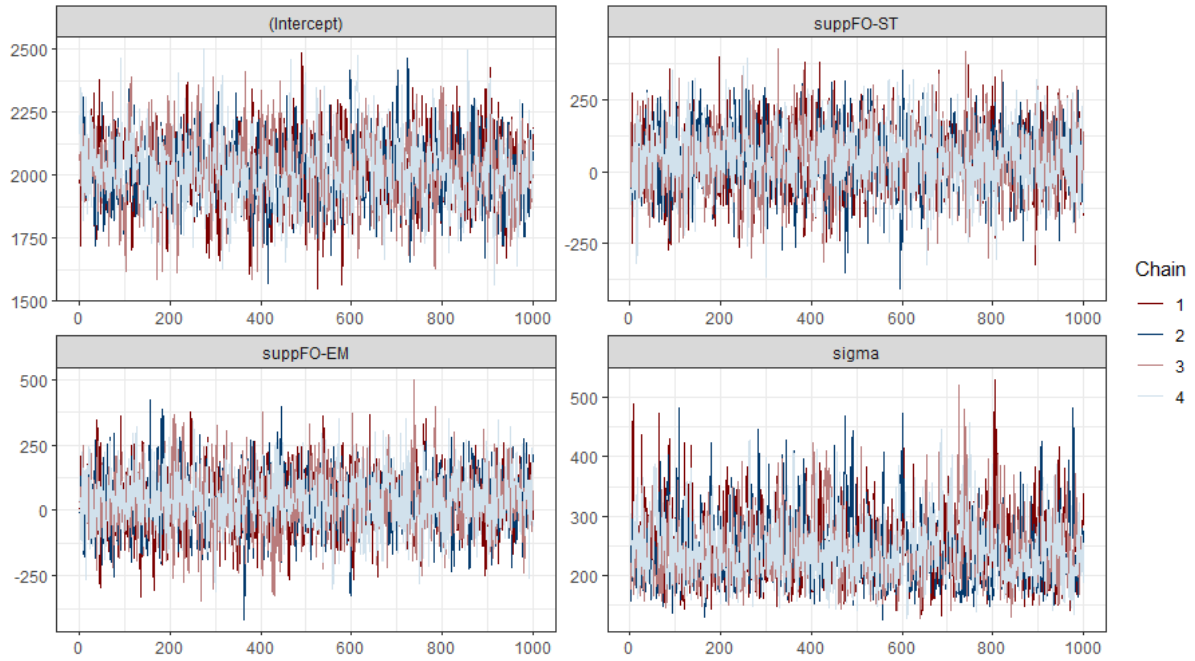

Figure A4.1.1 – MCMC trace plots of the Bayesian model for time trial performance (TT). The formula for the linear model of TT was: TT ~ supp + (1|id).

