The effects of bioavailable omega-3 polyunsaturated fatty acids, delivered at bedtime, on brain function, sleep, cognition and mood in healthy adults

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Abstract

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are 'essential' fatty acids that cannot be manufactured *de novo* within the body and must be obtained via the diet, with the n-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) previously being found to have a wide range of bioactive properties within the body and particularly on brain function. However, in the UK and several other western nations, n-3 PUFA intake is found to fall below government and health organisation guidelines. Although previous research into the effects of n-3 PUFA intake and physiological and psychological outcomes is extensive, the previous research can be criticised for several methodological limitations relating to supplementation lengths, population samples, formulations of the supplements and placing little focus on the bioavailability and uptake of the fatty acids across the supplementation period. Therefore this thesis aimed to address the previous limitations identified within the research area to more accurately examine the effects of both a DHA- and EPA-rich supplement on measures of cerebral blood flow, sleep, memory consolidation, cognition and mood.

Chapter 3 describes an intervention study that employs a near-infrared spectroscopy (NIRS) neuroimaging technique capable of measuring gross changes in cerebral haemodynamics across a 26 week supplementation period, a factor missing from the previous research conducted thus far. Chapter 4 describes a 26 week intervention study that measured parameters of sleep via subjective rating scales, actigraphy watches and urinary 6-sulphatoxymelatonin (aMT6s). Chapter 5 describes a novel 26 week intervention study that aimed to measure overnight memory consolidation via completion of learning and recall tasks before and after sleeping. Finally, Chapter 6 describes a 26 week intervention study measuring aspects of episodic memory, working memory, information processing speed, executive function, attention and global cognition. The intervention studies that comprise this thesis include the first investigations that administer self-micro-emulsifying delivery system (SMEDS) formulated DHA- and EPA-rich supplements, designed to increase fatty acid bioavailability, dosed at bed-time, in healthy, young adults aged 25-49.

Notably, there is evidence to suggest that supplementation with both the DHA-rich and EPA-rich treatments show a trend towards reducing the quantities of oxygenated haemoglobin during completion of serial subtraction tasks, interpreted as an increase in neural efficiency. N-3 PUFAs, particularly DHA, also improved objective measurements of sleep whilst the EPA-rich treatment improved global cognitive function and improved reaction times during both executive functioning and episodic memory tasks. Taken together, these findings suggest that 26 weeks' n-3 PUFA supplementation is beneficial for a number of psychological and physiological brain parameters in healthy, young adult samples.

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Author's Declaration

I declare that no outputs submitted for this degree have been submitted for a research degree of any other institution. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others. The author, supervisors and external collaborators worked together to design the methodology for each experimental chapter. For these chapters, the data collection, statistical analysis and interpretation was the work of the author, except if acknowledged elsewhere. Additionally, analysis of all biological samples collected throughout the thesis was completed by collaborators at Southampton University.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted for all studies presented throughout this thesis by the Faculty of Health and Life Sciences Ethics Committee at Northumbria University. Furthermore, approval has been sought and granted by the NHS REC (Yorkshire and The Humber – Bradford Leeds Research Ethics Committee) on the 12th September 2016.

I declare that the Word Count of this PhD thesis is 67,786

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Date: 25 / 10 / 2019

CHAPTER 1. INTRODUCTION

1.1. Background

Dietary supplements such as vitamins, minerals and amino acids are often used in conjunction with a regular diet, usually when that nutrient is void or deficient in the diet (Aina & Ojedokun, 2014). Sales of these supplements contribute to the economy, with total sales of such products in the USA increasing by 7.7% in 2016 (Izzo, 2018). An estimated $7.45 billion was spent on herbal supplements in 2016 in the US alone (Smith, Kawa, Eckle, Morton & Stredney, 2017) with global sales estimated at $108 billion in 2014 ("Supplement Business Report", 2016). Several benefits, such as reducing the risk of developing age related diseases, lessening the symptoms of non-deficiency diseases and boosting athletic performance or the immune system are often listed as motivations for dietary supplementation (Webb, 2011). One of the most widely used dietary supplements are fish oils rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs). Around 282,000 tons of fish oil were consumed by humans in 2014 with this estimated to increase to 711,000 tons by 2025 (Grand View Research, 2014; Bianchi et al., 2012). This increase represents an annual growth rate of around 8% and should this trend continue the market for fish oil supplements will increase from $1.69 billion in 2013 to $5 billion by 2025 (Grand View Research, 2014).

The n-3 PUFA α-linolenic acid (ALA) is known as essential fatty acid (EFA) as it is needed for survival in humans and other mammals, but cannot be synthesised within the body and therefore must be obtained from the diet (Innis, 2007). The more bioactive n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are naturally enriched in the blubber and tissues of sea mammals such as whales and seals as well as in oily fish (Calder, 2017). A review by Jones and Jew (2016) summarises the approved health claims for n-3 PUFAs from international food agencies stating how, across Europe, Australia, New Zealand and Canada, n-3 fatty acids benefit several cardiovascular parameters, maintenance of blood triglyceride levels and normal brain, eye and foetus development. However, benefits of n-3 PUFAs on cognitive outcomes are still unknown due to the overall inconclusive data that is available to fully support such claims.

This introduction aims to outline the various functions of n-3 PUFAs in the body and the impact these PUFAs have on behaviour and mood. This will include an evaluation of several factors involved with n-3 PUFA supplementation, such as bioavailability issues, oxidation, time of supplementation and comparative effects of DHA and EPA. The basic chemical structure, dietary sources and the metabolism of fatty acids will also be discussed.

1.2 Essential Fatty Acids, Chemical Structure and Metabolism

Previously, Jackson (2010) described how there are several types of fatty acids in the diet, which can be metabolised to provide energy, stored as fat deposits or are incorporated into cell membranes (Surette, 2008). Fatty acids are hydrocarbon chains flanked by a methyl group (CH_3) at one end and a carboxyl group (COOH) at the other (Calder, 2017). These fatty acids can be either saturated, having only single bonds between their carbon atoms making them rigid in nature, or unsaturated, having one (monounsaturated) or more (polyunsaturated) double bonds between the carbon atoms. The position of the first double bond in relation to the methyl end determines whether the PUFA is termed an n-3, an omega-6 (n-6) or an omega-9 (n-9) fatty acid.

Mammals are capable of synthesising every fatty acid required for biological processes within the body except ALA (n-3) and linoleic acid (LA, n-6) making them EFAs as they must therefore be derived from the diet (Innis, 2007). As all n-3 and n-6 PUFAs can be metabolised from LA and ALA these are sometimes referred to as “parent” fatty acids. Calder, (2017) describes how arachidonic acid (AA) is the chief metabolite of LA whilst EPA and DHA are the most functionally important metabolites of ALA (Calder, 2016). The pathway for the conversion of ALA to EPA involves three steps, catalysed in turn by delta-6 (Δ^6) desaturase, elongase 5 and delta-5 (Δ^5) desaturase. Further conversion to DHA, via docosapentaenoic acid (DPA), occurs by a complex pathway involving chain elongation catalysed by elongase 5, a second chain elongation catalysed by elongase 2 or 5, desaturation by Δ^6 desaturase and finally the removal of two carbon atoms by limited β-oxidation in peroxisomes (Calder, 2017). Although the conversion pathways of n-3 and n-6 are independent, the enzymes that are required during each stage are actually shared between both pathways (Figure 1.1). The metabolism of n-3 and n-6 PUFAs are mainly carried out in the endoplasmic reticulum of the liver and in glial cells (Moore, 2001).

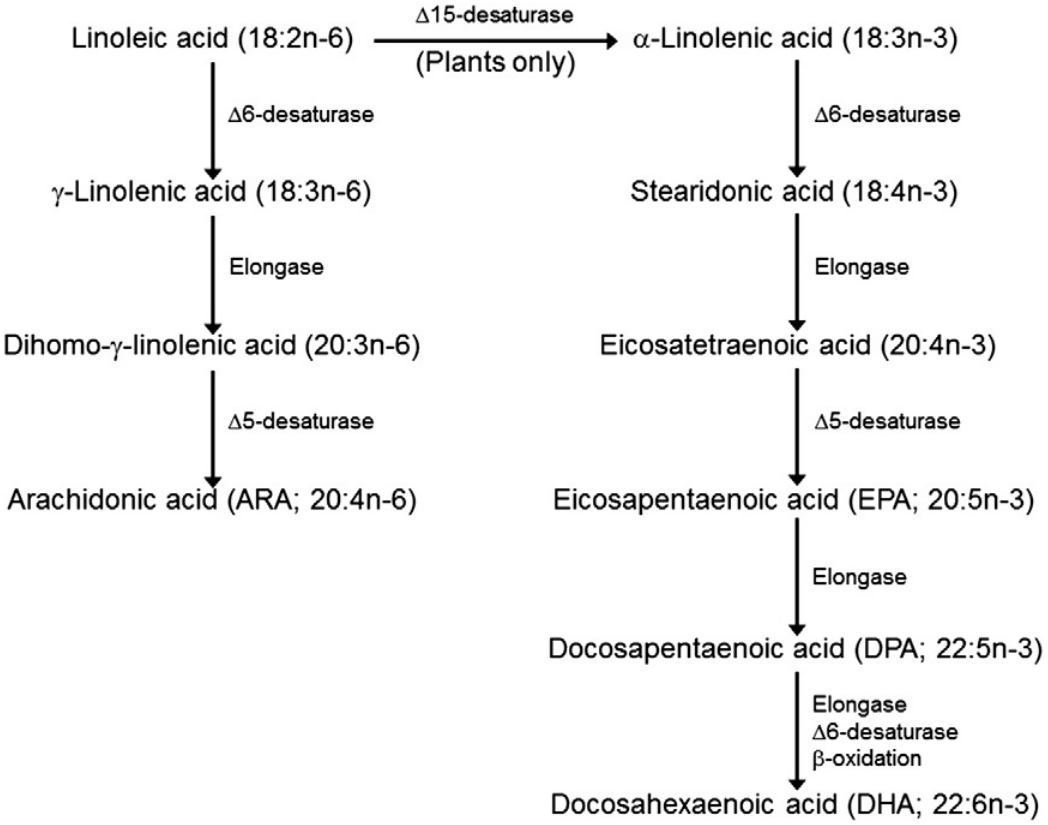

Figure 1.1 Pathways of biosynthesis n−6 and n−3 fatty acids. ARA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid. From “Marine omega-3 fatty acids and inflammatory processes: effects, mechanisms and clinical relevance” by P. Calder, *Biochimica et Biophysica Acta, 1851*(4), p. 471.

Figure 1.2 shows the route that long-chain n-3 PUFAs take once they have entered the body to incorporation into tissues. After the fats are ingested they are emulsified in the stomach before entering the small intestine and are then cleaved off from their various types of bonds to form free fatty acids (FFA) and 2-monoacylglycerides (2-MAG). The FFA and 2-MAG are then taken up by enterocytes (intestinal absorptive cells) as mixed micelles via passive diffusion. In the enterocytes, the n-3 PUFAs are re-esterified to triglycerides and then incorporated into chylomicrons (fat globules composed of protein and lipids) and transferred to the lymph and thus systemic circulation. Finally, blood circulation then transports the n-3 PUFAs to the target tissues where they are primarily incorporated into membranes.

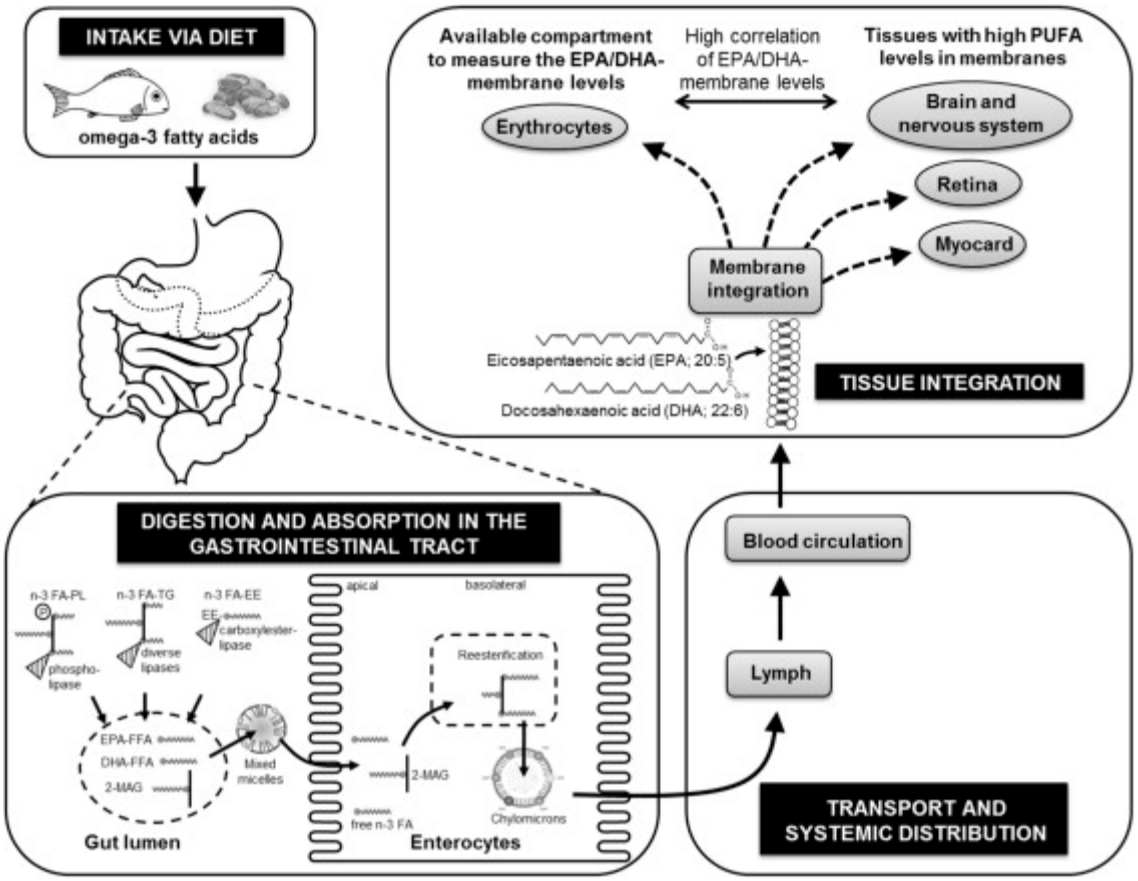

Figure 1.2. Digestion and uptake of long chain n-3 fatty acids. From "Bioavailability of long-chain omega-3 fatty acids" by J.P. Schuchardt & A. Hahn, *Prostaglandins, Leukotrienes and Essential Fatty Acids (PLEFA), 89*(1), p. 3.

The high intake of n-6 relative to n-3 in many western diets favours the conversion of LA over that of ALA due to the increased competition over the desaturases used to convert these PUFAs to their longer chain derivatives (Blasbalg et al., 2011). This increased competition from n-6 PUFAs for the enzymes required for metabolism of n-3 PUFAs may explain the low levels of conversion of ALA to EPA and DHA observed previously (Burdge & Calder, 2006). Although, conversion of ALA to EPA and DHA is also known to be influenced by several factors including stage of life, age, sex, hormones, genetics and disease (Baker et al., 2016). As a result, conversion of ALA to DHA has been found to be extremely inefficient in humans with rates of less than 1% being reported (Kidd, 2007; Plourde & Cunnane, 2007). This conversion is further limited by the fact that 25% of ALA is converted to carbon dioxide for energy within 24 hours of consumption (Brenna, 2002). In light of the abundant issues with metabolism of ALA to EPA and DHA, it is more efficient for these n-3 PUFAs to be supplied directly via dietary sources.

1.3 Dietary Sources, Consumption and the Importance of the n-3/n-6 ratio

The main dietary sources of LA include cereals, eggs, poultry, most vegetable oils, whole-grain breads, baked goods, margarine, sunflower oil, saffola oil and corn oils (Das, 2002; Meyer et al., 2003). Whereas the main dietary sources of ALA include canola oil, flaxseed oil, linseed and rapeseed oils, walnuts and leafy green vegetables (Das, 2006). A variety of marine microalgae are rich sources of both DHA and EPA (Adarme-Vega et al., 2012) and as fish consume algae in their diets, they too are rich in both DHA and EPA, which leads to some fish being known as “oily” fish. Oily fish that are rich in EPA and DHA include mackerel, salmon, trout, fresh tuna and sardines. In smaller quantities, both EPA and DHA can also be found in some livestock and both chickens and their eggs if they have been fed a diet enriched with n-3 PUFAs.

The minimum recommended daily intake of EPA and DHA is between 200-450mg, depending upon the organisation making the recommendation (EFSA Panel on Dietetic Products, 2012). The Food and Drug Administration (FDA) consider an intake up to 3 g/day safe and the American Heart Association recommends an intake of two servings of fatty fish e.g. cold water salmon (3.5-oz each serving) twice a week for the prevention of cardiovascular disease (Lichtenstein et al., 2006). In the UK, it is estimated that only 25% of the adult population regularly consume fatty fish with the remaining 75% consuming fatty fish rarely or never (Calder, 2017).

Over the past 100-150 years, the consumption of n-3 PUFAs has been falling gradually in western diets which now contain excessive levels of n-6 PUFAs compared to n-3 PUFAs, leading to an unbalanced n-6/n-3 ratio of 20:1 in favour of n-6 (Simopoulos, 2008). There is evidence to suggest that humans evolved on a diet where n-6 and n-3 PUFAs were consumed in equal amounts (Simopoulos, 2016). It has been argued that the fall in the intake of biologically active n-3 PUFAs has contributed to the increasing incidence of atherosclerosis, cardiovascular heart disease, hypertension, metabolic syndrome X, obesity, and potentially cancer (Das, 2006).

1.4 The Functions of n-3 PUFAs

Human cell membranes are composed of phospholipids that contain various types of fatty acids. The length and saturation of the fatty acids in these phospholipids is believed to affect the properties of cell membranes with incorporation of n-3 PUFAs into the phospholipids resulting in more fluid cell membranes and affecting lipid microdomain formation and signalling across the membranes (Endo & Arita, 2016). It is this concept by which n-3 PUFAs are

believed to impact brain function and hence behaviour and mood as well as having impacts on the cardiovascular system via modulation of certain cardiac ion channel functions, neurotransmission, inflammation from eicosanoid production, gene expression and tumour suppression.

1.4.1 Role of n-3 PUFAs in Cell Membranes and Abundance in the Brain

The cell membrane, composed of a phospholipid bilayer and several proteins, constitutes the outer boundary of the cell. Not only does the cell membrane control molecular transport, but it also regulates communication between the cell and its environment by transducing signals across the membrane (Hou, McMurray & Chapkin, 2016). EFAs are known to influence membrane fluidity, determining and influencing the behaviour of membrane bound enzymes and receptors (Das, 2006). The brain contains a higher concentration of lipids than any other organ, excluding adipose tissue (Heinrichs, 2010). Fifty percent of the dry weight in an adult brain is composed of lipids (Hamilton et al., 2007; Bruce, Zsombok & Eckel, 2017), 35% of which are n-3 and n-6 PUFAs (Haag, 2003). When the consumption of fish or fish oil in the diet increases, the EPA and DHA from these sources are known to partially replace the n-6 fatty acids, especially AA, in the membrane phospholipids of all cells, but especially in the membranes of platelets, erythrocytes, neutrophils, monocytes, neurons and liver cells (Simopoulos, 2008). These increases in EPA and DHA and decreases in AA are reflected in membrane levels (Marteinsdottir, 1998).

When n-3 PUFAs are incorporated into the phospholipid bilayer of cell membranes they can affect signalling across membranes and also modulate the function of membrane ion channels (Endo & Arita, 2016). The unique structure of n-3 PUFAs, particularly EPA and DHA, allows for increased membrane fluidity when incorporated into the membranes of cells due to the specific positioning of the double bonds between the carbon atoms that results in a “kink” of the fatty acid that then takes up more space in the membrane (Figure 1.3; Heinrichs, 2010; Hussein, 2013). Considering that neurotransmission relies on ion channels embedded in membranes, neurons composed of insufficient levels of PUFAs can be expected to exhibit signalling abnormalities (Zimmer et al., 2000).

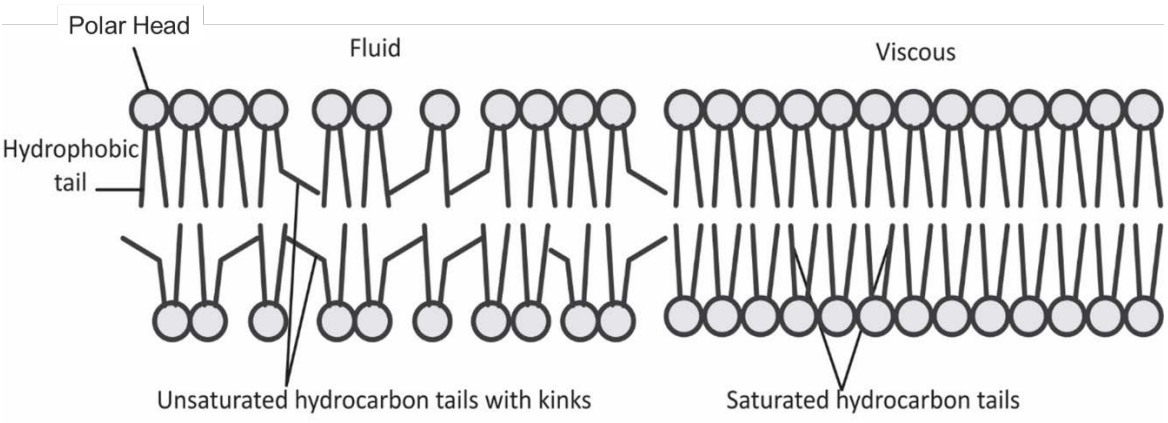

Figure 1.3. The difference between "fluid" and "viscous" cell membranes caused by an increased incorporation of unsaturated fatty acids into the phospholipid bilayer. Adapted from *Membrane Fluidity of Biomembrane,* in *Let's Talk Academy*, n.d., retrieved May 12, 2019, from https://www.letstalkacademy.com/publication/read/membrane-fluidity-of-biomembrane.

Evolutionary perspectives on fatty acid consumption suggest that long chain (LC) PUFAs may have played a role in the enlargement of the human brain. The increased size of the hominid cerebral cortex over the last two million years, when homo first emerged, is correlated with a large increase in fish consumption (Heinrichs, 2010), and some researchers have proposed that LC PUFAs were crucial in the development of human intellect (Chamberlain, 1996). As a large proportion of the mammalian brain is composed of lipids it does advocate the importance of lipids in maintaining the structure and function of the mammalian brain. Indeed, Kaplan, Gimbel & Harris, (2016), provide evidence for the role that n-3 PUFAs played in the development and morphology of the modern human and mammalian brain. This idea is also consistent with evidence from experiments with animals, which demonstrate that n-3 deficiency depletes brain fatty acid composition in rhesus monkeys (Neuringer et al., 1986) which then results in serious behavioural derangements (Reisbick et al., 1994) and neuronal atrophy in the dorsolateral and dorsomedial prefrontal cortex of mice (Larrieu et al., 2014). The effects of n-3 PUFA deficiencies have also been seen to be reversed when consumption of n-3 PUFAs is introduced back into the diet (Connor, Neuringer & Lin, 1990). This evidence again suggests that n-3 PUFAs play a vital role in the structure and functioning of the mammalian brain.

1.4.2 N-3 PUFAs Modulate Neurotransmission

Although no neurotransmitters themselves are directly derived from n-3 PUFAs, variation of dietary intake of these has been shown to influence levels of dopamine (Zimmer et al., 2000; Song et al., 2003; Song et al., 2007; Forster et al., 2008), serotonin (Hibbeln et al., 1998; Grosso et al., 2014; Patrick & Ames, 2015; Sugasini & Lokesh, 2015) and acetylcholine (Aïd

et al., 2003; Taepavarapruk & Song, 2010). N-3 PUFAs are also known to play a role in various signalling pathways including activation of secondary messenger systems in response to neurotransmitters binding to extracellular receptors (Heinrichs, 2010). N-3 PUFAs have been shown to increase levels of protein kinase A, as well as adenylate cyclase, in neuronal tissue cultures *in vitro* (Haag, 2003). Protein kinase A and adenylate cyclase are both involved in the metabotropic-receptor mediated signalling pathways used by norepinephrine (ά-2 and β receptors), serotonin (5-HT_1 receptor) and dopamine (D_1 and D_2 receptors; Heinrich, 2010). A deficiency of n-3 PUFAs in neurons would also likely be matched with lower levels of adenylate cyclase and protein kinase A activity, which would in turn result in the need for a greater amount of neurotransmitter agonist activity to depolarize the cell once a receptor is activated (Heinrich, 2010).

Certainly, altered levels of neurotransmitters are related to several conditions. For example, the pathophysiology of depression has been dominated by the monoamine hypothesis, suggesting that an imbalance in serotonergic and noradrenergic neurotransmission is at the core of depressive symptoms (Grosso et al., 2014). Additionally, levels of dopamine are known to be related to both schizophrenia (Ohara, 2007) and ADHD (Huang et al., 2015) whilst Alzheimer's disease (AD) is related to levels of acetylcholine (Kumar & Singh, 2015). Changes in brain n-3 PUFA concentrations induced by chronic deficiency in dietary n-3 PUFA intake have previously been seen to increase serotonin 2 (5-HT_2) and decrease dopamine 2 (D_2) receptor density in the frontal cortex (Delion et al., 1994; Berg, Maayani & Clarke, 1996; Chalon et al., 2001), changes thought to play a role in the pathophysiology of depression (Fakhoury, 2016). Human plasma n-3 levels in cerebral spinal fluid have also previously been positively correlated with 5-hydroxy-indoleacetic acid (5-HIAA), a metabolite of serotonin and an indicator of brain serotonin turnover (Hibbeln et al., 1998) with increased cerebrospinal fluid 5-HIAA release commonly associated with the improvement of depression and anxiety symptoms (Su, Matsuoka & Pae, 2015).

Pre-clinical studies have also shown that n-3 PUFAs can promote hippocampal neurogenesis in adult animals (Kawakita, Hashimoto & Shido, 2006; Beltz et al., 2007), potentially through their ability to modulate neurotrophins (Wu, Ying & Gomez-Pinilla, 2004; Venna et al., 2009; Blondeau et al., 2009). Brain-derived neurotrophic factor (BDNF), is known to be associated with neurogenesis and the survival and growth of many types of neurons (Hearing et al., 2016) and could be involved in improving cognition by enhancing learning and memory functions of the hippocampus (Schinder & Poo, 2000; Binder & Poo, 2004). Experimental studies in rats have demonstrated that n-3 PUFAs can modify BDNF levels (Rao et al., 2007; Vetrivel et al., 2012; Ferreira et al., 2013) with Ferreira et al., (2014) also identifying a correlation between

n-3 PUFA intake and peripheral BDNF levels in humans, providing support for the effects of n-3 PUFAs on BDNF levels in human samples. Together, these findings suggest that many neurotransmitter pathways can be influenced via dietary intake of n-3 PUFAs, which can then also have an impact upon neuronal activity, neurotransmission, neuroplasticity, mood and behaviour.

1.4.3 N-3 PUFAs Modulate Inflammation

Inflammation is a crucial part of the defence mechanism against pathogenic organisms, which forms an environment that is hostile to pathogens, inducing changes in the metabolism of the host and initiating pathogen killing (Calder, 2015). During the inflammation process, leukocytes migrate from the blood stream into the surrounding tissue, releasing lipid derived mediators called eicosanoids. Eicosanoids, further categorised as leukotrienes, thromboxanes and prostaglandins (Figure 1.4), are a class of biological compounds responsible for mediating many aspects of the inflammatory response (Calder, 2006; Calder, 2009) and are mainly derived from 20 carbon PUFAs, either AA (n-6) or EPA (n-3), liberated from cell membranes. As the consumption of n-6 PUFAs is far greater than n-3 PUFAs in western diets, AA is usually the dominant substrate for eicosanoid synthesis (Calder, 2017b), and AA derived eicosanoids are, by definition, pro-inflammatory (Schmitz & Ecker, 2008). Although, research has actually shown AA-derived eicosanoids as having a mixed response with both pro- and anti-inflammatory roles (Levy et al., 2001; Vachier et al., 2002; Miles, Allen & Calder, 2002; Gewirtz et al., 2002; Serhan et al., 2003).

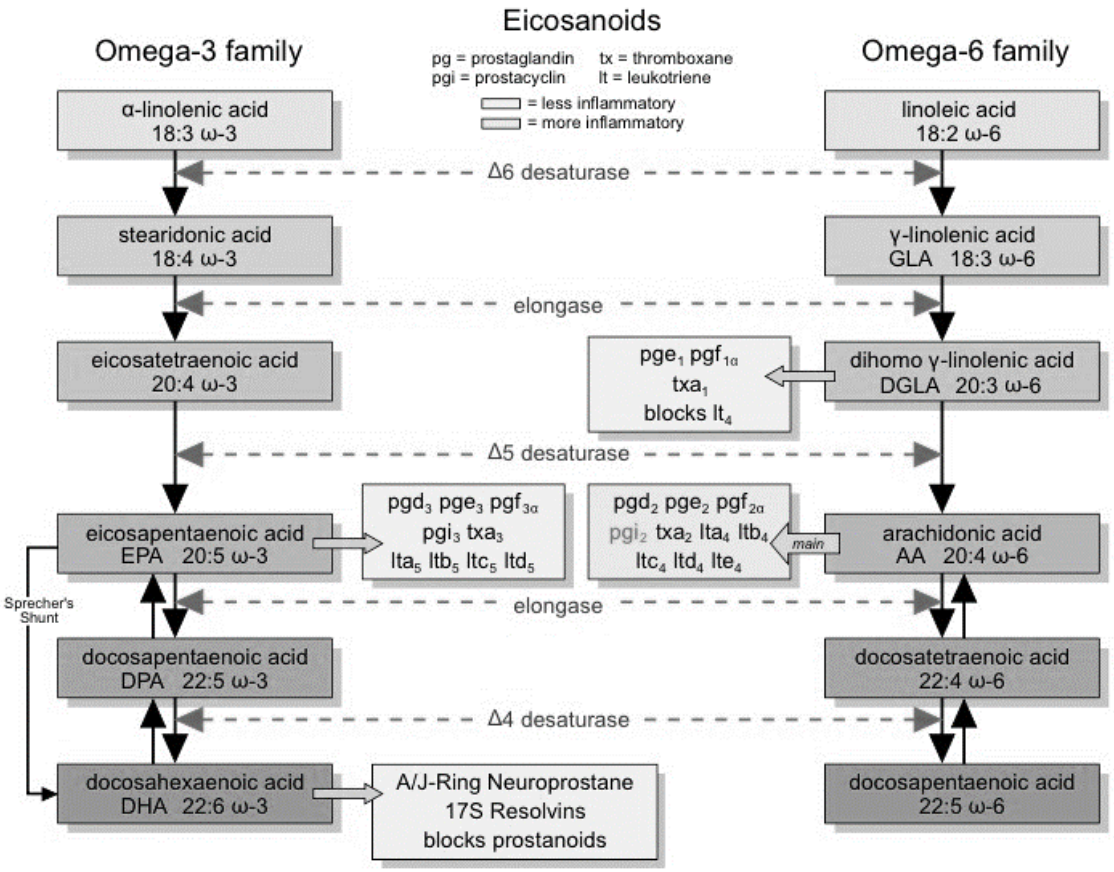

Figure 1.4. EFA metabolism and production of eicosanoids. Adapted from *Eicosanoids*, by D. R. Throop, 2018, retrieved May 22, 2019, from https://me-pedia.org/wiki/Eicosanoid.

As stated previously, increased consumption of both DHA and EPA results in an increased incorporation of n-3 PUFAs into inflammatory cell phospholipids, often displacing AA (Rees et al., 2006; Walker at al., 2015). This results in a decrease in the synthesis of AA-derived eicosanoids and an increased production of EPA-derived ones, believed to be more anti-inflammatory than AA-derived eicosanoids. Several animal studies have shown that feeding with EPA and DHA decreases production of PGE_2, an AA derived eicosanoid (Chapkin, Akoh & Miller, 1991; Yaqoob & Calder, 1995; Peterson et al., 1998). In line with this, a number of studies in healthy human volunteers or in patients with chronic inflammatory diseases, such as rheumatoid arthritis or inflammatory bowel disease, have also identified decreased production of AA-derived 2-series prostaglandins and 4-series leukotrienes by inflammatory cells following supplementation with high doses of EPA and DHA for a period of weeks to months (Meydani et al., 1991; Caughey et al., 1996; Varghese & Coomansingh, 2000; Trebble et al., 2003; 2005; Mas et al., 2012). Originally, it was believed that EPA-derived eicosanoids were less potent than AA-derived ones. However, studies have demonstrated that both PGE_2 and PGE_3 have equivalent inhibitory effects upon production of TNF-α and IL-1 β (Miles, Allen

& Calder, 2002) suggesting that EPA- and AA-derived eicosanoids do not always have different properties.

Like eicosanoids, docosanoids are also chemical signalling molecules, produced via controlled oxidative degeneration of DHA within or adjacent to cell phospholipids (Kidd, 2007). DHA-derived trihydroxydocosahexanoic acid mediators, termed D-series resolvins, have been shown to exhibit neuroprotective qualities. For example, neuroprotection D1 (NPD1) is known to promote amyloid-β phagocytosis while decreasing inflammatory cytokine production in microglia and peripheral mononuclear cells (Zhao et al., 2011; Zhu et al., 2016; Mizwicki et al., 2013), as well as repressing the expression of pro-inflammatory amyloid-β activated genes (Hopperton et al., 2018). Moreover, the anti-inflammatory functions of n-3 PUFAs appear not only to be the result of changes in patterns of lipid mediator synthesis, but also via inhibiting the expression of pro-inflammatory cytokine genes, such as nuclear factor $_{K}$B (Calder, 2006a; Calder, 2009).

Atherosclerosis, an inflammatory disease of the vascular wall primed by endothelial dyscfunction, is also known to occur from interactions between lipoproteins, immune cells and the endothelium (Glass & Witzum, 2001; Blake & Ridker, 2001; Hallenbeck, Hansson & Becker, 2005; Calder, 2012), resulting in a thickening of the arterial wall. In normal conditions, the endothelium contributes to vascular homeostasis via regulating vascular tone but this is negatively impacted in those suffering from atherosclerosis (Baker et al., 2018). Both animal and human studies have demonstrated that n-3 PUFAs can improve the function of endothelium via influencing the nitric oxide (NO) synthesis pathway and via competing with n-6 PUFAs resulting in reduced production of pro-inflammatory eicosanoids (reviewed in Colussi et al., 2017). These effects of n-3 PUFAs on cardiovascular function could be why increased intake of these fatty acids has previously been associated with reduced risk of cerebrovascular disease (Chowdhury et al., 2012). Positive effects of n-3 PUFAs on cerebrovascular functioning may be why intake of these fatty acids are also associated with promoting the brain's overall structural integrity and function (McNamara et al., 2010; Hamazaki-Fujita et al., 2011; Patan & Jackson, 2016) and help to prevent cognitive decline (Zhang et al., 2016; Cederholm, 2017).

Overall, the evidence suggests that eicosanoids derived from AA play a role in certain health conditions, such as inflammatory bowel disease, and that increased intake of the n-3 PUFAs DHA and EPA in the diet promotes the production of less inflammatory eicosanoids and of anti-inflammatory resolvins and similar mediators (Calder, 2008). These anti-inflammatory properties of n-3 PUFAs may then explain how DHA and EPA can potentially help to prevent

or ameliorate the symptoms caused by inflammation in certain diseases including AD (Zhang et al., 2015), rheumatoid arthritis (Lee, Bae & Song, 2012), inflammatory bowel disease (Calder, 2008) and atherosclerosis (Baker et al., 2018) in human populations throughout the lifespan. Furthermore, as n-3 PUFAs show beneficial effects on the prevention of inflammatory cardiovascular diseases, such as atherosclerosis, this may potentially be one of the reasons why intake of these fatty acids are also associated with lower incidences of cerebrovascular disease (Chowdhury et al., 2012) and cognitive decline (Zhang et al., 2016; Cederholm, 2017). As western diets in general are seen to favour consumption of n-6 PUFAs over n-3 PUFAs at a ratio of 20:1 (Simopoulos, 2008), it would appear that even the general healthy population could still benefit from increased dietary intake or supplementation with n-3 PUFAs with regards to achieving a more balanced inflammatory response.

1.4.4 Role of n-3 PUFAs on Gut Function and Immune Response

Increasing amounts of evidence have begun to reveal the relationship between the brain and microbiota (Borre et al., 2014, Sampson & Mazmanian, 2015; Gondalia et al., 2019). For instance, Mayer et al., (2014) describe how the microbiota–gut–brain axis, referring to the bidirectional network of communication between the gut microbiota, the gut, and the brain, interacts through endocrine, inflammatory and nervous systems to modulate immunological, gastrointestinal (GI) and CNS functions. As well as improving neurological outcomes, n-3 PUFA have also demonstrated a beneficial impact on the microbiota in the gut, by increasing bifidobacteria growth (healthy bacteria), reducing enterobacteria growth, common causes of stomach upset (Kang et al., 2018) and subsequently dampening inflammatory responses associated with metabolic endotoxemia (Kaliannan et al., 2015). ALA is also known to promote adhesion of *Lactobacillus casei* to mucosal surfaces which augments their growth and *Lactobacillus casei*, in turn, suppress the growth of pathogenic bacteria like *Escherichia*, *Salmonella typhimurium, Helicobacter pylori, Pseudomonas aeruginosa, Clostridium difficile* and *Shigella flexneri* (Das, 2010). As microbiota, the gut and the brain are all connected via microbiota–gut–brain axis, it seems reasonable that the impact of n-3 PUFAs on microbiota may also impact upon the brain and behaviour. Certainly, microbiota can influence brain and behaviour by direct and indirect pathways including activation of the immune system via cytokine release, neurotransmitter production, hypothalamic-pituitary-adrenocortical (HPA) axis, tryptophan metabolism and entero-endocrine activation and stimulation of neural pathways such as the vagus nerve (Cryan & Dinan, 2012; Sampson & Mazmanian 2015; Mayer et al., 2015). As a result, gut microbial alteration has emerged as a potential mediator in the aetiology of certain mental health issues such as bipolar depression (Evans et al., 2017). This may potentially explain previous findings that identify the beneficial effects of n-3 PUFAs

on depression (Mocking et al., 2016; Hallahan et al., 2016; Deacon et al., 2017), possibly a result of the effect n-3 PUFAs have on gut microbiota. Furthermore, investigations into the impact of diet on gut microbiota show an interactive relationship between altered microbiota composition and the development of neurodegenerative diseases (Friedland & Chapman, 2017; Byerley et al., 2017; Cenit, Sanz & Codoñer-Franch, 2017). As n-3 PUFAs have previously been found to be beneficial in patients suffering from AD (Shinto et al., 2014; Eriksdotter et al., 2015; Hooper et al., 2018) this again may suggest that the relationship between n-3 PUFA intake and protection against neurodegenerative diseases may be influenced, at least in part, by changes to gut microbiota (La Rosa et al., 2018).

Similarly, macrophages, neutrophils and T-cells are known to release PUFAs on stimulation, meaning it is possible that release of PUFAs may be one of the defence mechanisms of the body to fight viral and bacterial infections (Das, 2006). Indeed, n-3 PUFAs have been seen to enhance humoral immunity through enhancing B cell activation and antibody production (Rockett et al., 2010; Rockett et al., 2012; Gurzell et al., 2012), with B cells being critical for both innate and adaptive immune responses and for the hosts defence response against pathogens, protection against reinfection and tissue homeostasis (Whelan, Gowdy & Shaikh, 2016). Therefore, boosting B cell antibody production or activities via n-3 PUFA supplementation may have clinical relevance, particularly in populations with weakened immune responses or decreased humoral immunity, such as obese populations (Sheridan et al., 2012; Shaikh et al., 2015). Moreover, research has also shown that EPA and DHA ethyl esters can differentially enhance splenic B cell activation and upregulation of activation markers *ex vivo* (Teague et al., 2014; Gurzell et al., 2015). These data highlight that, although very similar, differential effects of EPA and DHA do exist and that the specific EPA or DHA content of a diet can influence immunological outcomes, emphasising the importance of understanding the independent effects of dietary intakes higher in EPA or DHA (Gurzell et al., 2015). Overall, these findings suggest that n-3 PUFAs can show antibiotic-like actions, impact on gut microbiota and beneficially impact humoral immune response, potentially helping to further explain some of the previously identified beneficial functions on the brain and behaviour.

1.4.5 N-3 PUFAs Modulate Gene expression

Both n-3 and n-6 PUFA families have been shown to control gene expression in a variety of tissues (Clarke, 2001; Duplus, Glorian & Forest, 2000) and several studies have confirmed the modulatory actions of PUFAs on gene expression in the brain (Kitajka et al., 2002; Barcelo-Coblijn et al., 2003a) and in certain brain regions such as the cerebrum (Barcelo-Coblijn et al.,

2003b) and hippocampus (Puskás et al., 2003; Berger et al., 2002). PUFA regulated gene expression can occur through interactions with specific and nonspecific ligands that bind to response factors and turn mRNA synthesis on or off (Kitajka et al., 2004). For example, PUFAs can directly interact with transcription factors, like peroxisome proliferator-activated receptors (PPAR) that directly modulate the expression of target genes (Kliewer et al., 1997; de Urquiza et al., 2000; Lee, Olson & Evans, 2003). In rats fed either a diet high in ALA or EPA + DHA it is found that expression levels of a number of genes involved in synaptic plasticity, ion channel formation, signal transduction and regulatory proteins were modulated compared to controls in DNA microarrays (Kitajka et al., 2002). Barcelo-Coblijn et al., (2003b) have also identified that the n-3/n-6 ratio in the experimental diets of rats can impact on cellular function at the genetic level in the brain, suggesting that the n-3 PUFA-induced alterations in expression of neuronal genes may be the link between dietary intake and improvements in cognition and/or the breakdown of these processes may offer insights into some neuropsychiatric or developmental conditions.

A recent systematic review of the literature by de Groot, Emmet and Meyer (2019) has also identified sixteen studies have been conducted thus far on the association between genetics and levels of n-3 PUFAs. These papers describe the effects of the fatty acid desaturase (FADS), elongation of very-long-chain fatty acid (ELOVL) 2 and apolipoprotein E (ApoE) 4 genotypes on levels of n-3 PUFAs. Minor allele carriers of a FADS single-nucleotide polymorphism (SNP) have previously been negatively associated with levels of both EPA (Baylin et al., 2007; Tanaka et al., 2009; Mathias et al., 2010; Al-Hilal et al., 2013; Gillingham et al., 2013) and DHA (Baylin et al., 2007; Mathias et al., 2010; Al-Hilal et al., 2013) in plasma. Minor allele carriers of the ELOVL2 genotype have also been previously identified as having lower plasma DHA levels (Tanaka et al., 2009; AlSaleh et al., 2014). However, Tanaka et al., (2009) also observed higher EPA levels in minor allele carriers whilst Gillingham et al., (2013) identified no association between ELOVL2 and plasma fatty acid levels. Triglyceride EPA and DHA was found to be higher in ApoE4 carriers than non-carriers at baseline by Plourde et al., (2009) although no differences between carriers and non-carries have also been observed (Whalley et al., 2008; Samieri et al., 2011; Chouinard-Watkins et al., 2013; Yassine et al., 2016). However, Stonehouse et al., (2013) has previously reported significant interactions between ApoE4 status, sex and treatment group during a 6 month clinical trial, which might suggest that the interaction between ApoE4 status and n-3 PUFA levels may be influenced by or dependent on sex. Indeed, sex differences in terms of gene expression patterns between male and female ApoE4 carriers have previously been identified, with female carriers appearing to be effected more than male carriers (Hsu et al., 2019). For example, female carriers show an overall heightened immune response and increased risk of developing AD

compared to male carriers (Klein, 2012; Taneja, 2018). As ApoE genotype has emerged as the strongest identified common genetic predictor of longevity (Beekman et al., 2013; Broer et al., 2015) as well as being associated with increased triglyceride levels (Plourde et al., 2009) cardiovascular disease (Bennet et al., 2007; Khan et al., 2013) and AD (Belloy, Napolioni & Greicus, 2019; Arnold et al., 2019), n-3 PUFA based interventions may be of interest to ApoE4 carriers specifically in an attempt to offset the increased risks aforementioned. However, screening for ApoE genotype is currently not freely available to the general population and further research into the effects of supplementation with n-3 PUFAs in ApoE4 carriers is still required to fully assess the efficacy of n-3 PUFA supplementation as a potential treatment for the negative effects experienced in this population.

1.4.6 Cardiovascular Effects of n-3 PUFAs

Consumption of fish is generally associated with a reduced risk for sudden cardiac death and cardiac disease (Harris, 2005; Kandasamy, Joseph & Goenka, 2008) as well as lowered heart rate and lowered incidences of atrial fibrillation (Mozaffarian et al., 2004; Mozaffarian et al., 2005). N-3 PUFAs have also found to diminish fatal myocardial events in patients after infarction (Marchioli et al., 2002). These effects may be attributed to membrane-stabilizing effects in cardiac muscle cells (Leaf, Kang, Xiao & Billman, 2003) as well as fast plaque stabilizing effects (Thies et al., 2003). In addition to the lowered cardiovascular risk, n-3 PUFAs have also been found to drastically reduce serum triglycerides (Nestel et al., 2015), a known biomarker for increased cardiovascular disease (CVD); have antiarrhythmic (Christensen et al., 2001) and anti-inflammatory effects (see 1.4.3); improve arterial and endothelial function via increased nitric oxide synthesis (Colussi et al., 2017); have positive actions on oxidative stress (Mori et al., 2000; Mabile et al., 2001), as well as reducing cardiovascular risk factor concentrations of lipoprotein(a) (Rahmani et al., 2017) and oxidised low density lipoprotein (Pizzini et al., 2017), which are known to promote the build-up and progression of plaques (Hasanally, Edel & Chaudhary, 2017).

Current evidence supports the hypothesis that increased dietary intake of n-3 PUFAs can decrease blood pressure in hypertensive patients (Colussi et al., 2017). EPA and DHA have also been seen to lower blood concentrations of norepinephrine, a vasoconstrictor agent (Hamazaki et al., 2005), while improvements in haemorheological parameters through fish oils have also been shown (Kobayashi et al., 2005). Growing evidence has linked n-3 intake with an improved endothelial function (Wang et al., 2012) potentially via reducing production of pro-inflammatory cytokines (He et al., 2009; Egert & Stehle, 2011) and increasing endothelium-dependent vasodilation through enhancing the release of NO (Abeywardena & Head, 2001;

Colussi et al., 2017). Given these observed effects of n-3 PUFAs on cardiovascular parameters, it could be that n-3 PUFAs may exert secondary effects on brain function via modulation of cerebral haemodynamics. Indeed, previous research has identified that increased intake of these fatty acids is associated with reduced risk of cerebrovascular disease (Chowdhury et al., 2012) and several studies have identified increases in cerebral blood flow (CBF) and increased cerebral blood oxygenation in relation to n-3 PUFA intake (Hamazaki-Fujita et al. 2011; Jackson et al., 2012a; 2012b; 2016). As brain activity is known to depend on a consumption of oxygen and glucose delivered via the blood to the sight of neural activation in a phenomenon known as neurovascular coupling (Denfield et al., 2016), it seems reasonable that any compound found to modulate cardiovascular activity may also exert secondary effects on brain function and behaviour. Indeed, several studies have investigated the effects of n-3 PUFA intake or supplementation on measures on neuronal activity and CBF via various neuroimaging techniques (Jackson et al. 2010; McNamara et al., 2010; Hamazaki-Fujita et al. 2011; Jackson et al., 2016). However, one mutual methodological limitation that exists within the literature concerns the use of neuroimaging techniques that only measure relative changes in cerebral activation and CBF as opposed to the measurement of absolute, quantifiable, amounts of haemoglobin present within the cortex. Consequently, there is still a need to further investigate this relationship with novel neuroimaging techniques that are capable of measuring absolute values of CBF, thus allowing for the measurement of gross changes that occur throughout the supplementation period to be captured. A feat that is possible with the employment of frequency domain near-infrared spectroscopy (NIRS).

1.4.7 Summary of Functions

N-3 PUFAs, especially EPA and DHA are involved in a plethora of fundamental functions at a cellular level. In the brain, DHA is greatly enriched in the cerebral cortex where its incorporation into the neuronal phospholipid bilayer helps to promote optimal membrane fluidity, resulting in improved membrane functioning in relation to neurotransmission and signal transduction. Additionally, there is a large body of evidence that describes the anti-inflammatory and secondary signalling effects of both EPA and DHA via the eicosanoids and docosanoids that are metabolised from them, helping to maintain a balanced immune response to pathogens and have been seen to possess neuroprotective properties. Furthermore, there is evidence to suggest that the numerous cellular effects of n-3 PUFAs may also be a result from their roles in gene expression, transcription factors and the turning on or off of certain genes. Research has also begun to show the beneficial effects of n-3 PUFAs on microbiota, the gut and how this may impact brain function via the microbiota–gut–brain axis and antibiotic like actions. Finally, dietary intake of n-3 PUFAs has also been seen

to modulate a number of cardiovascular parameters which seem to contribute to a modest decrease in the risk of fatal cardiac events. It is through these fundamental biological parameters that n-3 PUFAs, particularly EPA and DHA, are believed to impact upon brain function, behaviour and mood.