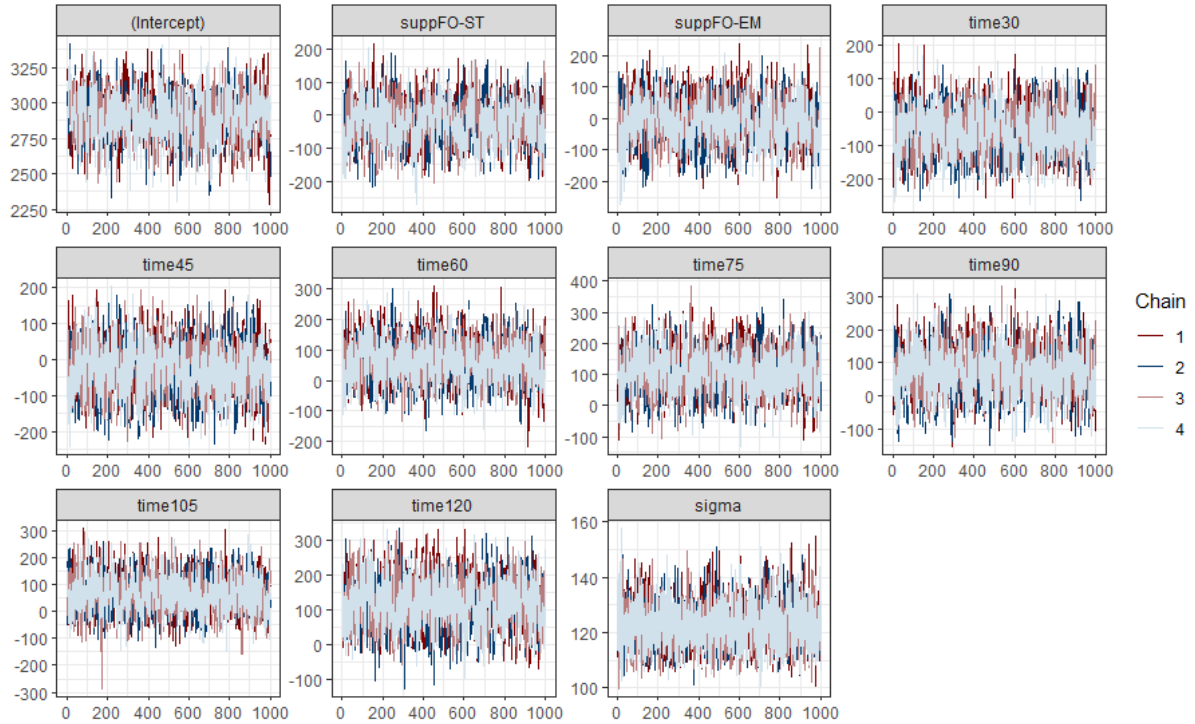

Figure A4.1.2 – MCMC trace plots of the Bayesian model for oxygen consumption (VO_2). The formula for the linear model of VO_2 was: VO_2 ~ supp*time + (1|id).