1.5 The Behavioural Effects of n-3 PUFAs

The impact of dietary n-3 PUFAs on brain function, and particularly cognition, has been largely based on the investigation of their effects in animals, quite often in rodents specifically. In these animal studies the role of n-3 PUFAs are usually measured in one of two ways: (1) involves the complete removal of n-3 from the maternal diet throughout gestation and lactation, resulting in a dramatic reduction of DHA in the nervous system of the offspring (Fedorova & Salem, 2006); (2) involves dietary supplementation with n-3 PUFAs either to the mother during gestation or directly to the offspring after birth. These studies are necessary as they allow for a level of control over the dietary intake of n-3 that is not possible with human trials, allowing for the measurement of the effects of extreme n-3 deficiencies that are almost never observed in humans. However, in humans n-3 status can be determined by measuring the concentrations of ALA, EPA and DHA in peripheral tissues such as serum/plasma and erythrocytes and so human trials are therefore capable of assessing various physiological and psychological parameters and their relationship to levels of n-3 within the blood. As a result of human trials, n-3 PUFA status has been associated with a range of neuropsychiatric, neurodevelopmental and cognitive parameters with evidence beginning to show the importance of maintaining appropriate n-3 PUFA intake across the lifespan to maintain optimal brain health. Therefore, the following sections will review the current literature on the behavioural effects of n-3 PUFAs in both animals and humans.

1.5.1 The Role of n-3 PUFAs on Mood

Intake of n-3 PUFAs has previously been associated with positive mood effects and potential therapeutic effects in the treatment of mood disorders such as anxiety and depression. Anxiety disorders are a common class of mental illness characterised by an inappropriate or exaggerated fear response leading to distress and impairment. The current edition of the Diagnostic and Statistical Manual for Mental Disorders (DSM-IV) includes amongst the anxiety disorders; generalised anxiety disorder, social phobia, other phobias such as agoraphobia, panic disorder and obsessive compulsive disorder (Ross, 2009). Some preclinical data support the role of n-3 PUFA as an effective treatment of anxiety disorders. For instance, Song et al., (2003) have identified that an EPA-rich diet can reduce the development of anxiety-like behaviours in rats as well as normalising dopamine levels in the ventral striatum.

Regarding therapeutic interventions, Fux et al., (2004) conducted a placebo-controlled cross-over trial of adjunctive EPA treatment in 11 participants with OCD but found no significant benefit of 6 weeks' supplementation with EPA of relieving symptoms of anxiety, depression or obsessive compulsiveness. However, in another randomised-controlled trial 2.25g/d of EPA with 0.5 g/d of DHA for 3 months was accompanied by significant decreases in anger and anxiety scores compared to placebo in a sample of 22 substance abusers (Buydens-Branchey, Branchey & Hibbeln, 2008). Additionally, Kiecolt-Glaser et al., (2011) have also identified that 12 weeks' supplementation with 2,085 mg/d of EPA and 348 mg/d of DHA can not only reduce inflammation but also reduced anxiety among 68 healthy, young adult medical students who faced stressful major examinations. A 14% decrease in stress-stimulated interleukin 6 production and a 20% reduction in anxiety symptoms were identified in the active treatment group compared to placebo. In summary, these studies seem to suggest that EPA rather than DHA might be efficacious in selective prevention of anxiety under serious physical conditions or stressful situations (Su, Matsuoka & Pae, 2015). This evidence also shows that the effects of EPA on anxiety symptoms are detectible even in healthy populations free from mood disorders, potentially due to the fact that western diets are seen to favour n-6 PUFAs over n-3 PUFAs (Simopoulos, 2008) and the general population are seen to fall below the recommended intake of n-3 PUFAs (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018). Suggesting that improving n-3 PUFA intake in otherwise healthy populations can still help to alleviate symptoms of anxiety.

As well as displaying beneficial effects on anxiety, n-3 PUFA intake has also been associated with improvements in those suffering with depression. Depression is part of a group of mental and behavioural problems termed "affective" mood disorders, with the world health organisation estimating that more than 300 million people worldwide suffer from depression, making it the world's leading cause of disability (WHO, 2018). Much of the previous research into understanding the aetiology and pathophysiology of depression has been focussed on environmental and genetic influences, while pharmacotherapeutic treatments are based on the monoamine hypothesis of depression (Hirschfeld, 2000). As a result, selective serotonin reuptake inhibitors (SSRIs) are still the most widely prescribed class of drug for depression (Young & Martin, 2003; Andrews et al., 2012). Recently, there has also been a considerable effort made to determine whether diet and nutritional factors play an important role in depression (Crowe, 2007; Martins, 2009; Akbaraly et al., 2013) with n-3 PUFAs, in particular, emerging as a potential agent in the treatment of depression (Logan, 2004; Martins, 2009).

Two main neurophysical mechanisms have been proposed to explain the association between n-3 PUFAs and depression: effects on pro-inflammatory cytokines and effects on inflammatory

eicosanoids. A number of studies provide support for the association between depression and production of pro-inflammatory cytokines (Parker, 2006; Chang et al., 2018). Effects of these pro-inflammatory cytokines include activation of the HPA axis, lowered neurotransmitter precursor availability and altered neurotransmission metabolism (Logan, 2004). Additionally, pro-inflammatory cytokines are also known to act as indicators of the severity of the disease (Suarez et al., 2003). Research has shown that patients with major depressive disorders (MDD) are also more likely to have elevated levels of inflammatory eicosanoids, particularly thromboxane B_2 and PGE_2, and n-3 PUFAs are known to inhibit these pro-inflammatory cytokines and eicosanoids, as outlined earlier in this Chapter (Logan, 2003; Kiecolt-Glaser et al., 2007). A second potential mechanism is via the importance of n-3 PUFAs in maintaining membrane integrity and fluidity, which is crucial for neurotransmitter binding, and signalling within the cell (Su, 2009). For example, a previous study using gas chromatography to measure fatty acid content within the anterior cingulate cortex of both depressed and healthy controls has shown significantly lower concentrations of several saturated and PUFAs including both the n-3 and n-6 fatty acids (Conklin, 2010).

Empirical observations have also shown that societies with a higher consumption of n-3 PUFAs are seen to have a lower prevalence of depression (Su, 2009). In Japan, where annual fish consumption rates are estimated at 70kg per person, the prevalence rates of depression are low at 0.12% when compared to Germany, where the annual fish consumption is less than 14kg per person and the prevalence rate of depression is 5% (Young & Martin, 2003). Additionally, an analysis of the published results from several countries has identified a positive correlation between DHA concentration in human mother's milk, seafood consumption and lower prevalence of postpartum depression (Hibbeln, 2002). However, a recent cross-sectional study by Sánchez-Villegas et al., (2018) in 6587 participants identified that only moderate fish and n-3 PUFA intake, but not high intake, was associated with lower odds of depression suggesting that a U-shaped relationship could actually exist. If this U-shaped relationship does exist, it may suggest that incidences of depression could potentially be reduced within the general population if dietary intake of n-3 PUFAs was increased, as consumption of n-3 PUFAs is known to fall below the recommended intake within the general population (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018). However, research thus far on the effects of n-3 PUFA supplementation and mood within general populations of healthy adults is conflicting (Fontani et al., 2005a; 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011; Kiecolt-Glaser et al., 2012; Giles et al., 2015; Watanabe et al., 2018) and still requires further investigation in rigorous, large scale, randomised controlled trials (RCTs).

A systematic review of published RCTs investigating the effects of n-3 PUFAs on depressed mood by Appleton et al., (2006) including 12 trials concluded that the evidence thus far was fairly limited and difficult to summarise due to the results varying considerably. Subsequently, Appleton et al., (2010) presented an updated systematic review and meta-analysis of the effects of n-3 PUFAs on depressed mood. Thirty-five RCTs, including seventeen that were not included in the previous review, were identified. In the more recent review, the authors concluded that whilst trial evidence of the effects of n-3 PUFAs on depressed mood has increased, it still remains difficult to summarise because of heterogeneity. However, the evidence does suggest that there appears to be some benefit of n-3 PUFAs in individuals with a diagnosis of depressive illness but limited evidence of benefits in those without a diagnosed depressive illness. Another meta-analytic review of placebo-controlled trials, including fourteen studies concluded that lower levels of EPA, DHA and total n-3 PUFA were found in patients with depression (Lin, Huang & Su, 2010). This supports the phospholipid hypothesis of depression, which describes how depression may be a result of abnormalities in phospholipid metabolism which may then further affect fatty acid and eicosanoid metabolism. If the phospholipid hypothesis of depression is accurate, then it provides a rationale for using n-3 PUFAs as an alternative treatment for depression (Lin, Huang & Su, 2010).

To summarise, whilst the findings from clinical trials remain inconclusive, there does appear to be adequate evidence to suggest that n-3 PUFAs can play a role in depression and deserves greater research (Deacon et al., 2017). Such research should include; a focus on whether EPA, DHA or a combination of both is the most clinically active compound; whether n-3 PUFA supplementation alone has antidepressant effects or has the greatest potential in conjunction with standard antidepressants; identifying a clinically appropriate dose (Deacon et al., 2017); and further investigation into the potential mood enhancing effects within the general healthy population who show inadequate dietary intake of n-3 PUFAs.

1.5.2 The Role of n-3 PUFAs in Sleep and Neurogenesis

While animal and human models have both demonstrated the role that n-3 PUFAs have in cognitive processes, our understanding of the mediating role of sleep within the n-3 PUFA-cognition relationship remains fairly limited (Liu et al., 2017). Sleep is well studied in its association with cognitive function in both children (Dewald et al., 2010; Gruber et al., 2010; Kopasz et al., 2010) and adults (Harvey, Tang & Browning, 2005; Goel, Durmer & Dinges, 2009), with insufficient or poor quality sleep being associated with poor school performance and objective measures of learning and memory (Dewald et al., 2010; Liu et al., 2012). N-3 PUFAs have previously been found to affect sleep via several mechanisms. Animal studies

have suggested the potential role of DHA in regulating endogenous melatonin production (Catalá, 2010; Peuhkuri, Sihvola & Korpela, 2012) which has been shown to regulate the sleep/wake cycle and improve sleep organisation (Turek & Gillette, 2004). Furthermore, n-3 PUFAs are involved in the production of prostaglandins which are believed to be one of the most potent endogenous sleep-promotion substances and are well known to mediate the sleep/wake cycle (Urade & Hayaishi, 2011) and responses of synaptic circuitry to sleep deprivation (Chen & Bazan, 2005). Epidemiological studies have also demonstrated significant associations between increased fish intake and improved sleep measures in adults (Christian et al., 2016; Del Brutto et al., 2016), children (Montgomery et al., 2014) and infants (Cheruku et al., 2002; Judge et al., 2012). RCTs that assess the effects of n-3 PUFA supplementation on aspects of sleep are sparse within the research area. However, from the studies that have been conducted improvements in sleep quantity and decreases in waking periods have been observed in 43 children (Montgomery et al., 2014) and overall positive impacts on sleep and on daily functioning in 95 adults aged 21-60 years have been observed (Hansen et al., 2014). As the population in general reports insufficient amounts of sleep or a high prevalence of sleep problems (Cirelli et al., 2016; Rössler et al., 2017; Kerkhof, 2017) as well as intakes of n-3 PUFAs that do not meet the recommended doses (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018) the healthy general population may benefit from increased n-3 PUFA intake or supplementation in terms of improvements in sleep.

One potential factor that may further explain the relationship between improved sleep parameters and improvements in cognitive function could be the process of neurogenesis, which is the process by which new neurons are generated from neural stem cells and progenitor cells (Dyall, 2015). Neurogenesis has been consistently shown to occur in two areas of the adult brain, the subgranular layer of the hippocampal denate gyrus and the subventricular zone of the lateral ventricles (Ehninger & Kempermann, 2008). Many studies on rodents have reported that instrumental sleep disruption (Guzmán-Marín et al., 2003; Guzmán-Marín et al., 2005; Tung et al., 2005; Sompol et al., 2011), rapid eye movement (REM) sleep deprivation (Mirescu et al., 2006; Guzman-Marin et al., 2008; Mueller et al., 2008), sleep restriction (Roman et al., 2005; Novati et al., 2011) and sleep fragmentation (Guzman-Marin et al., 2007; Sportiche et al., 2010) negatively affect neurogenesis in the hippocampus. Ageing is also known to be one of the greatest negative regulators of both sleep and neurogenesis and there is a strong correlation between age-related impairments in hippocampal-dependent memory tasks and the decline in neurogenesis (Drapeau et al., 2003). Increased neurogenesis has also been reported in rodents following ischemia (Takagi et al., 1999), stroke (Darsalia et al., 2005) and after seizures (Parent et al., 1997), with these observed increases in neurogenesis often interpreted as the brain attempting to repair itself,

raising the intriguing possibility that enhanced neurogenesis and the subsequent survival of new neurons may have significant therapeutic potential (Dyall, 2015). The existing evidence of the relationship between n-3 PUFA intake and improvements in sleep is also yet to be researched in terms of how n-3 PUFA intake may affect learning and memory consolidation and the processes which underpin these factors (Kitamura & Inokuchi, 2014).

1.5.3 The Role of n-3 PUFAs in Synaptic Plasticity & Memory Consolidation

Synaptic plasticity is a complex phenomenon of the brain that modifies the activity of neurons following sufficient stimulation, with plasticity changes being related to learning and memory (Palmeri et al., 2017). Mechanisms underpinning synaptic plasticity and memory are mostly focused on long-term potentiation (LTP), a cellular learning and memory mechanism in the brain (Ostroff et al., 2018) and a form of long-lasting synaptic strengthening that is thought to be an electrophysiological correlate of memory (Palmeri et al., 2017). LTP can be readily induced by theta-burst stimulation (Watson et al., 2016), a pattern that mimics naturally occurring neuronal firing patterns (Buzsakie, 2002), and is believed to consist of two fundamental phases: an early phase dependent on protein synthesis that can last minutes up to hours, and a more persistent protein-synthesis late phase (Kelleher, Govindarajan, & Tonegawa, 2004; Alberini, 2008). Protein synthesis is necessary to stabilise enlargement of dendritic spines induced by LTP and supports the role of protein synthesis in the remodelling of synapses during the consolidation from early phase LTP to late LTP (Tanaka et al., 2008). Protein synthesis is seen to appear locally in dendrites, where it may potentially serve as a source of new proteins during synaptic plasticity and the strengthening of neuronal connections (Martin & Ephrussi, 2009; Holt & Schuman, 2013).

In the dentate gyrus (DG) of the hippocampus, there is evidence to suggest that the mechanisms underpinning LTP in excitatory synapses are activated during learning and are required for several forms of hippocampal dependent memories (Lynch, 2004). Neurogenesis, is also another form of neural plasticity that may contribute to hippocampal-dependent memory function (Bruel-Jungerman, Laroche & Rampon, 2005; Winocur et al., 2006). Reports have demonstrated that specific hippocampal-dependant learning tasks (Snyder et al., 2005) can regulate neurogenic activity in the DG, but that learning itself also induces LTP-like mechanisms (Martin & Morris, 2002; Lynch, 2004). This may suggest that, although neurogenesis and synaptic plasticity contribute independently to hippocampal functions, it is possible that synaptic plasticity may also influence adult neurogenesis. Indeed, Bruel-Jungerman et al., (2006) have shown that electrically induced LTP in the DG *in vivo* provides

a cellular/molecular environment that favours both proliferation and survival of adult-generated neurons.

Research has identified that neurotrophins, a small class of secreted proteins, are potent regulators of synaptic plasticity (Leal et al., 2015). BDNF stands out amongst other neurotrophins due to its excessive expression in the mammalian brain, its potent effects on synapses and the release of BDNF being regulated by neuronal activity (Leal et al., 2015). BDNF supports neurogenesis and the survival and growth of many types of neurons (Hearing et al., 2016) and the regulation of activity-dependent changes in synapse structure and function (Park & Poo, 2013). The existing evidence suggests that BDNF is a major regulator of LTP in the hippocampus and other brain regions (Bramham & Messaoudi, 2005; Lu et al., 2008; Minichiello, 2009; Edelmann et al., 2014) and could be involved in improving cognition by enhancing learning and memory functions of the hippocampus (Schinder & Poo, 2000; Binder & Poo, 2004). The effects of BDNF on LTP and neurogenesis is one potential way in which n-3 PUFAs may exert beneficial impacts upon learning and memory functions. Experimental studies in rats have previously demonstrated that n-3 PUFAs can modify BDNF levels (Rao et al., 2007; Vetrivel et al., 2012; Ferreira et al., 2013) with Ferreira et al., (2014) also identifying a correlation between n-3 PUFA intake and peripheral BDNF levels in a sample of 137 adolescents, providing support for the effects of n-3 PUFAs on BDNF levels in human samples. Additionally, Abdel-Maksoud et al., (2017) have recently found that n-3 PUFAs reversed the negative effects of obesity on BDNF gene expressions *in vivo* and identified a dose-dependent increase in BDNF gene expressions in obese rat hypothalamus cultures *in vitro.*

Processes that may underpin synaptic plasticity and later memory consolidation have previously been identified to be influenced by n-3 PUFAs. For instance, Cao et al., (2009) revealed that DHA supplemented neurons have been seen to show more spontaneous synaptic activity than neurons treated with n-6 AA and that deficiency during development is associated with decreased expression of synapsin-1, which is critical to optical synaptic vesicle formation and release (Milovanovic & De Camili, 2017). Similarly, Aryal et al., (2019) found that dietary n-3 deficiency for 5 months in rats, post weaning, lead to a 65% reduction of DHA in whole brain synaptosomal phospholipids as well as reducing LTP in stratum oriens of the hippocampal CA1 region. Additionally, Robson et al., (2010) have compared the neurite promoting effects of DHA with EPA in neuronal cultures from young, adult and aged rats finding that both EPA and DHA increased neurite outgrowth in developmental stages, but only DHA produced positive effects in tissue from aged rats. This evidence appears to support the role of n-3 PUFAs, particularly DHA, in the processes that underpin synaptic plasticity and

memory consolidation which may be one potential way in which n-3 PUFAs could exert beneficial effects on learning and memory consolidation. Furthermore, this evidence shows the detrimental effects of n-3 deficiency on LTP within the brain, which can be seen as concerning when the population in general is seen to fall below the sufficient recommended intakes of n-3 PUFAs (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018).

Additionally, DHA *in vitro* has consistently been seen to promote differentiation of neural stem cells into neurons (Kawakita et al., 2006; Katakura et al., 2009, Ma et al., 2010; Rashid et al., 2013). Furthermore, (Kawashima et al., 2010) identified that dietary EPA can modulate synaptic plasticity and Katakura et al., (2013) have shown that both DHA and EPA appear to enhance differentiation of neural stem cells at similar amounts. However, Dyall (2015) reports how there is evidence to suggest that DHA and EPA have independent and divergent effects in the brain with Mandhair et al., (2013) identifying that EPA, but not DHA, significantly increased proliferation in neural stem cells suggesting that EPA may stimulate neurogenesis and DHA has more important roles in differentiation. Dyall et al., (2016) have also shown that DHA and EPA have differing effects on proliferation of neural stem cells and that these effects appear to be mediated via different cell signalling pathways. Together, the research suggests that both DHA and EPA are important in the mechanisms that underpin neural plasticity but in distinctly separate ways. Consequently, there appears to be a need for research that assess the potentially separate effects of intakes that are higher in EPA or DHA in relation to the processes that underpin neural plasticity.

Although there is evidence to suggest that the positive effects of DHA and EPA on plasticity and LTP, observed in animal studies, also exists in the human brain (Beck et al., 2000). The direct assessment of the effects of EPA and DHA supplementation on synaptic plasticity in human samples has yet to be conducted. Additionally, although a large number of studies assessing cognition have been conducted, only a small number have employed learning-memory tasks likely to induce LTP and memory consolidation (Dalton et al., 2009; Yurko-Mauro et al., 2010; Dangour et al., 2010; Kulzow et al., 2016). Collectively, there is a distinct lack of human data in the literature regarding the individual effects of dietary DHA and EPA on learning and memory consolidation and this area requires further investigation.

1.5.4 The Role of n-3 PUFAs in Maintaining Brain Health across the Lifespan

Brain health can refer to both long and short term brain health with the former relating to maintaining optimal functioning throughout the entire lifespan and delaying cognitive decline and neurodegeneration. However, it can also refer to maintaining optimal structure and functioning over shorter time frames via neuronal plasticity and maintaining optimal neuronal

efficiency. Poor brain health can manifest itself in numerous ways but is characteristically associated with cognitive impairments such as dementia (Ray & Davidson, 2014). A host of underlying molecular and cellular changes within the brain that include nerve inflammation, alterations in glucose-energy metabolism, mitochondrial impairments and oxidative damage contribute to cognitive decline and brain ageing (Poddar et al., 2018). It is now fully appreciated that a healthy brain is required to live a richer and longer life and that brain health fully supports planned actions, thought processes and emotional connections in humans (Gorelick et al., 2017). Together these factors influence the daily lives and indeed the quality of the lives of individuals, families and communities, as maintaining brain health across the lifetime can help to optimize levels of independence (Gorelick et al., 2017). Preserving brain health is also necessary to limit substantial economic and healthcare costs that poor brain health can cause, with the cost of dementia estimated to more than double in the next 20 years from £26 billion to £50 billion by 2040 in the UK alone (Prince et al., 2014). Living a healthy active lifestyle with regular exercise has already been shown to help maintain brain health across the lifespan (Cotman & Berchtold, 2002; Cotman, Berchtold & Christie, 2007; Gomes-Osman et al., 2018; Tyndall et al., 2018). Furthermore, the effects of diet on maintaining brain health have also been identified (Castelli et al., 2018; Croll et al., 2018; Tengeler, Kozicz & Kiliaan, 2018). As the abundance and importance of n-3 PUFAs in the brain has been made clear throughout the previous sections of this Chapter, it stands that optimal dietary intake throughout the lifespan should also be beneficial to maintaining brain health throughout the lifespan. Therefore, the following sections will aim to describe the effects of n-3 PUFA intake and supplementation on brain health throughout the lifespan.

1.5.4.1 The Role of n-3 PUFAs in the Neurodevelopment of Children

Brain accumulation of DHA starts *in utero* with a noticeable deposition in the second half of gestation coinciding with a growth spurt in grey matter (Innis, 2009). DHA levels in the brain are known to increase during development (Martinez, 1992; Lauritzen et al., 2016) and decrease with ageing (Guisto et al., 2002). Imbalances and deficiencies of n-3 PUFAs are also associated with impairments in behavioural measures and cognitive performance (Williams, Birch & Emmett, 2001; Innis, 2009). Additionally, fish intake during pregnancy and a higher n-3 PUFA status at birth has previously been associated with better visual development in infants born at term (Williams, Birch & Emmett, 2001; Innis, Gilley & Werker, 2001; Hibbeln et al., 2007).

Several studies have measured the effects of maternal intake of DHA during pregnancy on infant development and a consistent pattern seems to emerge in these studies, in which the

infant or maternal circulating levels of DHA at birth are associated with enhanced infant neural and visual development (Helland et al., 2001; Malcolm et al., 2003; Colombo et al., 2004; Lauritzen et al., 2004; Colombo et al., 2017), although these effects are not unequivocal (Dziechciarz, Horvath, & Szajewska, 2010; Saccone, Saccone & Berghella, 2016). However, some studies do suggest the maternal DHA provided during lactation and gestation has long-term benefits to the development of motor and cognitive skills in early childhood (Helland et al., 2003), which is consistent with epidemiological evidence that links a lowered risk of poorer IQ scores in young children to maternal n-3 PUFA intake in pregnancy (Oken et al., 2005; Hibbeln et al., 2007). However, it should be noted that the effect observed by Helland et al., (2003) were seen to disappear by 7 years of age (Helland et al., 2008). Additionally, recent follow up studies have failed to identify a positive impact of intake of n-3 PUFAs during pregnancy and childhood IQ (Gould et al., 2017; Crozier et al., 2018), perhaps suggesting that increased intake of n-3 PUFAs during pregnancy only results in shorter term benefits. Indeed, Judge et al., (2007) have reported a positive effect of n-3 PUFAs on performance on the 2 step problem solving test at 9 months old. Moreover, Dunstan et al., (2008) have reported improved hand-eye coordination during the Griffiths Mental Development Scales (GMDS) in a group who received a fish oil supplement (2.2g/d DHA + 1.1g/d EPA) from 20 weeks' gestation until birth, with a significant positive association with n-3 PUFA and an inverse correlation with AA in cord blood at 2.5 years. Additionally, Braarud et al., (2018) have identified that maternal DHA status during pregnancy was found to be positively associated with infants' problem-solving skills at 12 months and the infants' DHA status at three months was also associated with problem solving abilities at 12 months.

Increasing evidence also suggests that a relative lack of n-3 may contribute to a number of psychiatric and neurodevelopmental disorders including dyslexia, attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) (Richardson, 2006). Evidence has shown that visual and auditory symptoms in dyslexia have been related to physical signs consistent with fatty acid deficiency (Taylor et al., 2000) and the dietary intake of PUFAs, such as EPA and DHA, can affect the development and maturation of low-level auditory processing systems, known to be affected in those with dyslexia (Haubner et al., 2002; Unay et al., 2004). Recent evidence has also suggested that n-3 PUFA homeostasis may be altered in those with ASD, either as a result of nutritional imbalance or a genetic defect (Brown & Austin, 2011). A decrease in total n-3 PUFAs in the plasma of children with autism has also been identified without any changes in the n-6 PUFA family (Vancassel et al., 2001; Jory, 2016). A 12-week n-3 PUFA supplementation study has also identified improvements in hyperactivity in children with autism (Bent et al., 2011) whilst another study using a DHA, EPA and AA dietary supplement for 3 weeks reported improved behavioural performance in two-

thirds of the children with autism (Meguid et al., 2008). A pilot study in Singapore has also identified a positive association between changes in core symptoms of ASD and blood fatty acid levels following a 12-week n-3 PUFA dietary supplementation (Ooi et al., 2015). Conversely, several interventional studies with n-3 PUFAs have failed to reproduce these beneficial effects (Politi et al., 2008; Dubnov-Raz et al., 2014; Mankad et al., 2015) and so as a result larger cohorts and accurate ASD behavioural phenotypes are needed to clearly decipher the potential beneficial effects of n-3 PUFAs on behavioural deficits (Madore et al., 2016).

There is also promising evidence that n-3 PUFAs may be relevant to ADHD (Chang et al., 2018). In epidemiological studies, the children of mothers who have lower seafood intake during pregnancy have been identified as being at risk of suboptimal outcomes for fine motor coordination, prosocial behaviours, social development and verbal communication (Hibbeln et al., 2007). Furthermore, Chang et al., (2016) have also shown that children with ADHD have a greater severity of EFA deficiency, comprising symptoms such as dry eyes, eczema and dry, scaly skin. Some RCTs supplementing with n-3 PUFAs in participants with ADHD have also shown improvements in clinical symptoms (Richardson & Puri, 2002; Manor et al., 2012; Perera et al., 2012) and cognitive performance (Sinn et al., 2008; Vaisman et al., 2008). For instance, Chang et al., (2018) recently conducted a systematic review and meta-analysis of 8 clinical trials and 12 biological studies that assess the n-3 PUFA status of youths with ADHD. The authors concluded that there is evidence that n-3 PUFA supplementation improves clinical symptoms and cognitive performances in both children and adolescents with ADHD, and that these youths also have an observable deficiency in n-3 PUFA levels.

Concerning the effects of n-3 PUFA intake in typically developing children, findings thus far appear to show limited or inconclusive observable effects on measures of cognitive function or academic performance. For instance, Kennedy et al., (2009) have previously identified that supplementation with both 400mg and 1000mg of DHA for 8 weeks had no beneficial effects on brain function in cognitively intact 10-12 year olds. Likewise, Kirby et al., (2010) identified limited cognitive effects of 16 weeks' supplementation with 800mg/d fish oil (400mg/d DHA + 56mg/d EPA) in 450 mainstream school children aged 8-10 years old. From the cognitive test battery employed no differences were observed within the intend-to-treat analysis although increased accuracy in the active group compared to placebo was observed within the per-protocol analysis during the Matching Familiar Figures Task. However, Portillo-Reyes et al., (2014) have previously identified improvements in processing speed, visual-motor coordination, perceptual integration, attention and executive function in 59 malnourished but otherwise typically developing children aged 8-12 years following 12 weeks' supplementation

with 180mg/d DHA and 270 mg/d of EPA. Similarly, Johnson et al., (2016) has identified improvements in reading ability, phonologic decoding time and visual analysis time in 154 mainstream school children aged 9-10 years following 12 weeks' supplementation with 558mg/d EPA, 174 mg/d DHA and 60mg/d gamma-linolenic acid (n-6) compared to placebo. However, the authors also identify particular benefits to the children with attention problems within their sample. Similarly, Tammam et al., (2016) have identified that n-3 PUFA supplementation for 12 weeks decreases scores on the Conner's disruptive behaviour scale in typically developing children aged 13-16 years old, but only in those who reported high-misbehaviour at baseline with a subgroup with low-misbehaviour at baseline actually increasing misbehaviour across the trial.

Together, this evidence shows us the impact n-3 PUFAs can have during neurodevelopment and childhood and the importance of n-3 PUFAs in maintaining typical development of the brain. Furthermore, the body of evidence that exists within young samples suggests that supplementation with n-3 PUFAs may be more beneficial in atypically developing children or those with low n-3 PUFA status caused by poor diet and/or are malnourished. As DHA levels in the brain are known to increase during development (Martinez, 1992; Lauritzen et al., 2016) and decrease with ageing (Guisto et al., 2002) this may provide insight into the importance of maintaining brain DHA levels throughout the lifespan. As DHA levels are seen to be ample during periods of neurodevelopment but lowered during periods associated with neurodegeneration, this could be one of the underlying causes of poor brain health in later life, especially when taken together with research that suggests the general population do not meet the recommended intakes of n-3 PUFAs in their diets throughout the lifespan (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018).

1.5.4.2 The Role of n-3 PUFAs in Early Adulthood

A recent systematic review by Derbyshire (2018) identified three RCTs that have examined the roles of n-3 PUFAs on outcomes related to brain health in young adult samples. One was a double-blind trial comprised of 72 healthy, young adults (mean age 20 years) and observed that 2.8g/day of fish oil (1.12g/d DHA + 1.68g/d EPA) for 25 days kept feelings of anger and confusion stable compared to increases in the placebo group (Giles et al., 2015). Another trial in 13 healthy, young adults aged 20-34 year olds provided with varying ratios of DHA and EPA (Bauer et al., 2014a). The study identified that supplementation with an EPA-rich treatment enhanced neurocognitive function via lower levels of neural activation, measured via functional magnetic resonance imaging (fMRI), paired with improvements in reaction times during the Stroop task. The authors therefore interpreted these findings as a potential increase

in neural efficiency. Other research using DHA specifically (1.16g daily) over a longer 6 month span (mean age 33 years) observed that DHA significantly improved episodic memory in young women and the reaction times during working memory tasks in men, indicating that there are observable cognitive benefits in healthy, young adult samples whose diets are habitually low in n-3 PUFAs (Stonehouse et al., 2013). However, findings relating to cognitive outcomes are not unequivocal within young adult samples. For instance, Jackson et al., (2012c) have previously identified no effects on cognitive function following 12 weeks' supplementation with both 1g/d DHA-rich and 1g/d EPA-rich fish oils in healthy, young adults aged 18-35 years. The only finding to note revealed that the EPA-rich supplement may reduce subjective ratings of mental fatigue during time of high cognitive demand.

A body of work also exists within the research area utilising various neuroimaging methodologies to investigate the neurocognitive effects of n-3 PUFAs in healthy, young adults. For instance, Bauer et al., (2011) have identified reduced reaction times during completion of a Stroop and choice reaction task as well as enhanced neural recovery of the magnocellular visual system measured via fMRI. Additionally, Hamazaki-Fujita et al. (2011) have used continuous wave (cw) NIRS to identify that dietary intake of EPA was positively associated with tissue oxygen index (a proxy measure for CBF) in a sample of 54, healthy, young adults aged 20-49 years. Tissue oxygen index was also further associated with arousal level and inversely associated with negative mood measured via the profile of mood state (POMS) questionnaire. Both DHA and EPA were positively associated with arousal level and overall performance in the Uchida-Kraepelin Performance test of mental arithmetic and inversely associated with depression-dejection (POMS). Similarly, Jackson et al., (2012a) identified that both 1g/d and 2g/d DHA for 12 weeks' resulted in significantly increased concentrations of oxygenated haemoglobin and total levels of haemoglobin during completion of cognitive tasks. Additionally, both 1g/d and 2g/d DHA was found to significantly improve reaction times during completion of a choice reaction time task and 2g/d DHA improved reaction times during completion of the rapid visual information processing task.

Three RCTs that examined the roles of n-3 PUFAs on brain health in middle-aged samples have also been highlighted within the recent review by Derbyshire (2018). One 26-week trial providing 2.2g/day of n-3 PUFAs to 50-75 year olds (mean age 62 years) identified significant improvements in the recollection of object locations following supplementation, implying positive effects on memory function (Kulzow et al., 2016). Other research recruiting 65 adults aged 50-75 for 26 weeks showed that 2.2g/day of n-3 PUFAs improved brain structure, namely via white matter microstructural integrity and the volume of grey matter, measured via Magnetic Resonance Imaging (MRI) (Witte et al., 2014). Another trial focusing on adults with

loneliness-related memory problems (mean age 51 years) discovered that n-3 PUFA supplementation (either 1.25g/d or 2.5g/d) over 4 months attenuated verbal episodic memory declines that were linked to loneliness (Jaremka et al., 2014).

Overall, the n-3 PUFA supplementation studies that employ healthy, young or middle aged samples that have been conducted thus far are scarce and healthy young and middle aged samples appear to represent a section of the general population that have been under researched within the literature. Healthy adults may actually represent an interesting population with regards to increased n-3 PUFA intake or supplementation due to the fact that they are seen to fall short of the recommended intake of n-3 PUFAs (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018) and therefore could still potentially benefit from increased n-3 PUFA consumption. Taken together with the natural age-related declines this population may be beginning to experience (Salthouse, 2009), it appears that increased n-3 PUFA intake in otherwise healthy adults may be key in helping to maintain optimal brain health throughout the entire lifespan.

1.5.4.3 The Role of n-3 PUFAs in Neurodegeneration and Older Adults

N-3 PUFAs exhibit neuroprotective properties and represent a potential treatment for a range of neurodegenerative issues, yet, there has thus far been a lack of discrimination between the separate effects of DHA and EPA (Dyall, 2015). Cognitive function is known to naturally decline with age (Salthouse, 2009; 2016; 2019) and has been attributed to a number of factors including increased oxidative damage, decreased membrane fluidity and reduced synaptic plasticity (Willis et al., 2008). Additional widespread changes are characterised during normal brain ageing, such as damage to DNA (Canugovi et al., 2013), mitochondrial dysfunction and alterations in energy metabolism (Ames, 2004) and increased oxidative stress (Perluigi et al., 2014). Age-related structural changes also occur that include altered membrane lipid content (Svennerholm et al., 1997; Egawa et al., 2016) and a reduction in brain volume and weight (Anderton, 2002; Fiford et al., 2017). The ageing brain is susceptible to the development of neurodegenerative diseases, such as AD, compared to the young brain. AD is the most common form of dementia in older adults, with a prevalence of 4.4% in those over 65 years of age (Ward et al., 2012), with prevalence rates being seen to be higher in females than males (Niu et al., 2017) and affects a range of cognitive functions, moods and behaviours (Selkoe et al., 2012). One of the most distinctive features of AD is neurofibrillary tangles, which are largely composed of senile plaques containing amyloid β-protein and aggregates of hyperphosphorylated tau protein (Lloret et al., 2015). The aetiology of AD is still not well

understood, and management is mostly symptomatic, aimed at relieving the cognitive deficits (Selkoe et al., 2012).

A review by Dyall (2015) has identified that a number of studies have explored the associations between blood DHA and EPA levels and the risk of cognitive decline. Higher plasma EPA, but not DHA, has been seen to be associated with lower grey matter atrophy in the amygdala and the hippocampal/parahippocampal areas of community dwellers over 65 (Samieri et al., 2012). Furthermore, higher EPA, but not DHA, has previously been linked with slower cognitive decline (Samieri et al., 2011), lowered risk of dementia (Samieri et al., 2008), and lowered risk of depressive symptoms (Féart et al., 2008) in elderly demographics. Moreover, a meta-analysis identified that although blood EPA, DHA and total n-3 PUFA levels are significantly decreased in patients with dementia, only EPA was significantly lower in patients with pre-dementia syndrome. This suggests that EPA may potentially act as a biomarker for age-related cognitive impairments (Lin et al., 2012). Conversely, a cross-sectional analysis of 1575 dementia-free participants found that it was lower erythrocyte DHA, and not EPA, that was associated with lower scores in tests of visual memory, abstract thinking, executive function and smaller brain volumes (Tan et al., 2012). Dyall et al., (2015) has suggested that these inconsistencies between these observations could potentially be due to the issues of using blood fatty acid levels as a surrogate biomarker for CNS levels.

To date, clinical trials supplementing with n-3 PUFAs in healthy older adults with Mild Cognitive Impairment (MCI) or AD have tended to use EPA and DHA formulations at differing ratios (Jiao et al., 2014) with only a limited number of trials using either DHA- or EPA-rich preparations (Dyall, 2015). One study by Sinn et al., (2012) that directly compared the effects of EPA with DHA in participants with MCI was a 6-month, double-blind, randomised controlled trial, where participants received a supplement high in EPA (1.67g EPA and 0.16g DHA), high in DHA (1.55g DHA and 0.40g of EPA) or high n-6 PUFA (2.2g LA). It was found that in comparison to the LA group, both the DHA- and EPA-rich groups improved their Geriatric Depression Scores significantly. However, only the DHA-rich group had significant improvements in verbal fluency. Generally, where both EPA- and DHA-rich treatments have been investigated in samples of healthy aging, MCI or AD the main focus has been on DHA, which is fairly consistent with the pre-clinical evidence (Dyall & Michael-Titus, 2008) and treatment with DHA does appear to show the greatest promise compared to EPA for improving memory and learning in older populations.

A review by Cederholm, Salem and Palmbald (2013) describes how longitudinal observation studies on fish intake and DHA plasma concentrations in healthy older adults are mostly

positive concerning cognitive health. However, n-3 PUFA supplementation studies in healthy older adults are less conclusive as are studies that supplement n-3 PUFAs to individuals with established AD. This evidence may suggest that supplementation with EPA and/or DHA is not beneficial when administered to the oldest populations or those who have already developed neurodegenerative issues, potentially suggesting that supplementation at this stage of life is too late to ameliorate the effects of brain ageing and cognitive decline. It could be that increased n-3 PUFA intake or supplementation is more beneficial earlier in life and is required in younger populations to help to maintain long-term brain health that lasts into the final stages of the lifespan.

1.5.5 Summary of the Behavioural Effects of n-3 PUFAs

Research that has investigated the effects of n-3 PUFAs on behavioural outcomes in animals has demonstrated that brain depletion of n-3 PUFAs, as a result of removing them from the diet, results in a range of cognitive impairments and behavioural changes that can be ameliorated once n-3 PUFAs are reintroduced into the diet. However, human trials remain far less conclusive. Higher n-3 PUFA status in humans has been associated with beneficial outcomes on a range of mood disorders including depression (Appleton et al., 2010; Deacon et al., 2017) and anxiety (Su, Matsuoka & Pae, 2015) as well as ADHD (Chang et al., 2018), autism (Vancassel et al., 2001; Jory, 2016) and visual impairments (Williams, Birch & Emmett, 2001; Innis, Gilley & Werker, 2001; Hibbeln et al., 2007). The use of n-3 PUFA supplements as a treatment for these issues remains inconclusive. However, there are positive findings from a number of studies that do warrant continued investigation of the impact of n-3 PUFA intake or supplementation in these areas.

The importance of n-3 PUFAs throughout the lifespan and for maintaining brain health has also been shown from the research. The importance of n-3 PUFAs, particularly DHA, *in utero* during brain development is well described along with literature to support the importance of n-3 PUFAs during breastfeeding and infant visual and neural development. Research also appears to show the neuroprotective role of n-3 PUFA status against neurodegenerative disorders, such as cognitive decline in later life. However, research has also shown effects of n-3 PUFAs in early adulthood on cognitive function and increased neural efficiency but unfortunately, studies employing young adults are limited within the research area with this population being largely ignored. Additional research into the effects of n-3 PUFA intake and supplementation in healthy, young adult populations would be beneficial to the research area as the population in general is seen to fall short of the recommended dietary intakes of n-3 PUFA (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018). These factors, combined

with some limited findings from n-3 PUFA intervention studies in older populations and those already suffering with AD (Cederholm, Salem & Palmbald, 2013), might suggest that dietary interventions that aim to prevent neurodegeneration, brain ageing and cognitive decline may potentially not be beneficial in older samples and are being administered too late in the lifespan. Indeed, previous research has already shown the beneficial impact of people who frequently engage in mentally stimulating activities (e.g., learning, reading, or playing games) from young adulthood throughout their life, in relation to decreased likelihood of developing dementia (Yates et al., 2016). Thus, providing evidence for the impact of maintaining good brain health in early adulthood and the effects in later life. Therefore, it could be that n-3 PUFA supplementation is more beneficial earlier in life and is required in younger populations to help to maintain long-term brain health that lasts into the final stages of the lifespan. For these reasons, it appears that nutritional intervention studies supplementing with EPA and DHA that investigate a range of outcomes related to brain health are warranted in healthy, younger-adult populations.

1.6 Previous Limitations of Randomised Controlled Trials

1.6.1 Issues with n-3 PUFA Supplements and Bioavailability

There are several aspects that should be considered with n-3 PUFA supplementation before commencing human trials, most of which centre around absorption and bioavailability. Bioavailability refers to the extent by which a nutrient can be absorbed and transported to systemic circulation or the site of physiological activity (Schuchardt & Hahn, 2013). The bioavailability of EFAs has been regarded as of minor importance previously. However, it has been shown that the absorption of PUFAs is a much more complicated process than previously recognised and is subject to considerable variability (Schuchardt & Hahn, 2013). N-3 PUFA supplements are commonly in triglyceride form (TAG; natural sources typically derived from fish oil), free fatty acid form (FFA), or in ethyl ester form (EE), with both of the latter forms being derived from natural sources of TAG fish oil (Ghasemifard, Turchini & Sinclair, 2014). Some evidence suggests that TAG formulations can be utilised more effectively than EE (Lawson & Hughes, 1988; Beckermann et al., 1990; Hansen et al., 1993). However, factors such as dose, number of participants, study duration, and measurement parameters make it difficult to compare the findings from different studies.

1.6.1.1 Increasing the Bioavailability of n-3 PUFA supplements

The bioavailability of EPA and DHA can also be influenced by the food matrix with which they are consumed. Poorly water-soluble components such as n-3 PUFAs, show slow nutrient dissolution rates and poor absorption in the gastrointestinal tract when administrated orally. In

general, taking n-3 PUFA capsules with a meal is suggested, mainly because they are consequently more easily tolerated than when ingested on an empty stomach (Schuchardt & Hahn, 2013). The composition of the meal that the supplements are taken with, in particular its fat content, has previously been found to affect the bioavailability of n-3 PUFAs significantly. A study by Lawson and Hughes (1988) identified that three times more n-3 PUFAs as EE were absorbed when fish oil capsules were ingested with a high-fat meal than when taken with a low-fat meal. This effect was seen to be even more pronounced in the ECLIPSE studies (Kling et al., 2011; Davidson et al., 2012), which demonstrated that the bioavailability and uptake of n-3 PUFAs as EE and even FFA increased significantly when the capsules were ingested with high-fat meals. This positive effect on bioavailability and uptake of the fatty acids may be due to the stimulating effect of fat on the release of pancreatic lipases used to break down dietary fat molecules (Schuchardt & Hahn, 2013).

In addition to the food matrix with which supplements are consumed, several groups have also shown that emulsification of n-3 PUFAs can positively impact bioavailability (Garaiova et al., 2007; Müllertz et al., 2010; Wakil et al., 2010). This can be performed by applying a self-micro-emulsifying delivery system (SMEDS), containing a mixture of oils, surfactants, and co-solvents, which are spontaneously emulsified in the stomach and gastro-intestinal tract. SMEDS have attracted interest mainly because a drug containing (micro)-emulsion will result in a large surface area of the contents upon dispersion in the gastrointestinal tract. The emulsions further aid the absorption of the drug due to a faster digestion by gastrointestinal enzymes or via possible absorption directly from the emulsion particles (de Smidt, Campanero & Trocóniz, 2004). Lipid-based drug delivery systems, particularly SMEDS, offer a promising approach for improving the bioavailability of poorly soluble compounds, such as n-3 PUFAs. The mechanisms behind this improvement is attributed to a range of factors that include: easier partition of the drug into the mixed micelles believed to facilitate drug absorption, increased bile secretion, transporters systems and increased intestinal permeability, stimulation of lymphatic transport and modulation of enterocyte-based enzymes (Müllertz et al., 2010). Indeed, West et al., (2018) have specifically compared SMEDS formulated EPA- and DHA-rich to standard EE EPA- and DHA-rich oils identifying significantly higher EPA and DHA in plasma both 24 hours and 12 weeks after supplementation the SMEDS oils. These findings support the use of SMEDS formulated EPA and DHA oils in future interventions in an attempt to increase uptake and bioavailability of the PUFAs across the supplementation period.

1.6.1.2 Oxidation in n-3 PUFA supplements

N-3 PUFA oils are also known to be highly susceptible to oxidation and there has been concerns about the safety of oxidised fish oils since the 1950s (Matsuo, 1954). Although there is evidence that over-the-counter supplements are frequently oxidised, this has had no impact on the requirements for storage and labelling or on the design of human clinical trials. It is currently still unclear to what degree the oxidation of fish oils influences their efficacy or harm in humans (Albert et al., 2013). The rate of lipid peroxidation in supplements can be influenced by light, heat and oxygen concentration even at normal room conditions. Moreover, even oil stored in the dark at 4^{o}C can oxidise unacceptably within a month of storage (Pak, 2005). However, although not able to fully prevent oxidation, added antioxidants are known to reduce the oxidation process in n-3 PUFAs (Zuta et al., 2007). Antioxidants are generally classified as primary or secondary, based on their mechanisms of action (Reische, Lillard & Eitenmiller, 2008). Primary antioxidants or chain-breaking antioxidants are free radical scavengers that delay or inhibit the initiation step, or interrupt the propagation of autoxidation (Zuta et al., 2007). One commonly used primary antioxidants in foods is vitamin E (tocopherols). Tocopherols are naturally occurring phenolic compounds that can be found in a variety of plant-derived foods and are therefore of much interest as antioxidants because they are perceived as “natural”, with the antioxidant activity of tocopherols being due to their ability to donate their phenolic hydrogens to lipid free radicals (Kamal-Eldin & Appelqvist, 1996; Azzi, 2018). Various studies have reported the protective effects of α-tocopherols on oils (Fuster et al., 1998) with some studies reporting the stabilising effects on PUFA rich oils (Yuki & Ishikawa, 1976).

As a result of the high susceptibility of n-3 PUFA oils to oxidation, one factor that may have contributed to the mixed results within previous n-3 PUFA RCTs could potentially be the overlooked oxidation status of the supplements. For instance, Albert et al., (2013) describes how it must be recognised that the oxidative status of trial oils may have contributed to the previous mixed results if oxidized oils are less efficacious or may even potentially cause harm, for example, by contributing to the advancing of atherosclerosis. In light of the information on the high susceptibility of n-3 PUFAs to oxidation there seems a definite need in RCTs to actively prevent this process, ideally via ensuring that tocopherols are added into the supplements and proper storage is utilised in an attempt to stabilise the oils over the research period and maximise their efficacy throughout the trials.

1.6.1.3 Time of Dosage

The bioavailability of EPA and DHA may also be influenced by the time of dosing (Bray & Young, 2011). Gooley and Chua (2014) describe how circadian timing plays a key role in lipid metabolism, when synched with solar-cycles, and ensures daily rhythms in the absorption, transport and storage of lipids. At a cellular level, genes involved in fatty acid oxidation and lipid synthesis are activated and repressed by core clock proteins and consequent loss of the clock gene function results in impaired lipid homeostasis (Gooley & Chua, 2014). Concerning lipid metabolism, collective data from animal studies appears to suggest that under normal light-dark conditions the peak time for lipid absorption and digestion occurs during the very beginning of the active/awake phase (Bray & Young, 2011), suggesting that dosing of n-3 PUFAs should happen at this time. However, given the resistance of all long chain PUFA to intestinal lipases; it could be that a night-time dosing would be more appropriate, so that the fatty acids are at the site of action in the intestines at the time of peak digestion and absorption the following morning. Given the aforementioned efficacy of SMEDS formulations, it may be that a combination of both night-time dosing and SMEDS formulations will increase the fatty acid absorption of n-3 PUFA supplements. Moreover, night time supplementation with DHA, in particular, may acutely affect membrane composition of newly formed membranes during the process of neurogenesis (Beltz et al., 2007), known to occur during inactive/sleep phases (Lucassen et al., 2010), which is critical for the process of memory consolidation (Yamashima, 2012).