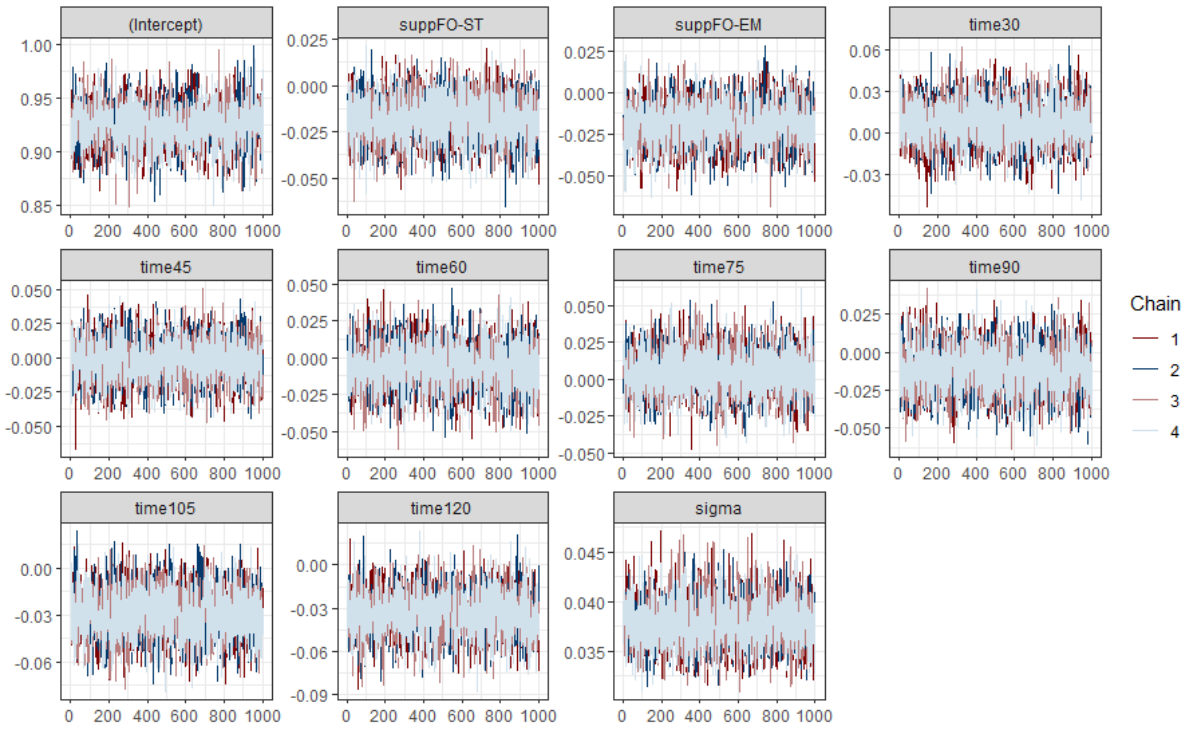

Figure A4.1.3 – MCMC trace plots of the Bayesian model for respiratory exchange ratio (RER). The formula for the linear model of RER was: RER ~ supp*time + (1|id).

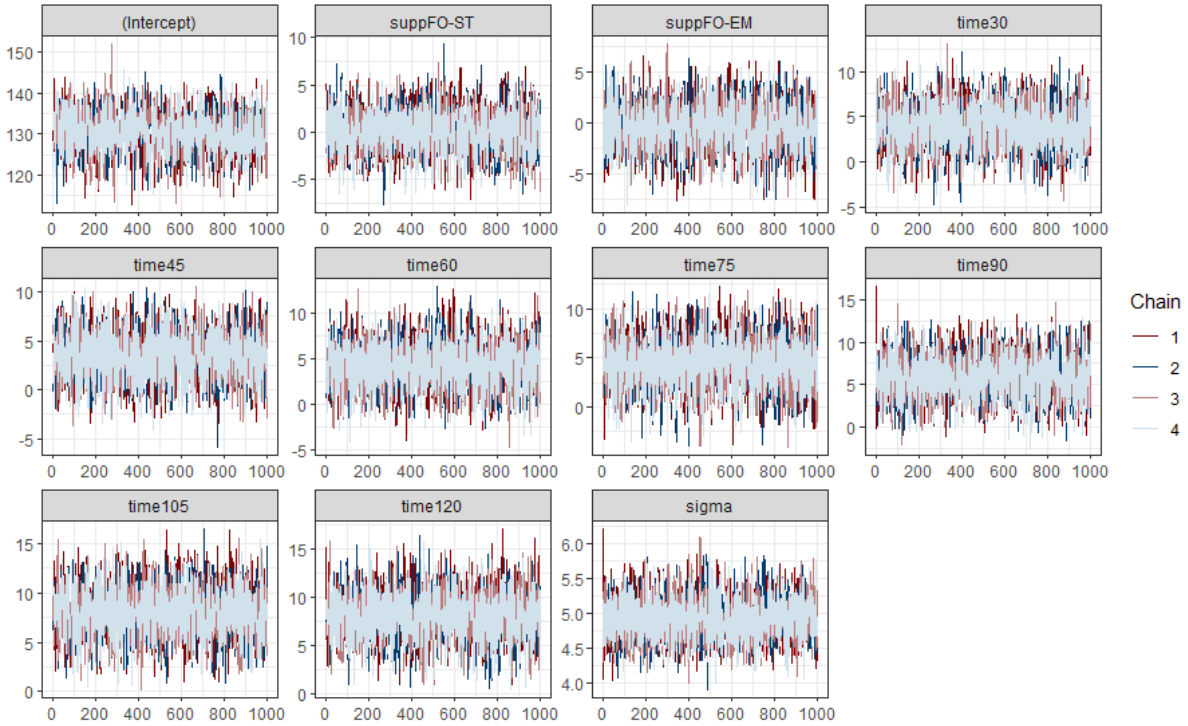

Figure A4.1.4 – MCMC trace plots of the Bayesian model for heart rate (HR). The formula for the linear model of HR was: HR ~ supp*time + (1|id).