Intervention studies employing a wide range of nutritional supplements have previously identified the importance of the time of supplementation with Haider and Bhutta (2017) showing differences between the timing of iron supplementation within pregnant women, with a significant reduction in perinatal mortality observed when supplementation occurred after 20 weeks of gestation as opposed to before 20 weeks. Additionally, Tipton et al., (2001) has identified that supplementation with an amino acid solution immediately before an acute bout of resistance training was seen to increase muscle protein synthesis compared to consumption immediately after the training, with Candow et al., (2006) also similarly showing that supplementation with protein immediately before resistance training was more effective for inducing hypertrophy of knee extensors than when consumed after the resistance training over a period of 12 weeks. Blumsohn et al., (1994) has also described how bone resorption in humans shows a circadian rhythm with a peak in the early morning, similar to the lipid absorption and digestion rhythm (Bray & Young, 2011), showing that night-time calcium supplementation was beneficial for bone resorption but showed no effect when taken in the morning. Recently, Jackson et al., (unpublished data; Appendix IX) have described the diurnal

variations of both EPA and DHA in a sample of healthy adults showing that circulating levels of these fatty acids within blood plasma do indeed appear to follow a diurnal pattern, falling during the overnight period and reaching their lowest point in the early morning. The authors suggest the potential for significant functional effects of consuming n-3 PUFAs at night time to counteract these natural decreases in fatty acid levels within the body. Upon investigation, Jackson et al., (unpublished data; Appendix X) observed that, when dosed at night time (22:00hr), there was a significant increase in DHA in blood plasma for 6 hours post dose compared to placebo, but in contrast, concentrations of EPA in plasma had a similar peak but were also significantly greater for the entire 24 hour period compared to placebo. The evidence from a range of nutritional intervention studies highlights the importance of correctly timing the intake of the supplements and Blumsohn et al., (1994) and Jackson et al., (unpublished data; Appendix X) specifically show the importance of night-time supplementation in circadian rhythms with peak absorption in the morning. In light of this evidence it appears that night-time supplementation may be beneficial for the increased efficacy of supplementation with n-3 PUFAs and should be considered in future studies in an attempt to maximise the absorption of the fatty acids.

1.6.2 Comparisons between DHA and EPA

Comparisons between, and the potentially separate effects of, DHA and EPA are often underreported or overlooked in the research area as a whole, with Drouin et al., (2019) describing how the full extent of both the separate and similar effects of DHA and EPA are still poorly understood. A recent systematic review on cardiometabolic risk factors in RCTs by Innes & Calder, (2018) describes how the majority of clinical trials that have been conducted thus far have been focused on administering both DHA and EPA together with only a small number of comparator trials existing within the literature, concluding that DHA and EPA actually appear to have different effects on cardiometabolic risk factors but further investigation in larger clinical studies is required. Additionally, Dyall (2011) has previously reported that a wide variety of enriched oil preparations have been used in human and animal studies, with earlier research tending to group ALA, EPA and DHA into one broad n-3 PUFA classification. However, clinical trials and recent reviews of the literature have actually begun to strongly refute this idea (Gorjão et al., 2009; Cottin, Sanders & Hall, 2011; Alexander et al., 2017; Innes & Calder, 2018; AbuMweis et al., 2018; Drouin et al., 2019), suggesting that these fatty acids can no longer be considered to have mechanistic equivalence and should be viewed as distinct, albeit related, chemical species (Dyall, 2011). As a results, a greater understanding of the individual roles of the n-3 PUFAs on brain health and function are still required (Dyall, 2015). As the ratios of EPA and DHA are seen to vary widely in different types

of fish in nature (Osman, Suriah & Law, 2001; Özogul, Özogul & Alagoz, 2007; Mohanty et al., 2016), it also means that dietary intake of EPA and DHA can vary widely. As the potentially separate effects of EPA and DHA or diets higher in EPA or DHA are not widely understood, it appears that there is a distinct requirement within the research area for studies that are capable of measuring and comparing the potentially independent effects of various formulations of n-3 PUFA supplements concerning the ratios between EPA and DHA. Therefore, studies that supplement with both EPA-rich and DHA-rich oils that aim to assess aspects of brain function, physiology and behaviour are still required within the literature to provide a greater understanding of the importance and effects of both EPA and DHA.

1.6.3 Sample Populations

A large majority of previous n-3 PUFA supplementation research focusses heavily on either prenatal, postnatal, children or older populations and quite often research uses clinical samples, putting very little emphasis on healthy, young and middle-aged adult populations (Derbyshire, 2018). Therefore, the effects of n-3 PUFA supplementation in this population are not actually well understood or described within the literature. These samples are of particular interest as research suggests that intake of n-3 PUFAs does not meet the recommended dose in the population in general (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018). As a result, healthy, young adults in general are likely be deficient in regards to their n-3 PUFA intake but otherwise healthy. This provides the opportunity to observe the effects of n-3 PUFA supplementation in a population that should require additional n-3 PUFA in their diets but report no other issues in relation to health or cognitive impairments. This effect of supplementation in deficient samples that are otherwise healthy is comparable to studies that investigate the effects of multivitamins on cognitive or physiological parameters as evidence also suggests that a considerable percentage of the general population of developed countries are deficient in one or more vitamins and minerals (Henderson et al., 2004; Kennedy & Haskell, 2011; Kennedy, 2016). For instance, Kennedy et al., (2016) has previously described how acute and chronic supplementation with micronutrients in healthy, young adults can modulate cerebral blood flow and metabolic parameters during cognitive task performance, suggesting that metabolism and brain function are responsive to micronutrient supplementation even in adults who have a nutritional status typical of the general population and who are otherwise healthy. As n-3 PUFAs have been observed to be crucial in a wide range of cellular processes and have links to a number of psychological and physiological functions, it stands that healthy, young adult, low consumers of fish should still benefit from increased intake of n-3 PUFAs.

Additionally, this sample is also of particular interest as research suggests that they may reportedly be undergoing natural age-related decrements in both overall cerebral blood flow, their ability to mobilise additional local blood flow during neural activity (the haemodynamic response) and overall cognition, that may begin in the 20's (Salthouse, 2009). Indeed, recent studies have reported that normal cognitive ageing is characterised by almost linear declines, from early adulthood, in measures of speed, and accelerated declines in memory and reasoning (Salthouse, 2016; Salthouse, 2019). Among the variables that have been found to exhibit nearly continuous age-related declines in cross-sectional comparisons beginning when adults are in their 20s are: measures of regional brain volume (Sowell et al., 2003; Allen et al., 2005; Fotenos et al., 2005; Kruggel, 2006; Pieperhoff et al., 2008), myelin integrity (Sullivan & Pfefferbaum, 2006; Hsu et al., 2008), cortical thickness (Magnotta et al., 1999; Salat et al., 2004), serotonin receptor binding (Volkow et al., 2000; Erixon-Lindroth et al., 2005), accumulation of neurofibrillary tangles (Del Tredici & Braak, 2008) and concentrations of various brain metabolites (Kadota et al., 2001). Currently, many n-3 PUFA interventions that aim to slow brain ageing and cognitive decline are implemented in older populations. However, the research evidence seems to suggest that neurobiological and cognitive changes actually begin far earlier in life, and so studies restricted to samples of older adults might have limited value for discovering the causes of a phenomenon that originated decades earlier, leading to the need for further investigation into young adult samples. Therefore, research into healthy, young adult populations may offer additional insights into when the optimum time is to implement interventions designed at preventing or reversing age-related declines (Salthouse, 2009). These factors, combined with some limited findings from n-3 PUFA intervention studies in older populations and those already suffering with AD (Cederholm, Salem & Palmbald, 2013), might suggest that dietary interventions that aim to prevent neurodegeneration, brain ageing and cognitive decline are not beneficial in older samples as they are being administered too late in the lifespan. It could be that n-3 PUFA supplementation is more beneficial earlier in life to help to maintain both long and short-term brain health that lasts into the final stages of the lifespan. Therefore, if RCTs can identify effects of n-3 PUFAs on brain function in healthy, young adults then it would be reasonable to assume that these effects will result in lower brain ageing later in life and could lead to the effects of n-3 PUFAs across the lifespan to be confirmed via longitudinal studies.

Overall, healthy, young adults appear to be a specific population that is largely ignored within the research area and should still benefit from increased n-3 PUFA intake or supplementation due to them falling below the recommended dietary intake. For these reasons, nutritional intervention studies employing both EPA-rich and DHA-rich supplements, representative of

the varying ratios that are seen in food sources, that investigate a range of outcomes related to overall brain health are warranted in healthy, young adult populations.

1.7 Rationale

The evidence outlined throughout this review of the literature shows the depth and breadth of knowledge regarding both the physiological and behavioural effects of n-3 PUFAs. The available evidence explains how these fatty acids are incorporated into every cell in the body and can only be acquired via the diet establishing their essential nature, with inadequate intake of the EFAs resulting in significant impacts to many physiological and behavioural parameters. However, previous RCTs within the literature are seen to be limited by several methodological issues including issues with n-3 PUFA treatments, such as formulations, bioavailability and sample populations, as well as often ignoring certain factors such as the time of supplementation, the stability of the oils in terms of oxidation and a lack of comparisons between the independent effects of DHA and EPA or the effects of diets higher in EPA or DHA.

One particular population that has been largely overlooked is healthy, young adults; very few data currently exist regarding the effects of n-3 PUFA supplementation on cognitive function, mood and neurophysiological measures such as cerebral blood flow in healthy, young adults. In addition, even less data currently exists in this population concerning the effects of n-3 PUFAs on sleep parameters and memory consolidation and in particular how both of these factors may be potential mediators in the relationship between n-3 PUFA status and cognitive function. Given the evidence provided throughout this literature review regarding the fundamental nature of n-3 PUFAs and adverse correlates of low intake, a population of healthy, young adults offers potential insights into the effects of n-3 PUFA supplementation in otherwise healthy populations whilst also providing information concerning when the most appropriate time to supplement with n-3 PUFAs may be in terms of maintaining brain health across the lifespan. Should positive behavioural or physiological changes be observed in this population then these results may offer insights into the early prevention of age-related cognitive decline as well as inform dietary advice. Taken together, there seems a clear need for research that investigates the effects of n-3 PUFA supplements on aspects of cognition, mood, neurophysiological measures such as cerebral blood flow, sleep and memory consolidation in healthy, young adults, whilst also taking the bioavailability, formulations, time and type of supplementation into account.

To this end: Chapter 3 will investigate the effects of two SMEDS formulated n-3 PUFA supplements (DHA-rich and EPA-rich), containing traces of the stabilising antioxidants

tochopherols (vitamin E) and ascorbyl palmitate (vitamin C), dosed at bed time, on aspects of cerebral blood flow and cognition in healthy, young adults aged 25-49; Chapter 4 will investigate the effects of the same SMEDS formulated supplements, dosed at bed time, on both objective and subjective sleep parameters in healthy, young adults aged 25-49; Chapter 5 will investigate the effects of the same SMEDS formulated supplements, dosed at bed time, on aspects of learning and memory consolidation in healthy, young adults aged 25-49; and Chapter 6 will investigate the effects of the same SMEDS formulated supplements, dosed at bed time, on aspects of cognition and mood in healthy, young adults aged 25-49. This thesis will therefore aim to address the following research questions:

1. What are the effects of night-time dosing with SMEDS formulated DHA-rich and EPA-rich supplements on neurophysiological parameters in healthy, young adult populations?

2. What are the effects of night-time dosing with SMEDS formulated DHA-rich and EPA-rich supplements on measures of sleep in healthy, young adult populations?

3. What are the effects of night-time dosing with SMEDS formulated DHA-rich and EPA-rich supplements on memory consolidation in healthy, young adult populations?

4. What are the effects of night-time dosing with SMEDS formulated DHA-rich and EPA-rich supplements on cognition and mood in healthy, young adult populations?

The studies that comprise this thesis will include the first investigations employing the methodological paradigm of SMEDS formulated oils, containing traces of the stabilising antioxidants tochopherols (vitamin E) and ascorbyl palmitate (vitamin C), dosed at bedtime, whilst comparing the effects of both DHA- and EPA-rich supplements. Furthermore, investigations into the effects of n-3 PUFA supplements on memory consolidation are novel to the research area.

CHAPTER 2. GENERAL METHODOLOGY

2.1. Overview of studies

Throughout the thesis four randomised, placebo-controlled, double-blind, parallel groups design studies were conducted. Although the data from the studies that comprise this thesis were analysed separately and each study had individual research questions, all participants were enrolled into the study presented in Chapter 6, as well as one of the other studies presented in either Chapter 3, 4 or 5 (Figure 2.1).

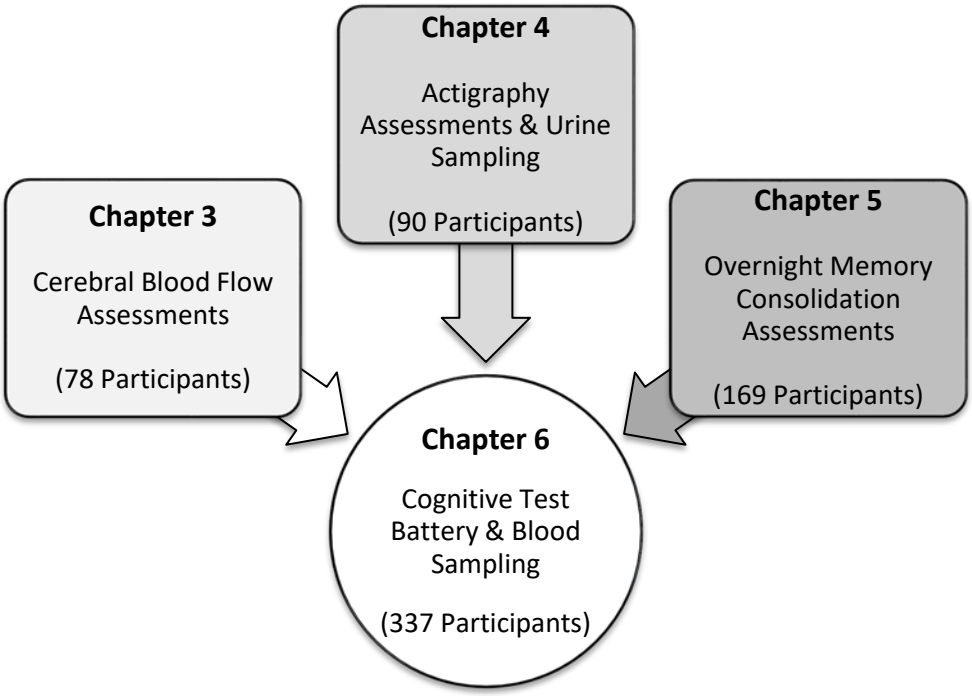

Figure 2.1. Diagram displaying how the studies were conducted throughout the thesis. All participants completed the requirements of the study presented in Chapter 6 in conjunction with the requirements of one of the additional studies presented in either Chapter 3, 4 or 5.

The two studies that each participant was enrolled on were run parallel to one another and this was made possible due to the similarities between the four separate studies concerning inclusion criteria, sample age, study duration, treatments provided and the short length of testing visits. To be eligible to participate in any of the studies participants had to consent to take part in both the study presented in Chapter 6, as well as, one of the additional studies presented in Chapter 3, 4 or 5. This was made fully clear to all participants and the individual requirements for each study were completed alongside one another, during the same visit to the lab, if possible, in an attempt to minimise the amount of times each participant was required to visit the lab. All participants across all studies completed a screening visit, a baseline testing

visit, a week 13 treatment refill and a 26 week post-supplementation visit. In addition to this participants enrolled in the studies presented in Chapter 4 and 5 also had two extra visits to the lab to collect either actigraph watches and urine sampling equipment or tablet computers respectively, in order to complete the study specific requirements. The approximate time required to attend the lab by participants, regardless of which studies were completed, was around 6 hours across the 6 month supplementation period. As the data from each of the respective studies was analysed independently and as each of the studies attempted to answer separate research questions, they will be reported as four separate studies throughout Chapters 3, 4, 5 and 6 of the thesis. All studies were pre-registered via www.clinicaltrials.gov with the following identifiers NCT03158545 (Chapter 3), NCT03559361 (Chapter 4), NCT03592251 (Chapter 5) and NCT02763514 (Chapter 6).

2.2. Treatments

Across all experimental chapters, participants were allocated to a single treatment throughout the course of the study according to a randomisation schedule. Treatments commenced on the night of each of the first respective testing visits (Baseline) and finished the night before the final testing visit (Week 26). As it is known that the bioavailability of n-3 PUFAs can be influenced by the food matrix with which they are consumed and due to the poorly water-soluble nature of n-3 PUFAs, they show slow nutrient dissolution rates and poor absorption in the gastrointestinal tract when administrated orally. Therefore, the active treatments used throughout the studies that comprise this thesis contained SMEDS formulations. In addition to the SMEDS formulation and due to the resistance of all long chain PUFA to intestinal lipase, participants were also instructed to take their capsules with a glass of water at their usual bed time, so that the fatty acids should be present in the intestines at the time of peak digestion and absorption the following morning. Treatments were consumed as three separate capsules taken each night before the participant went to bed. The breakdown of each of the treatments is provided in Table 2.1.

Table 2.1. Breakdown of the quantities of DHA and EPA in each of the three treatments. Quantities of DHA and EPA are presented as the total quantities from consuming three capsules.

Treatment (3 x 1g capsules)	DHA (mg)	EPA (mg)
Olive Oil Placebo	0	0
DHA-rich	900	270
EPA-rich	360	900

2.3. Participants

For the reasons outlined in section 1.6.3, all participants that were enrolled onto the studies that comprise this thesis were aged between 25-49 years and had to pass a physical/lifestyle screening to demonstrate they were in good health. Participants were self-reported low consumers of fish, measured via the DHA food frequency questionnaire (Benisek et al., 2002; Appendix I), reporting consumption of oily fish less than once per week. Having good health was identified as being; a non-smoker, free from prescription, herbal, illicit or recreational drugs (females taking the contraceptive pill were included), free from major illnesses, having a blood pressure lower than 159/99 and a BMI between 18.5 and 35. All participants were recruited via posters, adverts placed on social media websites or emails sent out to university staff and students and were either students or staff attending/working at Northumbria University or individuals living in the Newcastle-upon-Tyne surrounding area. Descriptions of how each sample size was determined via a priori calculations and accounting for any potential dropouts will be provided within the method section of each experimental chapter.

2.4. Blinding and Randomisation

To ensure blinding was maintained throughout the study a 3rd party researcher within the same university created a randomisation schedule. This schedule randomly assigned all 337 subject ID numbers evenly to either a placebo, DHA-rich or EPA-rich treatment. These treatment groups were then relabelled to either A, B or C and only the 3rd party researcher had access to the information which linked these letters to the respective treatment group. Upon delivery, all treatments were received by the same 3rd party researcher who removed any identifying information from the boxes containing the three separate treatments and relabelled said boxes with A, B or C according to their respective treatment code. Once the boxes containing the treatments had been relabelled as A, B or C and all other identifying materials had been removed they were then delivered to the researcher along with the randomisation schedule assigning each subject ID number to receive treatment from either A, B or C. The researcher then labelled the individual treatment bottles within each box with a subject ID (1-337) according to the randomisation schedule, information explaining how participants should consume the treatments and an expiry date then placed back into the respective boxes. Once labelling was completed, the principle supervisor checked that the subject ID's within each box correctly corresponded with the randomisation schedule. Treatment bottles were then assigned to participants in sequence as they enrolled onto the trials and until all 337 subject ID's had been dispensed. The researcher was only un-blinded with information regarding

which treatment A, B and C corresponded to once all analyses had been completed to allow for interpretations of the data to begin and for the study reports to be completed.

2.5. Blood Sampling & Analysis

Blood samples for all studies conducted throughout the thesis were collected via venepuncture into ethylenediaminetetraacetic acid (EDTA) vacutainers (6ml). The samples were stored in an ice box or at 5°C until they could be processed and were processed within 4-8 hours of collection. The samples containing whole blood were spun in a centrifuge at 2000 rpm (913 x g) for 10 minutes at room temperature. Following this, the top layer of plasma was removed using a plastic pastette and discarded. Once the plasma has been removed 1ml of red blood cells were collected from the bottom of the vacutainer and transferred into a 15ml centrifuge tube and made up to 15ml with phosphate-buffered saline (PBS). The mixture was then inverted and spun in the centrifuge at 1200 rpm (350 x g) for 10 minutes at room temperature with a low break. The PBS was then removed with a pastette and then this washing process was repeated for a second time. After the second wash the remaining blood cells were transferred into two labelled 1.5ml microtubes and immediately frozen at -80^{0}C prior to analysis which was carried out at Southampton University.

Following the guide created by Burdge, Cawood and Childs (2004) samples were analysed via Gas Chromatography (GC). The main principle underlying the separation of fatty acids by GC is that they differ in the temperature at which they become volatile depending upon carbon chain length, number and position of the double bonds. Increasing chain length results in an increase in the temperature at which fatty acids enter the vapour phase. During preparation the GC analysis 1.0ml of blood cells were pipetted into a screw-cap glass tube and 5ml of 0.9% sodium chloride (NaCl) mix was added before the sample was then inverted and placed in a centrifuge at 2,000 rpm. Following this supernatants were removed with a Pasteur pipette and discarded. This processes was repeated twice. Following this 1.0ml of 0.9% NaCl was added, vortex mixed and then spun in a centrifuge at 2,000 rpm for 10 minutes with a low break at room temperature before the lower phase was collected by aspiration with a Pasteur pipette and transferred to a screw-cap glass and dried under nitrogen at 40^{o}C. Lipid classes were then separated by solid phase extraction (SPE) and eluted phosphatidylethanolamine (PE) was collected. GC was then completed on the PE following the stages outlined in section 5 of the guide produced by Burdge, Cawood and Childs (2004).

2.6. Cognitive Assessments

All cognitive tasks conducted throughout the studies that comprise this thesis were delivered using the Computerised Mental Performance Assessment System (COMPASS, BPNRC, Northumbria University, Newcastle Upon Tyne, UK). This testing system is capable of delivering a tailored collection of tasks, with fully randomised parallel versions of each task being delivered during each assessment for each participant. COMPASS currently comprises over 20 cognitive tasks; many based on classic cognitive tasks, which measure attention, vigilance, working-, spatial- and secondary memory, executive function, mood and tasks which induce cognitive demand. COMPASS has been used within hundreds of published journal articles evidencing its sensitivity to a range of nutritional interventions.

2.7. Treatment Guess Questionnaire & Compliance

As each participant was provided with 600 treatment capsules throughout the supplementation period of each study, a treatment compliance percentage could be calculated for all participants in order to measure adherence to the study protocol concerning appropriate consumption of the study treatments. This treatment compliance percentage was calculated by comparing the number of treatments that were actually returned by each participant at the end of the study with the number of treatments that should have been returned. Therefore, compliance percentages were calculated as:

$$\text{Treatment Compliance (\%)} = \frac{\text{Number of treatments returned (not consumed)}}{\text{Number of treatments that should have been consumed (Days enrolled in study x 3)}} \times 100$$

Additionally, at the end of each respective study all participants were provided with a treatment guess questionnaire (APPENDIX II) and asked to choose between whether they had received an active or placebo treatment throughout the supplementation period, in an attempt to verify the blinding procedure. Responses from the treatment guess questionnaire were analysed via Chi-square test comparing the number of correct and incorrect responses given by each treatment group.

2.8. Data Cleaning Procedures

Before each analysis was conducted throughout the thesis the data sets were cleaned following the same procedures. These procedures included removing anomalous results and outliers from the raw data. All analyses were completed using SPSS (version 25) and box plots were generated for each outcome variable to identify potential outliers. These boxplots present five sample statistics - the minimum, the lower quartile, the median, the upper quartile and the maximum. SPSS has a two stage flagging process. Values which are between one and a half and three box lengths from either end are denoted by open circles and are interpreted as outliers. Values which are more than three box lengths from either end of the box are denoted by asterisks and interpreted as extreme values. Once any identified outliers had been removed, residual values were calculated and histograms produced to view the spread and distribution of the data. If any values were seen to be separate from the spread and distribution from the dataset then these values were also removed. Once these processes had been completed for each outcome variable the analysis commenced.

2.9. Linear Mixed Models Analysis

All analyses conducted throughout this thesis were from the intention-to-treat sample not the per-protocol sample. Intention-to-treat analysis is a comparison of the treatment groups that includes all patients as originally allocated after randomisation and is the recommended method in superiority trials to avoid any bias (Boutis & Willan, 2011). Throughout the thesis it was also decided that the most appropriate way to analyse the repeated measures data by treatment group was via linear mixed models (LMM). LMMs have several advantages over using repeated measures ANOVAs or ANCOVAs including the ability to accommodate missing data points often encountered in longitudinal datasets and the ability to model nonlinear, individual characteristics (Krueger & Tian, 2004). Therefore, data were analysed using the mixed models procedure in SPSS, unless another analysis is stated otherwise. For each model that was run the covariance matrix structure of said model was chosen based on the structure that produced the lowest Schwarz's Bayesian Criterion (BIC), an indication of the best fitting model for the data (Drton & Plummer, 2017). The fixed factors and covariates appearing in each model will be reported within each of the respective Chapters. However, for the analysis of the bloods data, an analysis for all participants is provided within Chapter 6, whilst analyses of the bloods data for the sub groups which formed the samples within Chapters 3, 4 and 5 are reported in Appendix XI.

2.10. Analysis between Treatments and Multiple Comparisons

The primary analysis throughout this thesis was focused on placebo comparisons with both the DHA- and EPA-rich treatments. However, the methodological design of each of the studies also offers the opportunity to directly compare the potentially separate effects between the DHA- and EPA-rich treatments, a factor that is often overlooked within the previous research. Therefore, comparisons between placebo and the active treatment groups, as well as, comparisons between the two active groups will be reported in each of the respective Chapters results sections. All post-hoc analyses reported throughout the thesis are Sidak corrected comparisons to counteract the problem of multiple comparisons.

CHAPTER 3. OMEGA-3 INDEX IS ASSOCIATED WITH CORTICAL BLOOD FLOW AND NEURAL EFFICIENCY DURING SERIAL SUBTRACTION TASKS

3.1 Introduction

Reviews of the literature have identified positive associations between levels of DHA in plasma phospholipids and erythrocytes and greater performance on tasks of visual memory, abstract skills, executive function as well as increased total cerebral volume (Cederholm and Salem, 2013). Additionally, Sinn et al., (2012) have previously identified significant benefits of 6 months' n-3 PUFA supplementation, predominantly with a DHA-rich oil, for both cognition and mood, in older adults with mild cognitive impairment leading to the hypothesis that such benefits might possibly be attributable to improvements in CBF. It appears reasonable, that these relatively swift changes in brain functioning, following just 6 months dietary supplementation, that have been observed in adults following n-3 PUFA supplementation were more likely to be a result of effects on the microvascular endothelium rather than on neuronal function and/or structural changes (Kuszewski, Wong & Howe, 2017). Furthermore, Wong, Evans and Howe (2016) have identified inverse correlations between markers of cerebrovascular compliance, vasodilator responsiveness and cognitive performance in postmenopausal woman using transcranial Doppler ultrasound (TCD) as a non-invasive surrogate measure of cerebrovascular function. The impact n-3 PUFAs could have on CBF is therefore suggested to underpin, at least in part, the relationship between n-3 PUFAs and cognition.

Augmented brain activity is known to result in an increased consumption of adenosine triphosphate (ATP), oxygen and glucose as well as an increased release of the neurotransmitters glutamate and GABA within the brain (Pasley & Freeman, 2008). These changes then influence the vasoactive chemical agents K+, nitric oxide (NO) and adenosine which leads to increased CBF to increase the delivery of oxygen and glucose to the sight of activation (Pasley & Freeman, 2008). This mechanism is known as neurovascular coupling. Under normal conditions, local CBF is related to the metabolic activity of specific brain regions and this neurovascular coupling relationship forms the basis of the signal changes used in certain neuroimaging techniques including fMRI and NIRS to localise brain activation in regions associated with specific cognitive tasks (Wong et al., 2019). Increased consumption of the n-3 PUFAs DHA and EPA may help to support the neurovascular coupling process via several cardiovascular processes. Evidence obtained from both human and animal studies suggests that n-3 PUFAs affect numerous cardiovascular processes including the promotion of vasodilation via relaxation of smooth muscle cells, delayed development of plaques,

improved endothelial function, antioxidant and anti-inflammatory properties, decreased stiffening of arterial walls as well as decreasing blood pressure in hypertensive patients (Colussi et al., 2016). Given these observed effects on cardiovascular parameters, it seems only logical that previous observations of the effects of n-3 PUFAs on brain function, cognition and overall brain health may be underpinned via modulation of cerebral haemodynamics via promotion of healthy regulation of neurovascular coupling. Indeed, Howe et al., (2018) have used TCD to measure cerebrovascular function in a sample of 38 mildly hypertensive older adults following 20 weeks' supplementation with 1600mg/d DHA and 400mg/d EPA compared to a corn oil placebo. The authors report significant increases in neurovascular coupling in males, measured as the cerebral vascular response to cognitive stimuli, correlated with an increase of EPA in erythrocytes, as well as increased cardiovascular responsiveness in females. However, these effects were found not to be paired with any significant improvements in cognitive function or mood.

The complex relationship between n-3 PUFA supplementation, CBF and cognition has also been investigated in healthy samples several times previously during nutritional intervention studies. For instance, McNarama et al. (2010) conducted an 8 week randomised, placebo-controlled, double blind intervention study with 3 treatment arms (placebo, 400 mg/d DHA, 1200 mg/d DHA) on a sample of 33 healthy male children (age^{m} 8.98 years). fMRI was employed to assess relative changes in cortical activation during a sustained attention task. Significantly greater changes from baseline in activation of the dorsolateral prefrontal cortex (PFC) were observed in both DHA groups compared to placebo and the high-dose and low-dose DHA groups had greater decreases in the cerebellar cortex and occipital cortex respectively. Erythrocyte DHA composition was also positively correlated with dorsolateral PFC activation and negatively correlated with reaction time. These findings provide support for the role of dietary intake of DHA as a modulator of cortical activity in healthy samples. However, the authors do recognise that an 8 week supplementation period is relatively short along with a sample size that is small and therefore future studies in this area must consider larger and longer controlled imaging studies.

Similar to McNamara et al., (2010), the findings presented by Hamazaki-Fujita et al. (2011) suggest that EPA alone may modulate the oxygenation level of the PFC, improving various psychological parameters and cognition. Although this study does provide interesting information surrounding the effects of EPA on PFC oxygenation, the study is limited due to the fact that it was not an intervention study and therefore may be limited by confounding factors. Additionally, the author's report that the sample size employed was not large enough which again suggests that there is still a need for larger scale nutritional intervention studies

in healthy, young adults to further investigate the potentially effects of dietary EPA on CBF. Additionally, Jackson et al. (2012a) have conducted a 12-week, randomised, double blind, placebo-controlled supplementation study with 3 treatment arms (placebo, 1g/d DHA, 2g/d DHA rich fish oil), using cw-NIRS. The authors identified that dietary intake of DHA was significantly associated with increased relative changes in CBF during performance of cognitive tasks in 65 healthy, young adults aged 18-29 years. However, it should be noted that no interpretable effects of the intervention were found on cognition in either Jackson et al. (2012) or McNamara et al. (2010) studies despite the observed changes in CBF between the active and control groups. Furthermore, Jackson et al., (2016) have also demonstrated no effect of 6 months' supplementation on any cw-NIRS measures or cognitive performance tasks, in a sample of 86 older adults supplemented with either 2g/d DHA-rich fish oil (896 mg DHA, 128 mg EPA) or a multinutrient containing 2g/d DHA-rich fish oil (946.4 mg DHA, 160 mg EPA) with added phosphatidylserine (PS, 88 mg), Ginkgo biloba (240 mg), folic acid (1 mg) and vitamin B12 (24 mg).

Overall, there has been a clear lack of consistent findings of the effects of n-3 PUFA supplementation on the relationship between CBF and cognition in healthy, young adults within the literature thus far. Additionally, the majority of research conducted to date has focused solely on the effects of varying quantities of DHA supplementation on CBF, even though Hamazaki-Fujita et al. (2011) suggest that it may be dietary intake of EPA alone that could modulate the oxygenation level of the PFC. In relation to this, there is also evidence that dietary intake of EPA in particular may be important for increases in neural efficiency which is an important feature of brain health. The neural efficiency hypothesis (Haier et al., 1988) claims that the brains of individuals with higher intelligence may require less neural energy resources than those of individuals with lower intelligence in order to perform higher order cognitive tasks. This effect has been seen previously and is shown as a more focused cortical activation and communication between brain regions, resulting in lower total cortical activation and suggesting that there is more efficient use of the brain in more intelligent individuals (Haier et al., 1992a; 1992b; Neubauer et al., 2005; 2006). Previously, Bauer et al., (2011; 2014a) conducted two intervention studies comparing the effects of 4 weeks' supplementation with either a DHA-rich or EPA-rich fish oil on a number of multifocal visual evoked potentials (mfVEPs), fMRI outcomes and cognitive tasks in a crossover study of healthy, young adults (20-34 years). The authors reported reduced reaction times during completion of a Stroop and choice reaction task as well as enhanced neural recovery of the magnocellular visual system (Bauer et al., 2011) and reduced activation in the anterior cingulate cortex (Bauer et al., 2014a) following supplementation with the EPA-rich fish oil supplement. Overall, the authors concluded that their data comprises evidence that increased dietary intake of EPA may result

in enhanced neural efficiency. The findings from both Hamazaki-Fujita et al., (2011) and Bauer et al., (2011; 2014a) provide support for the importance of increased dietary intake of EPA specifically in cortical activation and oxygenation. Furthermore, Howe et al., (2018) has recently concluded that there is still a clear need for studies that examine the differential effects of both EPA and DHA in this research area. Indeed, Bauer et al., (2011; 2014a) are the only groups to investigate the effects of both DHA-rich and EPA-rich supplementation on neural efficiency thus far. It therefore appears that further investigation into the differential effects of both increased dietary EPA and DHA is warranted.

A methodological limitation that also exists within the literature is the use of cw-NIRS that is only capable of measuring relative changes in cerebral activation and concentrations of haemoglobin as opposed to the measurement of absolute, quantifiable, amounts of haemoglobin present within the cortex. The data gathered from the cw-NIRS employed previously is baseline-adjusted to the concentration reading that is taken immediately before the first data point of the recording session. As a result, it is not able to quantify any gross changes in concentrations of haemoglobin that take place between separate recording sessions. However, the employment of frequency domain (fd) NIRS systems allows for the measure of absolute values and so allows for the assessment of gross changes that occur throughout the supplementation period. Employment of this fd-NIRS in the research area is crucial as previous studies have employed methodologies that are only capable of measuring acute changes in concentrations of haemoglobin within paradigms that are actually designed to measure the chronic effects of n-3 PUFA supplementation (Jackson et al. 2010; Jackson et al., 2016). As a result, there remains the clear need for the employment of neuroimaging techniques that are designed to measure the chronic changes that may occur in concentrations of haemoglobin following n-3 PUFA supplementation in order to more appropriately investigate the effects of n-3 PUFA supplementation on CBF and any potential relationships to cognition.

To date, no study has assessed the parallel effects of dietary n-3 PUFAs on CBF and cognition using a quantitative NIRS approach. Therefore, the present study aimed to investigate the effects of 26 weeks supplementation with 1.2g/d of either DHA-rich, EPA-rich or an olive oil placebo on absolute values of oxygen saturation %, total haemoglobin, oxygenated haemoglobin and deoxygenated haemoglobin in healthy, young adult, low consumers of oily fish. Performance was assessed using three numerical subtraction tasks that varied in difficulty while CBF parameters were measured using the fd-NIRS. Measurement of both cognitive performance and absolute values of haemoglobin then also allows for the potential

measurement of neural efficiency, an aspect that has been overlooked within the majority of the research mentioned previously.

3.2 Materials and Methods

3.2.1 Design

This study employed a randomised, placebo-controlled, double-blind, parallel groups design. With participants being randomly assigned to one of three treatment groups (placebo, DHA-rich, EPA-rich; see section 2.2).

3.2.2 Participants

Ninety males and females aged 25-49 years were recruited and of the ninety participants screened seventy-eight were enrolled into the study and seventy-five participants completed all requirements. The three participants who withdrew following randomisation reported no adverse events related to the treatments and left the trial due to time commitments or lack of continued interest in the study. This sample size was calculated based on a small effect size (d = 0.32) observed by (Jackson et al., 2016) for effects on total haemoglobin following 6 months' supplementation with a DHA-rich fish oil. Given this effect size, an a priori calculation of the size of sample required in order to detect a significant difference between the groups given 80% power and an alpha level of 0.05, is twenty-three participants per treatment arm. Additionally, in order to account for any potential dropouts the total sample size was increased by 10%. This resulted in twenty-six participants per treatment arm or seventy-eight participants overall. Power calculations were made using GPower 3.1.3. Participant disposition through the trial is displayed in Figure 3.1 and their demographic data in Table 3.1.

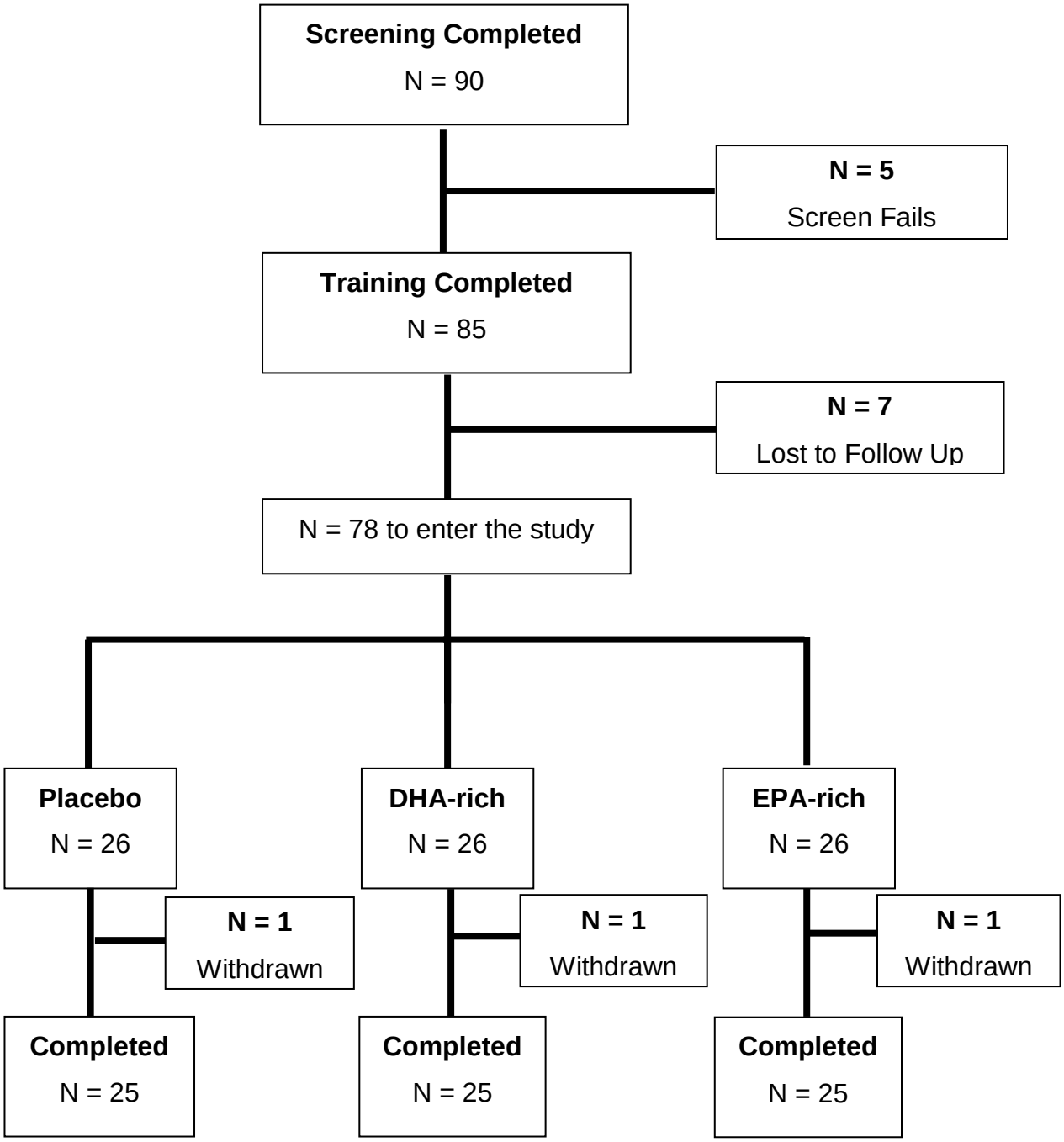

Figure 3.1. Participant disposition through the trial. Figure depicts the disposition of participants throughout the study, culminating in N = 75 of the 78 who were randomised.

Table 3.1. Participant demographic information and baseline characteristics for the 78 subjects enrolled in the study. Means and Std. Deviation (*sd*) are given where appropriate. *F* or χ^2 and *p* values are given for separate one-way ANOVAs or Chi-Square tests that were conducted on this baseline data by treatment group.

		Baseline		Main Effects		
		Mean	Sd		F / χ^2	*p*
N (Males/Females)	Placebo	14/12	-	Treatment	3.18	.204
	DHA-rich	9/17	-			
	EPA-rich	15/11	-			
% of EPA in RBC	Placebo	0.99	0.07	Treatment	2.06	.136
	DHA-rich	1.04	0.10			
	EPA-rich	0.82	0.06			
% of DHA in RBC	Placebo	5.63	0.23	Treatment	2.26	.112
	DHA-rich	5.62	0.25			
	EPA-rich	4.98	0.25			
n-3 Index	Placebo	6.62	0.28	Treatment	2.71	.074
	DHA-rich	6.67	0.31			
	EPA-rich	5.80	0.28			
Age (years)	Placebo	33.85	1.57	Treatment	.489	.615
	DHA-rich	35.08	1.72			
	EPA-rich	32.92	1.31			
Systolic BP	Placebo	126.25	2.81	Treatment	.625	.538
	DHA-rich	122.94	2.22			
	EPA-rich	122.90	2.21			
Diastolic BP	Placebo	81.67	1.78	Treatment	.872	.422
	DHA-rich	80.15	1.72			
	EPA-rich	78.52	1.57			
Heart Rate (BPM)	Placebo	72.23	2.01	Treatment	.220	.803
	DHA-rich	70.62	1.93			
	EPA-rich	70.27	2.68			
Weight (Kg)	Placebo	76.47	3.25	Treatment	.476	.623
	DHA-rich	72.64	2.95			
	EPA-rich	76.10	2.99			
Height (cm)	Placebo	172.69	2.32	Treatment	1.08	.344
	DHA-rich	171.31	1.68			
	EPA-rich	175.58	2.22			
BMI (Kg/m^2)	Placebo	25.45	0.72	Treatment	.618	.542
	DHA-rich	24.55	0.67			
	EPA-rich	24.51	0.65			
Years in Education	Placebo	18.08	0.45	Treatment	1.94	.150
	DHA-rich	18.38	0.49			
	EPA-rich	17.27	0.26			
Fruit & Vegetable (portions per day)	Placebo	3.40	0.30	Treatment	.349	.707
	DHA-rich	3.37	0.33			
	EPA-rich	3.67	0.21			
Alcohol (Units per day)	Placebo	1.31	0.15	Treatment	.131	.877
	DHA-rich	1.26	0.17			
	EPA-rich	1.38	0.20			

3.2.4 Physiological Measures

3.2.4.1 Near Infrared Spectroscopy (fd-NIRS)

Cerebral Haemodynamic response was measured using a fd-NIRS system (OxiplexTS Frequency-Domain Near-Infrared Tissue Oximeter; ISS, Inc., Champaign, IL, USA). This system provides absolute measurements of the absorption of the near-infrared light emitted at two distinct wavelengths by the device, which allows for the quantitation of oxygenated haemoglobin ($Hb0_2$) and deoxygenated haemoglobin (Hb) via their differing photon absorption properties. These values can then be used to determine total haemoglobin (THb; $Hb0_2$ + Hb) and oxygen saturation percentage (Ox%; $Hb0_2$/THb × 100%). This system is well designed for quantifying changes in haemodynamic response over both acute (i.e., changes in response to demanding cognitive tasks) and chronic (i.e., comparing between pre-dose and the 6 month post-dose assessments) time points.

Light was emitted at 691 and 830nm by optical fibres glued in pairs to four prisms (eight fibres in total) separated from the collector bundle, which was also glued to a prism, by 2.0, 2.5, 3.0 or 3.5cm. Each of the emitter and collector bundle prisms were embedded into a flexible polyurethane resin to form a sensor, with overall dimensions of 7.6cm × 2.5cm × 0.3cm. Identical sensors were attached to both sides of the forehead of the participants and secured in place with a self-adhering bandage. The sensors were positioned so that the bottom edge was level with the top of the participants' eyebrows and the middle edge touching at the midline of the forehead. Data were collected at a rate of 5Hz and quantities are measured in micro molars (μM). Increases in THb and $Hb0_2$ are usually interpreted as increased activation of the PFC and increased CBF (Jackson et al., 2012b).

3.2.5 Cognitive Tasks

3.2.5.1 Serial subtraction tasks (3s, 7s, 17s; 1 minute)

Computerised versions of the serial subtraction tasks were implemented using tasks of 1-minute durations. Participants were required to count backwards in either threes, sevens or seventeens from a given number as quickly and as accurately as possible using the number keys to enter each response. A random starting number between 800 and 999 is presented on the computer screen, which is cleared by the entry of the first response. In the case of an incorrect response, subsequent responses are then scored as correct in relation to the previous incorrect number. The task is scored for number of total responses and the number of errors.

3.2.5.2 'Task Difficulty' visual analogue scale

Participants rated how difficult they found the task they had just completed by making a mark on a line representing 0-100% with the end points labelled "not at all" (left hand end; 0) and "very much so" (right hand end; 100).

3.2.6 Procedure

All study visits took place at Northumbria University's Brain, Performance and Nutrition Research Centre (BPNRC). Potential participants attended the site for an initial screening visit. The principal investigator or designee discussed with each participant the nature of the trial, its requirements and restrictions in line with the participant information sheet previously given to the participant. No restrictions were placed on the participants prior to this visit. Following informed consent, eligible participants underwent training on the computerised cognitive tasks. The training session followed standard operating procedures to decrease the chance of learning effects during the main trials. This entailed the participants completing three shortened versions of the tasks to gain familiarity, followed by two full length versions of the tasks. Once this session was completed to the required standard according to performance norms for the participant's age group they were then eligible to be enrolled into the trial.

Prior to attending the baseline and week 26 assessments, participants were asked to avoid alcohol and refrain from intake of 'over the counter' medications for 24 hours and caffeine for 18 hours. On the morning of the baseline testing visit, participants were requested to eat their usual breakfast or no breakfast if they usually skipped breakfast at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products). Participants completed baseline assessments of the numerical subtraction tasks and ratings of task difficulty with concurrent NIRS measurements. This included a 5 minute rest measurement to collect resting baseline data followed by the completion of a 1 minute serial subtraction task and a rating of task difficulty. Each serial subtraction task (3s, 7s, or 17s) and a rating of task difficulty were repeated three times each, separated by a 2 minute resting period (see Figure 3.2 for schematic depicting the study testing visits). Task order was also fully randomised and counterbalanced between the groups. Participants were then provided with the first batch of capsules (3 bottles of 100 capsules each) and given a diary in which to record their daily consumption of the capsules along with any adverse events and concomitant medications, should there be any throughout the supplementation period.

Participants also reported to BPNRC during week 13 and brought with them their diary and any remaining unused treatment capsules, so that treatment compliance could be calculated.

Participants were provided with a second batch of capsules (3 bottles of 100 capsules each), along with a new diary to complete between weeks 13-26.

The week 26 assessment was identical to the baseline assessment in all aspects apart from collecting in the diaries, all remaining treatments, completion of a treatment guess questionnaire (see Appendix II) and finally a full debrief once all assessments were completed. During both the baseline and week 26 visits participants were also required to provide a 6 mL venous blood sample to determine blood fatty acid profile.

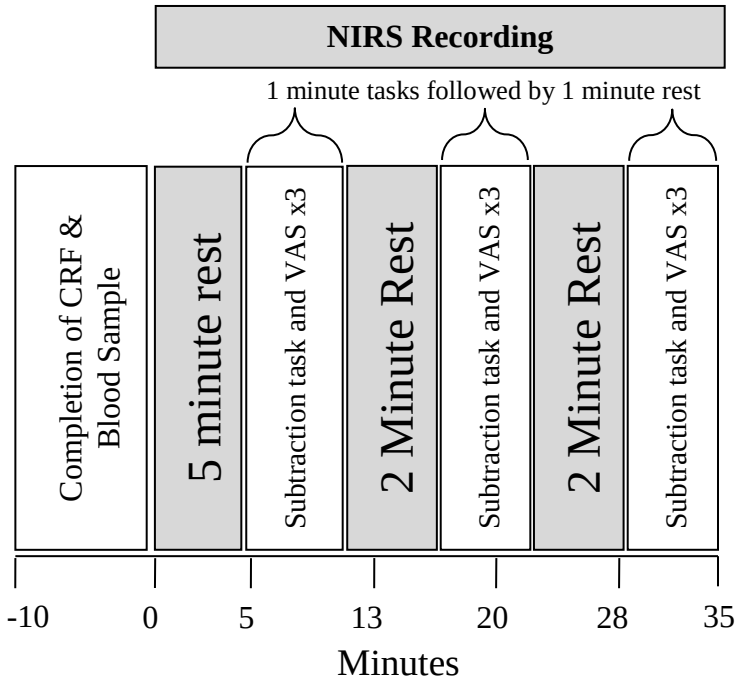

Figure 3.2 Schematic showing the procedures for the baseline and week 26 assessments. Each variation of subtraction task was completed three times for 1 minute, followed by a rating of task difficulty and then a 1 minute rest before the next repetition of the same subtraction task. A two minute rest was then provided after three repetitions of the same subtraction and before completing the next series of subtraction tasks. CRF, Case Report Form; 3s, Serial 3 subtractions; 7s, Serial 7 subtractions; 17s, Serial 17 subtractions; vas, visual analogue scales.