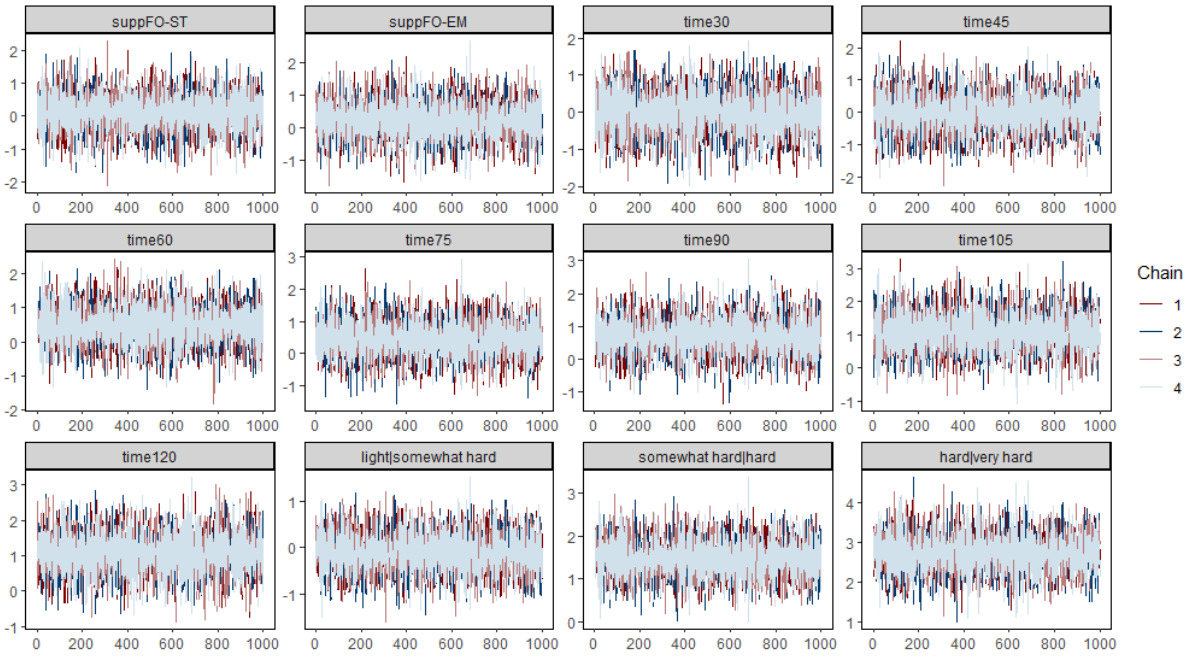

Figure A4.1.5 – MCMC trace plots of the ordered-logit Bayesian model for rate of perceived exertion (RPE). The formula for the ordinal-logistic model of RPE was: RPE Category ~ supp*time.

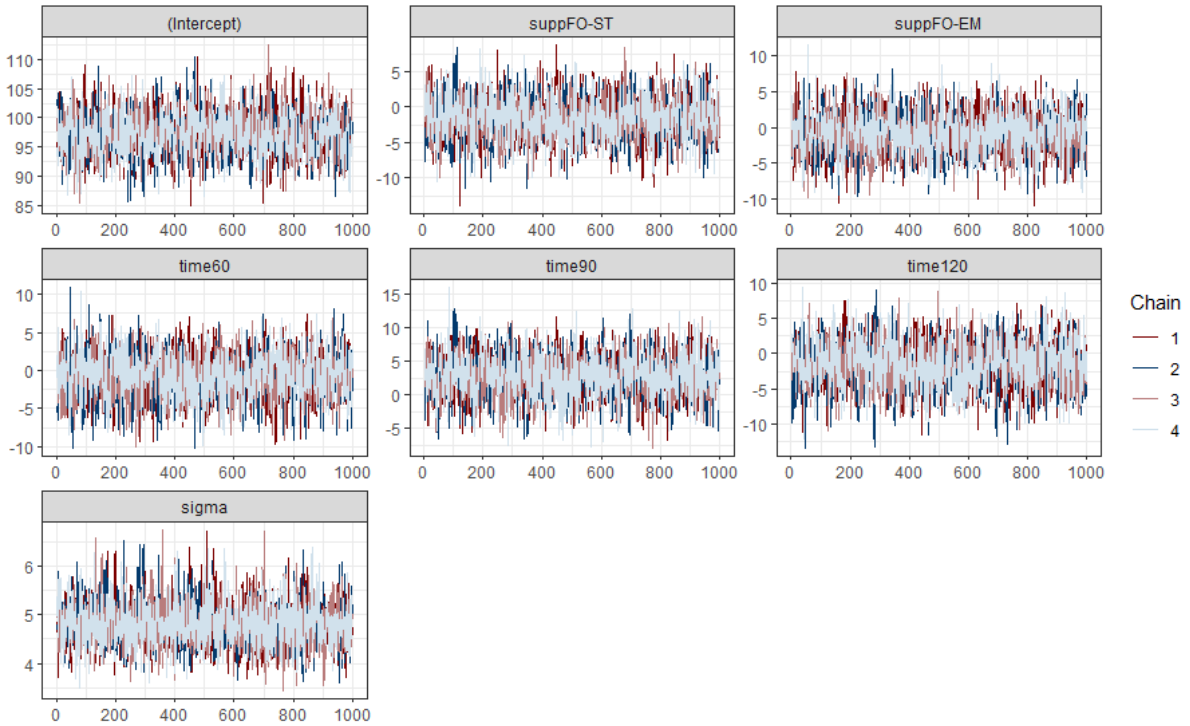

Figure A4.1.6 – MCMC trace plots of the Bayesian model for mean arterial pressure (MAP). The formula for the linear model of MAP was: MAP ~ supp*time + (1|id).

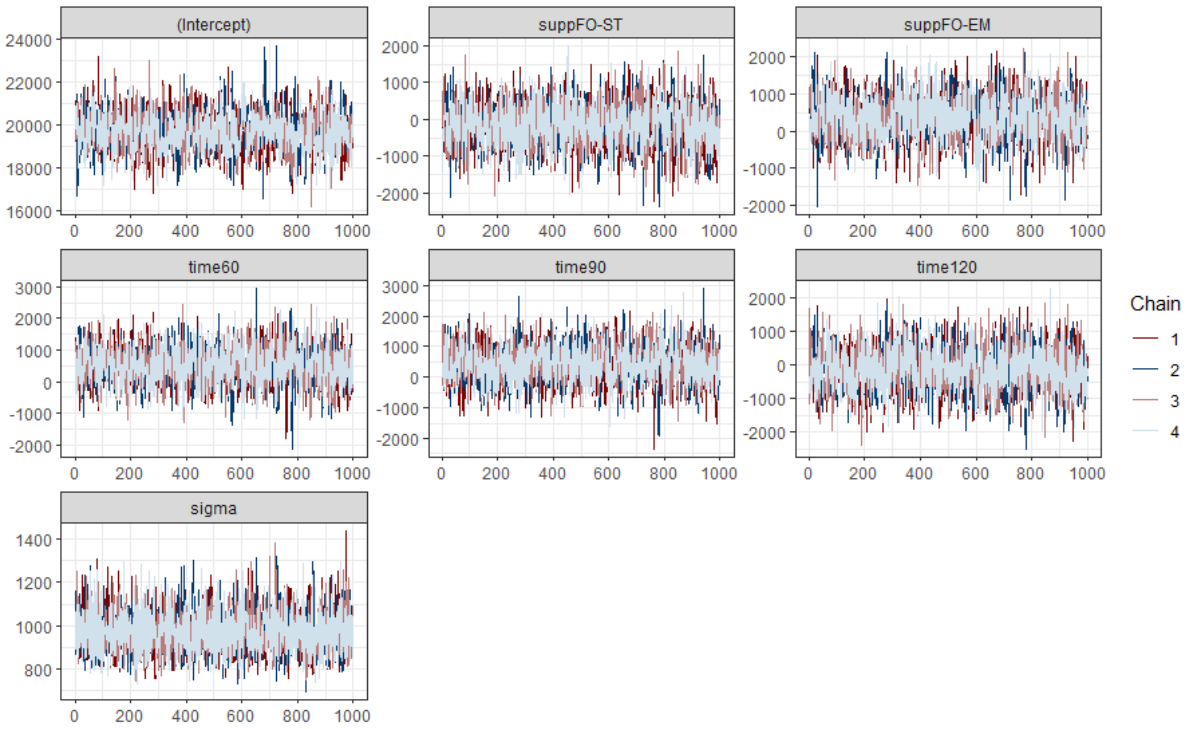

Figure A4.1.7 – MCMC trace plots of the Bayesian model for rate pressure product (RPP). The formula for the linear model of RPP was: RPP ~ supp*time + (1|id).