3.2.7 Statistical Methods

3.2.7.1 NIRS Data

NIRS data [oxygen saturation % (Ox%), total haemoglobin (THb), oxygenated haemoglobin ($Hb0_2$) and deoxygenated haemoglobin (Hb)] were split into two distinct periods; resting and active. The resting period contained the averaged data from the 5 minute resting period before the cognitive tasks began and the active period consisted of the averaged data from all three

repetitions of the serial 3s, serial 7s and serial 17s subtraction tasks. All NIRS data were analysed using the Mixed Models procedure in SPSS (version 25) with the covariance matrix structure being chosen from the model with the lowest Schwarz's Bayesian Criterion (BIC) indicating the best fitting model for the data. This resulted in an autoregressive covariance structure being used for the resting Ox%, resting Hb and active Ox% models and an identity covariance structure being used for the resting THb, resting $Hb0_2$, active THb, active $Hb0_2$ and active Hb models.

Fixed effects appearing in the resting NIRS models were treatment (DHA-rich, EPA-rich, Placebo) and hemisphere (left, right) whilst the fixed effects appearing in the active NIRS models consisted of treatment (DHA-rich, EPA-rich, Placebo), hemisphere (left, right), task (3s, 7s, 17s) and task randomisation order (1 – 6). Subject was also added into all models as a random factor and respective baseline values were entered as a covariate.

3.2.7.2 Cognitive Data

The cognitive data consisted of the averaged number of correct responses and accuracy % from all 3 repetitions of the serial 3s, 7s and 17s tasks. The data were analysed using the same Mixed Models procedure described previously with both the total number of responses and number of errors models using an autoregressive covariance matrix. The fixed effects appearing in both models were treatment (DHA-rich, EPA-rich, Placebo), task (3s, 7s, 17s) and task randomisation order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

3.2.7.3 Subjective Task Difficulty

The subjective task difficulty data consisted of the averaged task difficulty rating from all 3 repetitions of the serial 3s, 7s and 17s tasks. The data were analysed using the same Mixed Models procedure described previously and using an Identity covariance matrix. The fixed effects appearing in both models were treatment (DHA-rich, EPA-rich, Placebo), task (3s, 7s, 17s) and task randomisation order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

3.2.7.4 Efficiency Index

The efficiency index (EI) data were calculated by standardising the raw active NIRS data (Ox %, THb, $Hb0_2$, Hb) and the accuracy % for every task during each visit to a value of 0-1 (1= the highest accuracy % or highest respective NIRS value). Once these new standardised

values had been calculated each participant's standardised NIRS value was then subtracted from their standardised accuracy % for each task. This then provided an EI score of between -1 and 1 with higher scores indicating greater neural efficiency. Causse et al., (2017) recently employed this measure of neural efficiency in a similar paradigm.

These data were analysed using the same Mixed Models procedure described previously, using an Identity covariance matrix. The fixed effects appearing in both models were; treatment (DHA-rich, EPA-rich, Placebo), hemisphere (left, right), task (3s, 7s, 17s) and task randomization order (1 – 6). Subject was also added into all models as a random factor and respective pre-dose values were entered as a covariate.

3.2.7.5 Exploratory Correlations

Pearson's bivariate correlations were conducted post hoc in an attempt to further explore the relationship between n-3 index, CBF and efficiency index scores. These correlations were conducted between n-3 index and NIRS outputs (0x%, THb, $Hb0_2$ and Hb) during each of the subtraction task (3s, 7s or 17s), for each hemisphere (left or right) and for each of the visits (baseline or week 26). Additionally, correlations were conducted between n-3 index and efficiency index scores (0x%, THb, $Hb0_2$ and Hb) during each of the subtraction task (3s, 7s or 17s), for each hemisphere (left or right) and for each of the visits (baseline or week 26).

3.3 Results

3.3.1 Compliance

Two participants did not return their unused treatments and three participants withdrew from the study. For the remaining participants, compliance was observed to be very good in all three groups (96% Placebo, 97% DHA-rich, 98% EPA-rich) with a one way ANOVA identifying no significant difference for compliance percentage by treatment group [F (2, 72) = .445, p = .643]. A Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was completed at the end of the final visit and revealed no significant difference in participants' ability to correctly identify whether they had been administered an active or placebo treatment between the three groups [χ^2 (2) = .787, p = .675].

3.3.2 Mixed Models Analysis

Due to the number of statistical analyses performed, only those which revealed significant main or interaction effects including treatment are reported. A Sidak correction was applied to all pairwise comparisons.

3.3.2.1 Resting NIRS

The analysis revealed no significant main or interaction effects of treatment.

Table 3.2 Resting NIRS analysis outcomes, by hemisphere, for placebo, DHA-rich and EPA-rich treatment groups. Week 26 estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects and interactions.

			Post-dose			Main Effects		
			n	Mean	SE		*F*	*p*
Ox (%)	Left	Placebo	71	65.85	0.68	Treatment	.297	.744
		DHA-rich		65.06	0.69			
		EPA-rich		65.76	0.71			
	Right	Placebo	71	66.11	0.69	Treatment*Hemisphere	.871	.423
		DHA-rich		65.67	0.69			
		EPA-rich		65.17	0.72			
THb (µM)	Left	Placebo	71	41.78	1.10	Treatment	.834	.439
		DHA-rich		40.77	1.09			
		EPA-rich		39.99	1.12			
	Right	Placebo	71	39.84	1.09	Treatment*Hemisphere	.068	.935
		DHA-rich		38.53	1.10			
		EPA-rich		38.38	1.12			
$Hb0_2$ (µM)	Left	Placebo	71	27.72	0.91	Treatment	.711	.495
		DHA-rich		26.82	0.91			
		EPA-rich		26.37	0.93			
	Right	Placebo	71	26.57	0.91	Treatment*Hemisphere	.005	.995
		DHA-rich		25.63	0.91			
		EPA-rich		25.31	0.94			
Hb (µM)	Left	Placebo	71	13.97	0.32	Treatment	.363	.697
		DHA-rich		13.93	0.32			
		EPA-rich		13.55	0.33			
	Right	Placebo	71	13.30	0.32	Treatment*Hemisphere	.356	.702
		DHA-rich		13.00	0.33			
		EPA-rich		13.13	0.33			

3.3.2.2 NIRS: Active period

The analysis revealed no significant main effect of treatment. A significant interaction between treatment and hemisphere was identified for THb, [F (2, 251.24) = 3.65, p = .027]. However, post hoc comparisons revealed no significant differences between both the EPA-rich (38.78µM; p = .146) and DHA-rich (40.45µM; p = .807) treatments compared to placebo (41.35µM) in the left hemisphere and no significant differences between both the EPA-rich (37.69µM; p = .131) and DHA-rich (37.51µM; p = .137) treatments compared to placebo (40.34µM) in the right hemisphere. Additionally, no significant differences were identified in either hemisphere between the two active treatments [left hemisphere, p = .621; right hemisphere, p = .999].

A significant interaction between treatment and hemisphere was also identified for $Hb0_2$, [F (2, 256.55) = 4.95, p = .008], with both the EPA (24.97µM; p = .082) and DHA (24.62µM; p = .060) groups showing trends towards significance compared to the placebo group (27.36µM) in the right hemisphere during completion of the subtraction tasks. No significant differences were identified between the active groups in the right hemisphere or between any of the groups in the left hemisphere (Figure 3.3).

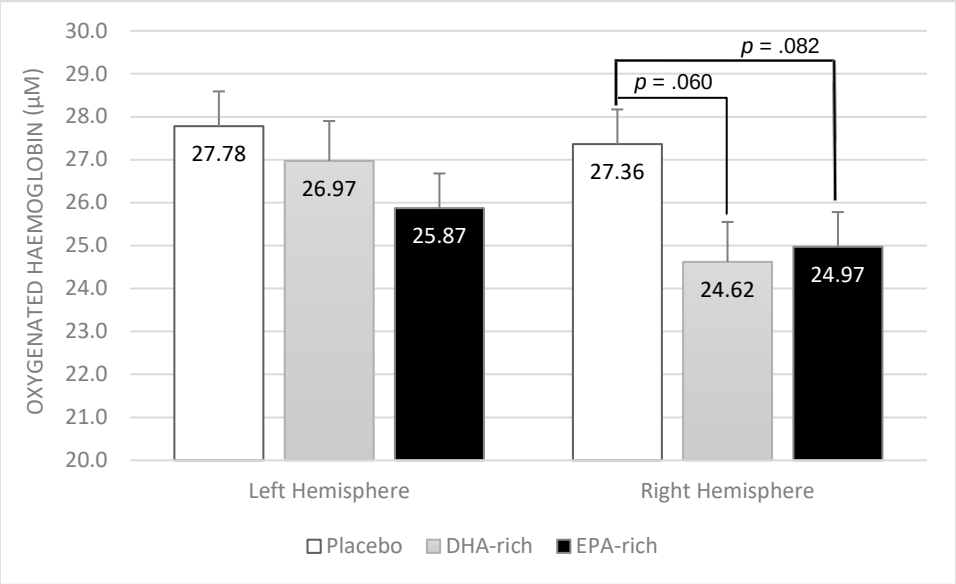

Figure 3.3. Estimated marginal means and standard errors (SE) for post-dose quantities (µM) of oxygenated haemoglobin, in the right prefrontal cortex, by treatment group during the serial subtraction tasks.

Table 3.3 NIRS: active period analysis outcomes by hemisphere for placebo, DHA-rich and EPA-rich treatment groups. Week 26 estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

			Post-dose			Main Effects		
			n	Mean	SE		*F*	*p*
Ox (%)	Left	Placebo	71	66.51	0.60	Treatment	1.30	.280
		DHA-rich		65.92	0.68	Treatment*Task	.946	.439
		EPA-rich		65.84	0.60	Treatment*Hemisphere	1.57	.213
	Right	Placebo		67.17	0.60	Treatment*Hemisphere*Task	.296	.938
		DHA-rich		65.67	0.68			
		EPA-rich		65.84	0.60			
THb (µM)	Left	Placebo	71	41.35	1.01	Treatment	1.90	.162
		DHA-rich		40.46	1.15	Treatment*Task	.023	.999
		EPA-rich		38.78	1.01	**Treatment*Hemisphere**	**3.65**	**.027**
	Right	Placebo		40.34	1.01	Treatment*Hemisphere*Task	.030	1.000
		DHA-rich		37.51	1.16			
		EPA-rich		37.69	1.00			
$Hb0_2$ (µM)	Left	Placebo	71	27.78	0.81	Treatment	2.13	.131
		DHA-rich		26.97	0.93	Treatment*Task	.034	.998
		EPA-rich		25.87	0.81	**Treatment*Hemisphere**	**4.95**	**.008**
	Right	Placebo		**27.36^T**	0.81	Treatment*Hemisphere*Task	.047	1.000
		DHA-rich		**24.62^T**	0.93			
		EPA-rich		**24.97^T**	0.81			
Hb (µM)	Left	Placebo	71	13.58	0.30	Treatment	.891	.418
		DHA-rich		13.51	0.34	Treatment*Task	.266	.900
		EPA-rich		12.91	0.30	Treatment*Hemisphere	1.08	.340
	Right	Placebo		13.01	0.30	Treatment*Hemisphere*Task	.031	1.000
		DHA-rich		12.91	0.34			
		EPA-rich		12.91	0.29			

^{T} = Trend towards a significant difference between active and placebo groups

3.3.2.3 Serial Subtraction and VAS Analysis

The analysis revealed no significant main or interaction effects of treatment for the serial subtraction tasks. However, a significant effect of treatment was identified for ratings of subjective task difficulty [F (2, 56.99) = 3.91, p = .026], with the DHA-rich (50.18; p = .028) but not the EPA-rich (43.65; p =.911) group rating the tasks more difficult than the placebo group (42.01) (Figure 3.4). Additionally, no significant difference between the active groups was observed (p = .098).

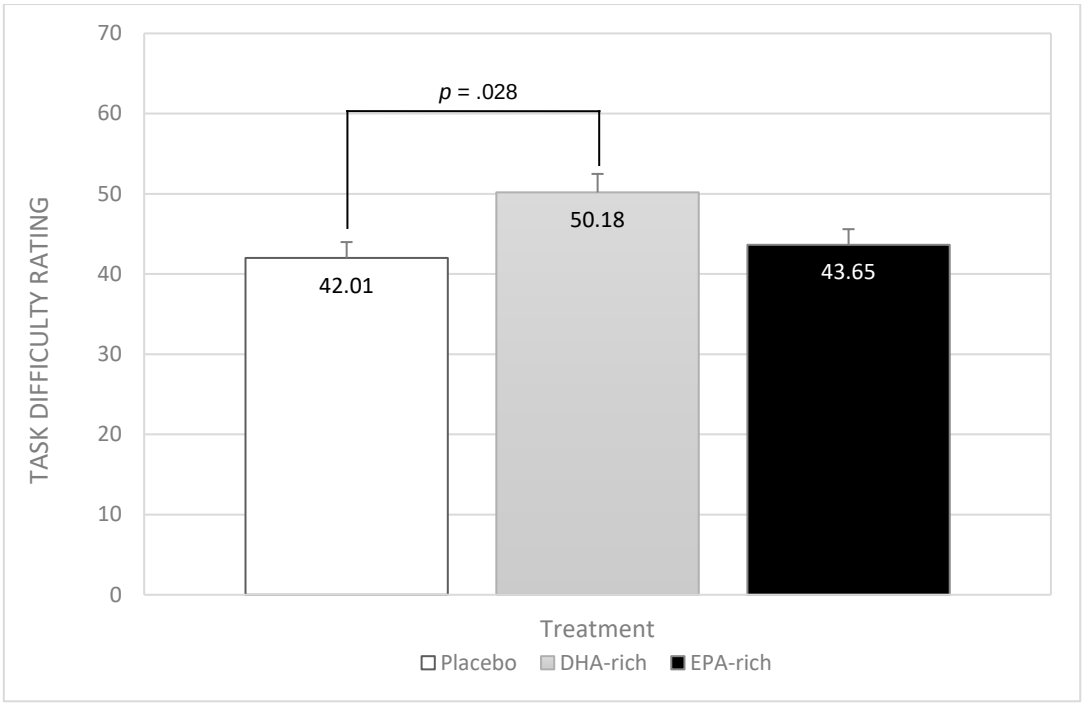

Figure 3.4. Estimated marginal means and standard errors (SE) for post-dose ratings of subjective task difficulty for the subtraction tasks by treatment group.

Table 3.4 Averaged serial subtraction task and task difficulty rating outcomes for placebo, DHA-rich and EPA-rich treatment groups. Week 26 estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Total Responses	Placebo	75	15.42	0.39	Treatment	.234	.792
	DHA-rich		15.82	0.46			
	EPA-rich		15.49	0.39	Treatment*Task	1.42	.235
Number of Errors	Placebo	75	1.21	0.11	Treatment	.336	.716
	DHA-rich		1.07	0.13			
	EPA-rich		1.19	0.11	Treatment*Task	.192	.942
Task Difficulty (0-100%)	Placebo	75	**42.01[a]**	1.97	**Treatment**	**3.91**	**.026**
	DHA-rich		**50.18[a]**	2.30			
	EPA-rich		43.65	1.94	Treatment*Task	.997	.412

[a] = significant difference between active and placebo groups below p <.05.

3.3.2.4 Efficiency Index

No significant main effect of treatment or interactions between treatment and any other factors were identified.

Table 3.5 Efficiency index analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Week 26 estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

			Post-dose			Main Effects		
			n	Mean	SE		*F*	*p*
Ox	Left	Placebo	71	0.07	0.04	Treatment	.312	.734
		DHA-rich		0.10	0.05	Treatment*Task	.096	.984
		EPA-rich		0.09	0.04	Treatment*Hemisphere	.390	.678
	Right	Placebo		0.15	0.04	Treatment*Hemisphere*Task	.978	.441
		DHA-rich		0.22	0.05			
		EPA-rich		0.18	0.04			
THb	Left	Placebo	71	0.27	0.05	Treatment	.167	.846
		DHA-rich		0.28	0.05	Treatment*Task	.080	.989
		EPA-rich		0.31	0.05	Treatment*Hemisphere	.272	.762
	Right	Placebo		0.33	0.05	Treatment*Hemisphere*Task	.079	.998
		DHA-rich		0.37	0.05			
		EPA-rich		0.36	0.05			
$Hb0_2$	Left	Placebo	71	0.29	0.04	Treatment	.168	.846
		DHA-rich		0.30	0.05	Treatment*Task	.076	.990
		EPA-rich		0.32	0.04	Treatment*Hemisphere	.362	.697
	Right	Placebo		0.34	0.04	Treatment*Hemisphere*Task	.113	.995
		DHA-rich		0.39	0.05			
		EPA-rich		0.37	0.04			
Hb	Left	Placebo	71	0.36	0.05	Treatment	.185	.832
		DHA-rich		0.35	0.06	Treatment*Task	.131	.971
		EPA-rich		0.39	0.05	Treatment*Hemisphere	.010	.990
	Right	Placebo		0.34	0.05	Treatment*Hemisphere*Task	.054	.999
		DHA-rich		0.33	0.06			
		EPA-rich		0.36	0.05			

3.3.3 Correlational Analysis

3.3.3.1 NIRS and Blood Fatty Acid Profile

Pearson's bivariate correlations found significant negative correlations in the right hemisphere between THb and n-3 index during the serial 3s (r = -.28, p = .017), serial 7s (r = -.29, p = .015) and serial 17s (r = -.29, p = .013) at baseline. Trends towards significant negative correlations were also seen at 26 weeks in the right hemisphere between THb and n-3 index during the serial 3s (r = -.24, p = .060), serial 7s (r = -.24, p = .056) and serial 17s (r = -.23, p = .060). No significant correlations were identified in the left hemisphere.

Pearson's bivariate correlations also identified significant negative correlations, in the right hemisphere, between $Hb0_2$ and n-3 index during the serial 3s (r = -.27, p = .024), serial 7s (r = -.28, p = .017) and serial 17s (r = -.29, p = .013) at baseline. Significant negative correlations

were also found at week 26, in the right hemisphere, between $Hb0_2$ and n-3 index during the serial 3s (r = -.25, p = .048), serial 7s (r = -.25, p = .041) and serial 17s (r = -.26, p = .038). No significant correlations were identified in the left hemisphere.

3.3.3.2 Efficiency Index and Blood Fatty Acid Profile

Pearson's bivariate correlations found significant positive correlations between THb efficiency index scores and n-3 index in both the right hemisphere (r = .32, p = .006) and the left hemisphere (r = .25, p = .040) during the serial 17s task at baseline. Trends towards significant positive correlations at week 26 were also found between THb efficiency index scores and n-3 index in both the right hemisphere (r = .22, p = .074) and the left hemisphere (r = .23, p = .065) during the serial 17s task. No significant correlations were identified in either hemisphere for the serial 3 or serial 7 tasks.

Pearson's bivariate correlations found significant positive correlations between $Hb0_2$ efficiency index scores and n-3 index in both the right hemisphere (r = .32, p = .006) and the left hemisphere (r = .24, p = .042) during the serial 17s task at baseline. Trends towards significant positive correlations at week 26 were also found between $Hb0_2$ efficiency index scores and n-3 index in both the right hemisphere (r = .24, p = .056) and the left hemisphere (r = .23, p = .072) during the serial 17s task. No significant correlations were identified in either hemisphere for the serial 3 or serial 7 tasks.

3.4 Discussion

Overall the results from the current study show a consistent positive relationship, both at baseline and week 26, between n-3 blood index and efficiency scores for THb and $Hb0_2$ during the serial 17 subtraction tasks, which was coupled with a consistent negative relationship, both at baseline and week 26, between n-3 index in the blood and quantities of THb and $Hb0_2$. These pre-existing and consistent relationships may provide promising insights into the role that EPA and DHA may play on neural efficiency. However, it must be noted that compared to placebo, no significant effects of treatment were found on any of the efficiency index score parameters.

Both active treatments revealed trends towards decreased quantities of $Hb0_2$ in the right prefrontal cortex during performance of the serial subtraction tasks, in comparison with placebo. These decreases in quantities of $Hb0_2$ seem to contradict previous findings, which identified increased activation (McNarama et al., 2010) or increased CBF (Hamazaki-Fujita et al., 2011; Jackson et al., 2012) in the PFC during performance on cognitive tasks following n-3 supplementation. This difference may be a result of the current study employing fd-NIRS rather than cw-NIRS or fMRI which both measure the relative changes in CBF rather than the absolute quantities of haemoglobin within the PFC. For example, the difference between participants resting values and active values may indeed have been greater in the n-3 PUFA groups within the current study, yet the total quantity of haemoglobin measured at both time points was significantly lower in the active groups compared to the placebo group. As the current study measured absolute values it therefore allowed for the direct comparison between baseline and week 26 values as opposed to just the differences between rest and activation during the week 26 assessment only. However, the current study's null findings in relation to the effects of treatment on cognition does seem to compare with the previous lack of observable effects within this research area. Neither, Jackson et al., (2012) or McNamara et al., (2010) observed any interpretable cognitive effects following supplementation with varying quantities of DHA-rich oils. One potential reason for these null findings may be due to the fact that the serial subtraction tasks may not be sensitive to n-3 PUFA supplementation. Although, these tasks are useful in activating the prefrontal cortex they utilise working memory when previous studies have identified effects on tasks related to executive function (Bauer et al., 2011; 2014a) and episodic memory (Stonehouse et al., 2013) in this population. Another potential explanation for the null findings concerning the cognitive outcomes, is a result of the study not being statistically powered to observe effects on cognition. Indeed, after supplementing older adults with *Sideritis scardica* and following a similar methodological paradigm to the current study Wightman et al., (2018) concluded that the reduced power in

RCTs that utilise neuroimaging techniques may be hindering the detection of cognitive effects. As a result of the decreased statistical power within studies employing neuroimaging methodologies, the null effects concerning cognitive outcomes observed within the current study should not be over interpreted.

Interestingly, the decrease in quantities of $Hb0_2$ following supplementation with both the DHA-rich and EPA-rich oils suggests that there was less activation in the PFC in these groups during completion of the serial subtraction tasks, as increases in $Hb0_2$ are usually interpreted as augmented activation within the brain region where the measurement is being made (Jackson et al., 2012b). However, this decrease in activation appeared to have no effect on performance during the serial subtraction tasks between the treatment groups. These findings may then be interpreted as a potential increase in neural efficiency within the DHA-rich and EPA-rich groups, as these groups were able to perform to the same level as the placebo group during the subtraction tasks with a lower activation in the PFC. Indeed, lower brain activation during completion of cognitive tasks has previously been associated with higher intelligence individuals (Neubauer & Fink, 2009) and fits with the neural efficiency hypothesis (Haier et al., 1992). Additionally, Schmithorst and Holland (2006) have identified associations between higher levels of intelligence and more efficient connections between different brain regions, rather than an increase in function of specific brain regions. Furthermore, this effect is accompanied by significantly lower brain activation within frontal brain regions in males but not females (Neubauer & Fink, 2009). Together, this evidence might suggest that supplementation with both the DHA-rich and EPA-rich treatment can result in localised increases in neural efficiency and/or supports increases to the efficiency of the connections between brain regions which then results in decreased activation within the brain.