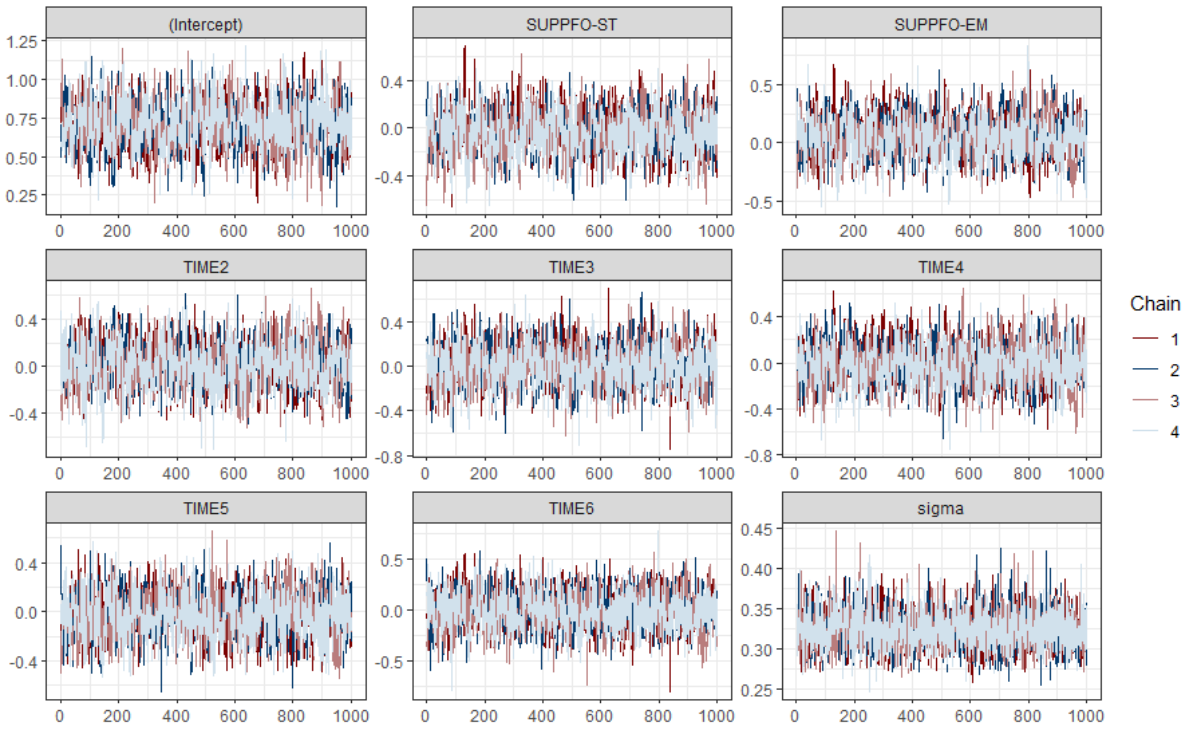

Figure A4.1.8 – MCMC trace plots of the Bayesian model for eicosapentaenoic acid (EPA). The formula for the linear model of EPA was: EPA ~ supp*time + (1|id).

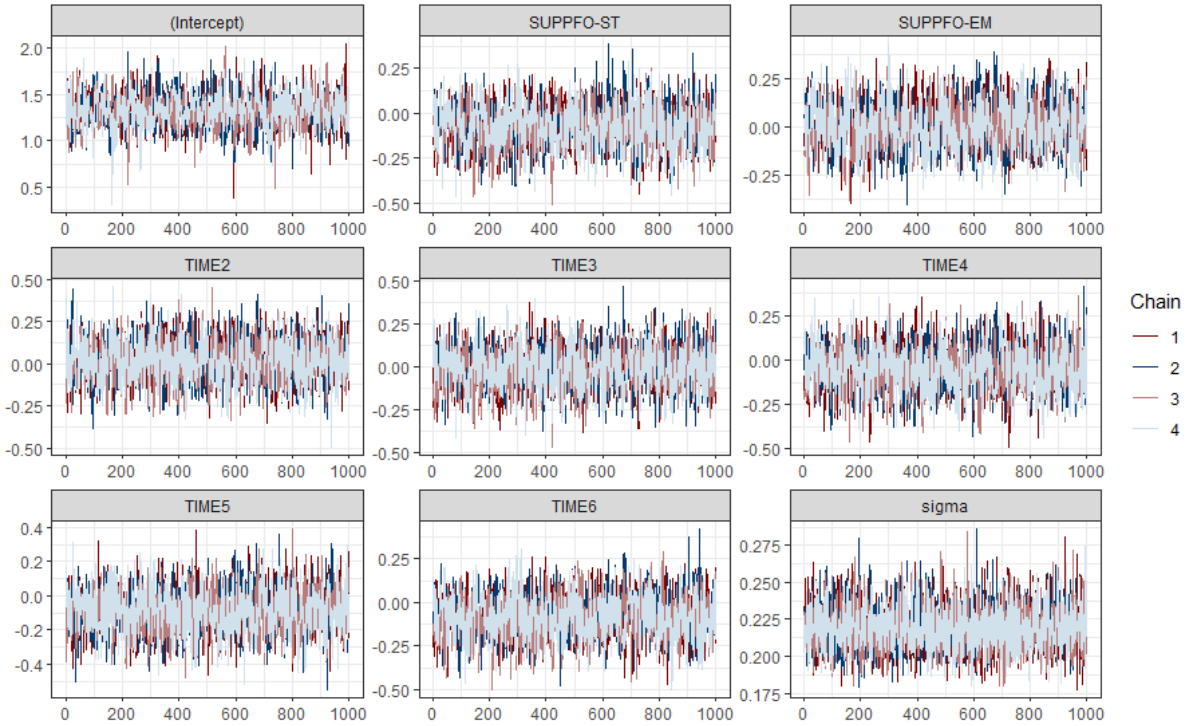

Figure A4.1.9 – MCMC trace plots of the Bayesian model for docosahexaenoic acid (DHA). The formula for the linear model of DHA was: DHA ~ supp*time + (1|id).

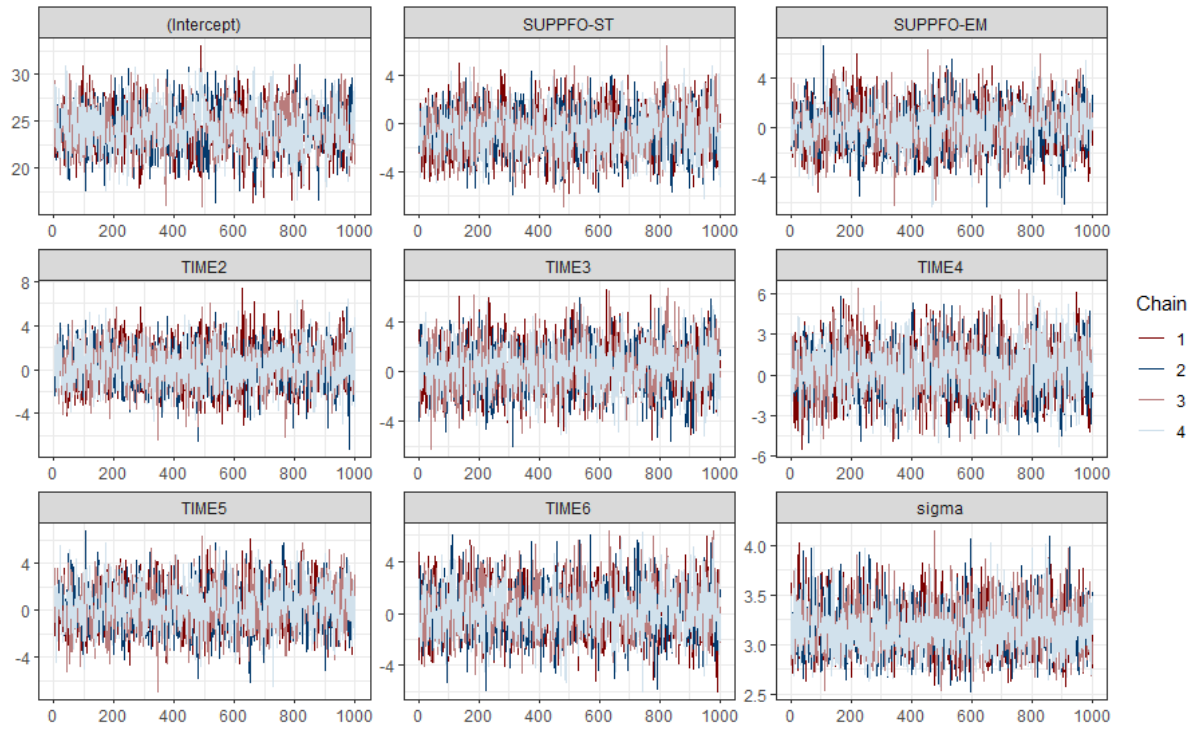

Figure A4.1.10 – MCMC trace plots of the Bayesian model for total n-3 PUFA. The formula for the linear model of total n-3 PUFA was: total n-3 PUFA ~ supp*time + (1|id).

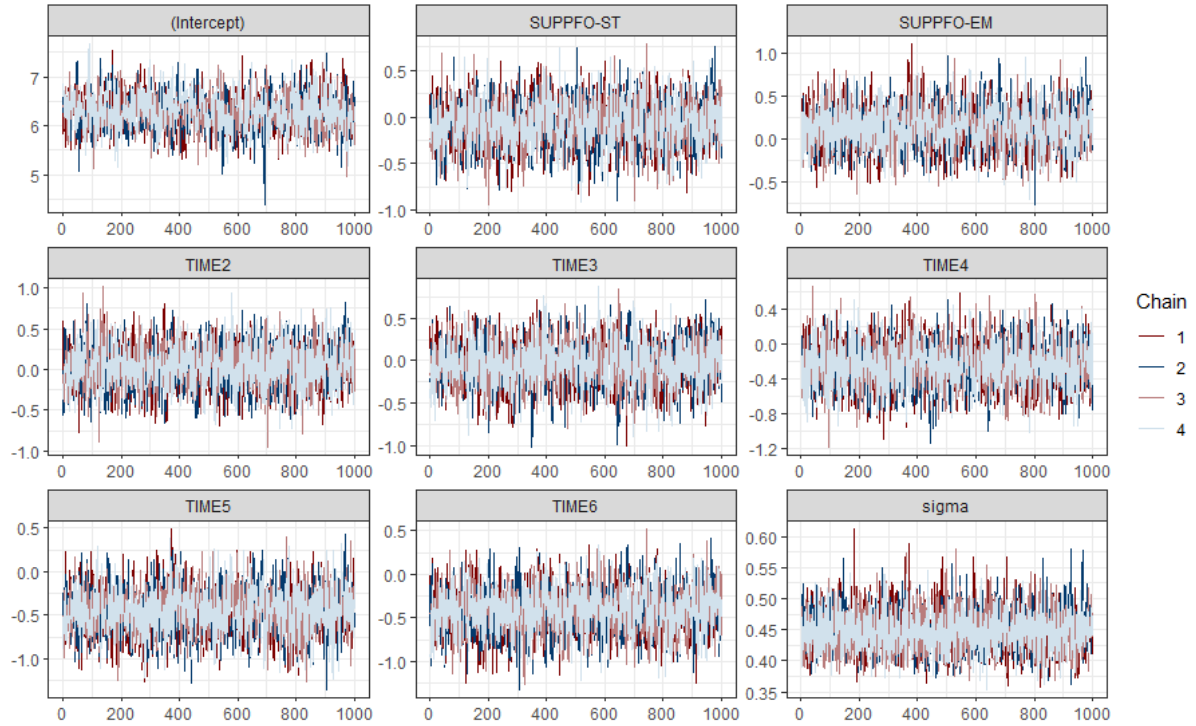

Figure A4.1.11 – MCMC trace plots of the Bayesian model for arachidonic acid (ARA). The formula for the linear model of ARA was: ARA ~ supp*time + (1|id).