Certainly, there is literature that exists which suggest that EPA in particular could increase neural efficiency as Bauer et al., (2011; 2014a) have identified decreased cortical activation paired with improvements in reaction times during completion of complex tasks. The author's report that these increases in neural efficiency may be due to the potential EPA has to facilitate enzymatic processes that are required to produce energy for the survival of cells and organs including the brain (Lonergan et al., 2002; Flachs et al., 2005). Additionally, increases in n-3 levels within the membranes of mitochondria (Sullivan et al., 2005) have been seen to help to protect mitochondrial function in the brain (Eckert et al., 2010), and EPA specifically may also have direct actions on the mitochondrial enzymes that produce ATP (Bauer et al., 2014b), which then supplies energy in neuronal cells (Illes, Nieber, & Nörenberg, 1995; Devine & Kittler, 2018). Indeed, Vaughan et al. (2012) have found that combinations of EPA and DHA significantly induce the gene PGC-1a, an essential precursor for mitochondrial biosynthesis,

supported further by the increase in total mitochondrial content observed within the study. Furthermore, Lemasters et al. (2009) describe how significant loss of mitochondrial membrane potential, an essential component in the process of energy storage during oxidative phosphorylation, can activate mitochondrial apoptosis pathways via opening of the mitochondrial permeability transition pore (MPTP). MPTP is a protein formed under certain conditions, such as traumatic brain injury and stroke, which reduces ATP production (Stavrovskaya & Kristal, 2005). Interestingly, DHA has been found to delay the opening of MPTP (O'Shea et al., 2009; Khairallah et al., 2012; Yeh et al., 2015), supporting the role of DHA in stabilising mitochondrial membrane potential. Together, the various effects of EPA and DHA on mitochondrial structure, content and function may offer explanations into how reductions of THb and $Hb0_2$ were observed in the active groups of the current study, whilst no reduction in cognitive performance was observed. This could suggests that increased dietary intake of EPA and DHA may result in neuronal mitochondria producing and storing similar amounts of ATP within neurons from fewer resources, such as oxygen and glucose, i.e. the neuronal cells become more efficient at synthesising and storing ATP. This idea is further supported by evidence that shows individuals who suffer from neurodegenerative diseases, experience serious mitochondrial dysfunction, increased oxidative damage and decreased ATP production which may contribute, at least in part, to the impaired cognitive functioning that is also observed within this population (Terman & Brunk, 2006; Moreira et al., 2007).

Similarly, prevention of oxidative stress and reactive oxygen species (ROS; which are highly toxic to cells) may also be a key component in the relationship between n-3 PUFAs and neural efficiency. Because of its high metabolic rate and relatively reduced capacity for cellular regeneration compared to other organs, the brain is particularly vulnerable to the damaging effects of ROS (Anderson, 2004). Increased consumption of n-3 PUFAs may result in decreased oxidative stress and neuroimflammation due to the production of resolvins, neuroprotectins and maresins (Farooqui & Farooqui, 2016). Moreover, Satyanarayanan et al., (2018) recently demonstrated that EPA provides effective neuroprotective and anti-oxidative effects against oxidative stress. These anti-inflammatory and anti-oxidant effects of n-3 PUFAs provide further evidence in support of the idea that they allow neuronal cells to function more efficiently by preventing apoptosis of healthy neuronal cells (Sinha et al., 2009). However, it must be noted that this effect would be expected to be more prominent in older samples or those who suffer from neurodegenerative disorders as these populations are seen to have increased neuroinflammation (Popp et al., 2017; Bowman et al., 2018; Gu et al., 2019) compared to healthy, young adults. Still, as healthy, young adults are known to have inadequate dietary intakes of n-3 PUFAs (Gopinath et al., 2017; Sioen et al., 2017; Yau et al.,

2018) they may therefore still receive the aforementioned benefits when n-3 PUFA intake is increased in the diet.

Surprisingly, the DHA-rich supplement was also found to lead to increased ratings of perceived task difficulty during the subtraction tasks when compared to the placebo group, with this effect being specific to the DHA-rich group and not present in the EPA-rich group. This increased reporting of task difficulty also appeared to have no effect on task performance. Negative effects of DHA-rich supplements are novel within the literature with negative effects rarely reported, which makes this finding difficult to interpret. However, using electroencephalographic (EEG), both Fontani et al., (2005b) and Sumich et al., (2009) have previously identified a positive association between EPA and lower waveband frequencies (theta), and between DHA and higher waveband frequencies (alpha and beta), suggesting that DHA-rich supplementation may result in increased "brain effort" as it also results in faster frequency activity (Bauer et al., 2014b). If the interpretation of these findings by Bauer et al., (2014b) is correct then Fontani et al., (2005b) and Sumich et al., (2009) may provide evidence that DHA-rich supplementation can result in increases in brain effort and may potentially explain why the DHA-rich group, within the current study, rated the subtraction tasks as more challenging to complete, even though they performed similarly to the other groups. In support of this, Craig et al., (2012) have identified significant increases in fast wave activity in frontal brain regions following a driving task designed to induce fatigue. This may again provide evidence that increases in fast wave activity that have been previously associated with DHA-rich supplementation are also associated with fatigue. Together, these findings may offer insights into why participants subjectively rated the subtraction tasks as more difficult to complete following DHA-rich supplementation compared to EPA-rich supplementation or to placebo. Future studies should aim to employ both NIRS and EEG measurements at the same time to investigate these findings further, as although both the EPA-rich and DHA-rich supplements decreased quantities of haemoglobin in the prefrontal cortex they may influence electrical activity in the brain differently which may then effect subjective feelings of difficulty or mental effort.

The current study aimed to build upon the designs of previous studies via employment of both a DHA-rich and EPA-rich oil, the measurement of absolute quantities of haemoglobin within the PFC, and by providing pre-treatment baseline measures of cognitive performance and cerebral haemodynamics, a feature that was absent in Jackson et al., (2012b). The measurement of absolute quantities of CBF also allowed for the possible investigation of the effects of n-3 PUFAs on neural efficiency, an aspect only measured by Bauer et al., (2011; 2014a) previously. However, one possible limitation of the current study is the length of both

the serial subtraction tasks and the intermittent rest periods. It is possible that 1 minute of subtractions was not a sufficient amount of time to both elicit an appropriate cerebral response and for CBF to return to resting levels between subtraction tasks. For example, Allen et al., (2007) has identified that a transcranial magnetic stimulation (TMS) induced neurovascular coupling response was evident immediately after TMS and remained elevated for ~60 seconds post TMS. These findings may show that the neurovascular coupling response induced via 60 seconds of subtractions may not have returned to resting levels by the beginning of the next subtraction task and perhaps a 90-120 second rest between repetitions would have been more appropriate. Furthermore, the fact that significant correlations were only observed for neural efficiency scores during the serial 17 subtraction tasks may suggest that the serial 3s and 7s subtractions may not have evoked a large enough cerebral response as they were simply not challenging enough for this population, or, may suggest that the effects of EPA and DHA on neural efficiency may actually be specific to cognitive tasks that demand higher levels of effort only. Indeed, neural efficiency (with respect to differentiation between higher and lower IQ individuals) has previously been observed when task difficulty fell in the moderate to high range of cognitive demand (Fairclough et al., 2019), suggesting that there is an optimum level of task difficulty needed to evoke an increased neural efficiency response. Future research should consider employing more challenging cognitive tasks for longer durations, to not only measure the initial CBF response but to also measure a sustained CBF response, as well as employing longer durations of rest between tasks to fully allow CBF to return to resting levels to avoid crossover noise between the tasks.

Another potential direction for future investigation may be the employment of indirect calorimetry (IC) in conjunction with NIRS to also measure cellular respiration and energy expenditure, from oxygen consumption and carbon dioxide production, during completion of cognitive tasks (Oshima et al., 2017). The combination of these devices would allow for the measurement of absolute quantities of CBF as well as metabolic rate/cellular respiration, potentially offering further insights into the measurement of neuronal efficiency. Additionally, the employment of other methodologies such as TMS may be a useful tool when measuring the efficiency of neural pathways as research has previously shown increases in cortico-cortical pathways associated with specific functions during TMS (Chiappini et al., 2018). Investigations incorporating transcranial direct-current stimulation (tDCS) may also provide valuable insight, as a growing body of evidence suggests that tDCS can have beneficial effects on long-term memory, synaptic plasticity and adult neurogenesis (Di Lazzaro et al., 2013; Coffman et al., 2014; Leone et al., 2014, 2015; Podda et al., 2014), which may all be relevant to neural efficiency. As a result tDCS could potentially be employed in future n-3 PUFA interventions to encourage LTP, synaptic plasticity and neurogenesis within the brain and then

measure the effects of increased dietary intake of DHA and EPA on supporting these processes.

The findings from the current study show that 26 weeks' supplementation with DHA-rich and EPA-rich SMEDS formulated oils, in healthy, young adults, showed a trend towards reducing quantities of $Hb0_2$ in the PFC during serial subtraction tasks, whilst having no effect on cognition, compared to placebo. These findings appear to support previous research by Bauer et al., (2011; 2014a; 2014b) that increases in n-3 index may be relevant to increases in neural efficiency, potentially through the various effects of EPA and DHA on mitochondrial function, ATP synthesis and the reduction of oxidative stress, but further investigation of this phenomenon is still required in larger samples, and via potential employment of other devices such as IC, TMS or tDCS.

CHAPTER 4: SUPPLEMENTATION WITH DHA- AND EPA-RICH OILS IMPROVES SLEEP EFFICIENCY AND EFFECTS OTHER PARAMETERS OF SLEEP IN HEALTHY, YOUNG ADULTS

4.1 Introduction

Sleep is essential for maintaining a wide range of functions including mood, motor function and cognitive performance (Durmer & Dinges, 2005) with sleep disorders being one of the earliest signs of neurodegenerative disorders such as AD (Rauchs et al., 2008). Additionally, deficits in daytime performance due to sleep loss or deprivation are seen to be associated with significant financial, social and human cost (Durmer & Dinges, 2005; Gardner, 2018; Barnes & Watson, 2019). Studies to date suggest that both quantity and quality of sleep are important for optimal performance in many aspects of cognition including executive attention, working memory and higher cognitive functions (Miller, Wright, Hough & Cappuccio, 2014). Previously, significant reductions in performance on verbal fluency and memory tasks have been identified when the tasks are completed on a Monday morning following delayed weekend sleep (Yang & Spielman, 2001), which refers to going to bed and getting out of bed later than usual on a weekend (Yang et al., 2001). Furthermore, sleep is known to have important roles within processes of memory consolidation. For instance, the formation of declarative memories appears to be benefited by slow wave sleep (SWS) and verbal memory retention has been correlated with an increased number of sleep spindles during stage 2 sleep (Born, Rasch & Gais, 2006).

Previous research has identified the importance of melatonin, a hormone produced by the pineal gland, as an endogenous regulator of circadian rhythms within the body, helping to coordinate the sleep-wake cycle (Claustrat, Brun & Chazot, 2005). Melatonin receptors are present in the superchiasmatic nuclei (SCN), known in mammals to govern circadian rhythms, supporting the influence of melatonin as an endogenous regulator (Bernard et al., 2007). The pinealocytes in the pineal gland are responsible for synthesising melatonin, a process that begins by synthesising serotonin from the amino acid tryptophan in serotonergic terminals (Rath et al., 2016). Melatonin is then synthesised from serotonin and eventually further metabolised to 6-sulfatoxymelatonin (aMT6s) in the liver before it is finally excreted in urine (Brojkowski et al., 1987; Hardeland, 2010). This process is displayed in Figure 4.1.

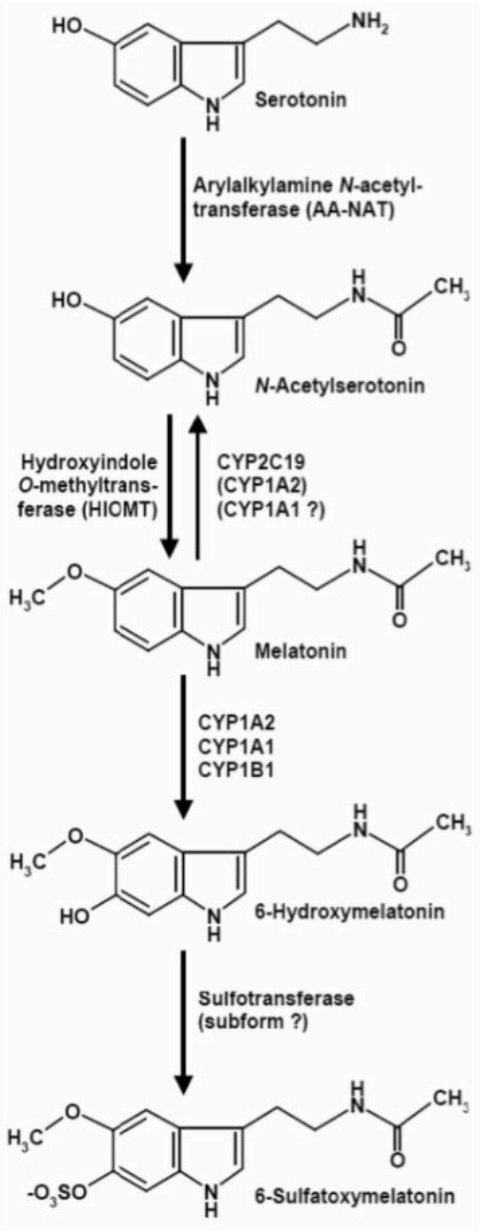

Figure 4.1. Melatonin metabolism pathway from serotonin in the central nervous system. From "Melatonin metabolism in the central nervous system" by Hardeland, (2010) *Current neuropharmacology*, *8*(3), p.169.

N-3 PUFAs, especially DHA, play important roles within the mammalian pineal gland through the conversion of PUFAs into bioactive lipid mediators, with evidence to suggest that two key pineal biochemical functions, lipoxygenation and melatonin synthesis, may be regulated by n-3 PUFA status (Catala, 2010). Animal studies provide evidence of the influence of fatty acid status on melatonin synthesis. In rodents, an n-3 deficient diet reduces night time melatonin secretion and weakens endogenous functioning of the circadian clock (Lavialle, 2008), which has also been found to return to normalised secretion of melatonin following DHA-enriched supplementation (Zaouali-Ajina et al., 1999). Further evidence provided from animal

experiments shows that the rhythm of aralkylamine *N*-acetyltransferase (AA-NAT; an enzyme that metabolises serotonin into N-Acetylserotonin, the precursor for melatonin) may be altered by n-3 status and the n-3/n-6 ratio and may also modify the activities of membrane-bound proteins including enzymes, receptors and transporter proteins (Catala, 2010).

N-3 PUFAs have also previously been observed to influence the release of serotonin (Patrick & Ames, 2015; Sugasini & Lokesh, 2015), which in turn stimulates the release of arachidonic acid (AA) from membrane phospholipids to act as a secondary messenger (Qu, 2019). Serotonin is then known to play major roles in sleep processes during both waking and sleeping periods. When released during waking it influences the synthesis of hypnogenic substances, such as Corticotropin-like Intermediate Lobe Peptides (CLIP), in specific brain regions and during sleep it contributes to the silencing of serotonin-containing neuronal perikarya (Cespuglio, 2018). In support of the role of serotonin in sleep, variations in the alleles of the serotonin transporter gene-linked polymorphic region (5-HTTLPR) have been seen to influence differences in the percentage of sleep that is REM sleep, with short-allele carriers showing lower percentages of REM sleep which may support the elevated risks for insomnia that are associated with this group (van Dalfsen et al., 2019). Certainly, populations suffering from depression are well known to report sleep disorders whilst also being a population seen to have disrupted serotonergic neurotransmission (Healy, 2015; Dell'Osso et al., 2016; Underwood et al., 2018). Additionally, the effects of acute administration of SSRIs, which inhibit the reuptake of serotonin and therefore enhance central nervous system (CNS) serotonergic neurotransmission, have been studied previously in lab animals, healthy adults and depressed patients (Armitage, 1996; Staner, Luthringer & Macher, 1999; Oberndorfer, Saletu-Zyhlarz & Saletu, 2000) and has been found to improve sleep disorders in depressed patients (Fava et al., 2006; Holshoe, 2009; AbdulRahman, Yaqoob & Bhatti, 2018). For instance, Monti and Jantos (2003) have previously demonstrated that systemic injection of flesinoxan, a potent agonist of the serotonin receptor 5-HT_{1A}, increases wakefulness and reduces SWS and REM sleep in rats, providing additional evidence for the importance of serotonin in regulating the sleep-wake cycle.

With these links between serotonin and SWS, DHA may be of particular interest due to its role in membrane fluidity and influence on serotonin receptor function (Patrick & Ames, 2015). The presence of DHA in lipid membranes is known to be necessary for adequate membrane fluidity (Salem et al., 2001; Wassall & Stillwell, 2009; Bradbury, 2011; Calder, 2012) and as the membrane becomes less fluid, the binding of serotonin to its receptor decreases significantly because serotonin receptors have lower accessibility (Heron et al., 1980; Paila, Ganguly & Chattopadhyay, 2010). This effect is not limited to the serotonin receptors but also affects

dopamine receptors and other neurotransmitter receptors (Heinrichs, 2010). DHA's role in membrane fluidity has also been shown to be important for synaptosomal membranes, which regulate neurotransmission (Jones, Arai & Rapoport, 1997; Pinot et al., 2014). Low n-3 fatty acid status has also been associated with decreased serotonergic neurotransmission and DHA deficiency decreases the concentration of serotonin in the frontal cortex (Owens & Innis, 1999; Chalon, 2006). Additionally, EPA may also contribute to serotonin functioning through regulation of serotonin release in the presynaptic neuron (Patrick & Ames, 2015). Serotonin release is known to be inhibited by the E_2 series prostaglandins generated from AA (Schlicker, Fink & Göthert, 1987; Günther et al., 2010). However, EPA has been found to help to inhibit the formation of these E_2 series prostaglandins (Rees et al, 2006; Vedin et al., 2010). Therefore, as the E_2 series prostaglandins inhibit serotonin release and EPA inhibits the generation of these prostaglandins, it seems likely that EPA in the brain would be important for normal serotonin release. Certainly, human plasma n-3 levels have previously been positively correlated with the serotonin metabolite 5-HIAA in cerebral spinal fluid (Hibbeln et al., 1998).

To date, very few studies have investigated the relationship between n-3 PUFA supplementation and sleep parameters in human populations and the majority of those conducted thus far are observational and/or focus on children or populations with existing sleep conditions. For example, Liu et al., (2017) have studied the mediating effect of sleep within the relationship between fish consumption and cognitive function in 541 Chinese school children aged 9-11 years old. Fish consumption and sleep quality was assessed at age 9–11 years, while IQ was assessed at age 12. Frequent fish consumption was related to both fewer sleep problems and higher IQ scores with sleep quality partially mediating the relationship between fish consumption and verbal, but not performance, IQ. These findings were also present after controlling for a multitude of sociodemographic covariates. These findings provide support for the beneficial role of n-3 PUFA intake on sleep parameters and cognition. However, as this study was an observational study causation cannot be inferred with the authors stating that RCTs supplementing with n-3 PUFAs are still required to further investigate the observed relationships between n-3 PUFA intake, sleep and cognition.

In an RCT, Montgomery et al., (2014) have examined associations between blood fatty acid concentrations from fingerstick blood samples and subjective sleep in a large epidemiological sample of healthy 7-9 year olds (n = 395) from mainstream schools. Participants were randomly assigned to either 600 mg/d of algal DHA or placebo supplementation for 16-weeks. Sleep was also assessed objectively via actigraphy in a small sub sample of forty-three children. Poorer total sleep disturbance was found to be related with lower blood DHA and a

lower DHA:AA ratio at baseline, yet there were no treatment-related effects on subjective sleep measures. However, in the sub-sample for whom sleep quality was measured via actigraphy, DHA supplementation led to an average of seven fewer wake episodes and 58 minutes more sleep per night. Again, although these findings provide support for the positive role of DHA on sleep parameters the authors concluded that the findings from the intervention must be taken as preliminary given that benefits of supplementation with DHA were only found for the objective measures of sleep in a small subset of the RCT sample. Additionally, 20% of the RCT population was found to have missing data for at least one sleep measure post intervention which, although in line with previous sleep studies (Price et al., 2012), still resulted in a significant reduction in statistical power.

Hansen et al., (2014) have previously investigated the effects of fatty fish consumption on sleep, heart rate variability (HRV) and vitamin D status in ninety-five male forensic patients, aged 21-60 years, from a secure forensic inpatient facility in the USA. Participants were randomly assigned to consume either Atlantic salmon three times per week from September to February or three alternative meals as their habitual diet. Actiwatch monitoring systems were used to measure objective sleep parameters, sleep diaries to assess subjective sleep parameters and Actiheart systems to measure HRV. Overall, it was concluded that fish consumption appeared to have a positive impact on sleep in general and on daily functioning. Although Hansen et al., (2014) did manipulate the diets of their participants well, the authors do conclude that their relatively small sample of male forensic patients is not necessarily generalisable to other populations and as the observed correlations between sleep parameters and fish consumption were weak, further studies are still required in order to validate their findings.

Additionally, Cornu et al., (2010) have previously evaluated the effects of a dietary supplement containing PUFAs on subjective sleep quality using the Leeds Sleep Evaluation Questionnaire (LSEQ) and objective sleep measures via melatonin and aMT6s assessments. The study was a randomised placebo-controlled trial in adults aged 25 – 65 years with chronic primary insomnia, defined according to the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the International Classification of Sleep Disorders. Participants were randomly assigned to either an olive oil placebo or an active PUFA treatment containing 260mg soya oil (*Glycine Max*), 173mg cade oil (*Cannabis sativa*), 50mg Houblon (*Humulus lupulus*) and 6mg Soya Lecithin for 4 weeks. No effect of treatment was found on perceived quality of sleep, urinary melatonin or aMT6s. Several other previous studies have verified and supported the assessment of the melatonin metabolite aMT6s as an objective measure of melatonin production (Brojkowski et al., 1987; Leger, Laudon, & Zisapel, 2004; Saksvik-Lehouillier et al.,

2015; Sigurdardottir et al., 2016). The measurement of aMT6s by Cornu et al., (2010) is a major strength of the study and allows for biological insights into the effects of PUFAs on aspects of sleep. Physiological measurements of sleep hormones and their metabolites, collected through blood or urine samples, are scarce within the research area. The metabolite of melatonin, aMT6s can be easily collected in urine samples as a measure of biological regulatory processes of sleep, providing additional insights into the regulation of melatonin synthesis from the pineal gland (Benloucif et al., 2008). However, as the treatment employed by Cornu et al., (2010) was not specifically an n-3 PUFA supplement, research employing similar biological measurements of aMT6s are still warranted in RCTs supplementing with EPA-rich and DHA-rich supplements. Additionally, in order to allow for a more holistic view of the relationship between n-3 PUFA and sleep, it is crucial that objective measures of sleep such as actigraphy are also employed alongside subjective and biological measures.

The aforementioned studies have either been observational, employ child samples or samples with pre-existing sleep issues. This has left a gap in the current knowledge of the effects of n-3 PUFA supplementation on sleep in the general healthy adult population. Healthy adults represent a population who could benefit from increased dietary intake of n-3 PUFAs whilst also being free from other health complaints or intakes of concomitant medications. Additionally, it appears that the designs of the n-3 PUFA RCTs conducted thus far could be improved via measurement of the melatonin metabolite aMT6s as it was in Cornu et al., (2010). The combination of subjective sleep questionnaires, objective actigraphy measurements and physiological measures of the regulatory processes of sleep will therefore allow for a more holistic measurement of the relationship between dietary intake of n-3 PUFAs and sleep in healthy, young adult populations. Finally, no study thus far has supplemented with an EPA-rich oil and as mentioned previously, EPA has been seen to impact serotonin functioning and release (Schlicker, Fink & Göthert, 1987; Rees et al, 2006; Günther et al., 2010; Vedin et al., 2010; Patrick & Ames, 2015) and therefore increased dietary intake of EPA specifically may also impact upon sleep and warrants investigation. Therefore, the present study will aim to investigate the effects of 26 weeks' supplementation with 1.2g/d of either a DHA-rich, EPA-rich or an olive oil placebo on subjective sleep parameters (LSEQ), objective sleep parameters (Actigraphy) and urinary aMT6s in healthy, young adult, low consumers of oily fish.

4.2 Materials and Methods

4.2.1 Design

This study employed a randomised, placebo-controlled, double-blind, parallel groups design. With participants being randomly assigned to one of three treatment groups (placebo, DHA-rich oil, EPA-rich oil; see section 2.2).

4.2.2 Participants

Ninety-five males and females aged 25-49 years were recruited and of the ninety-five participants screened ninety were enrolled into the study and eighty-four completed all requirements. From the six participants that did not complete all aspects four were lost to follow up after completion of the baseline testing visit, one withdrew consent and one was advised to stop adhering to the consumption of the supplements due to reporting minor adverse events. The sample size was calculated based on a medium effect size (d = 0.55) reported by Montgomery et al., (2014) for total minutes asleep measured via actigraphy following 16 weeks' supplementation with DHA. Given this effect size, an a priori calculation of the size of sample required in order to detect a significant difference between the groups given 80% power and an alpha level of 0.05, is twenty-seven participants per treatment arm. Additionally, in order to account for any potential dropouts the total sample size was increased by 10%. This resulted in thirty participants per treatment arm or ninety participants overall. Power calculations were made using GPower 3.1.3. Participant disposition through the trial is displayed in Figure 4.2 and their demographic data in Table 4.1.

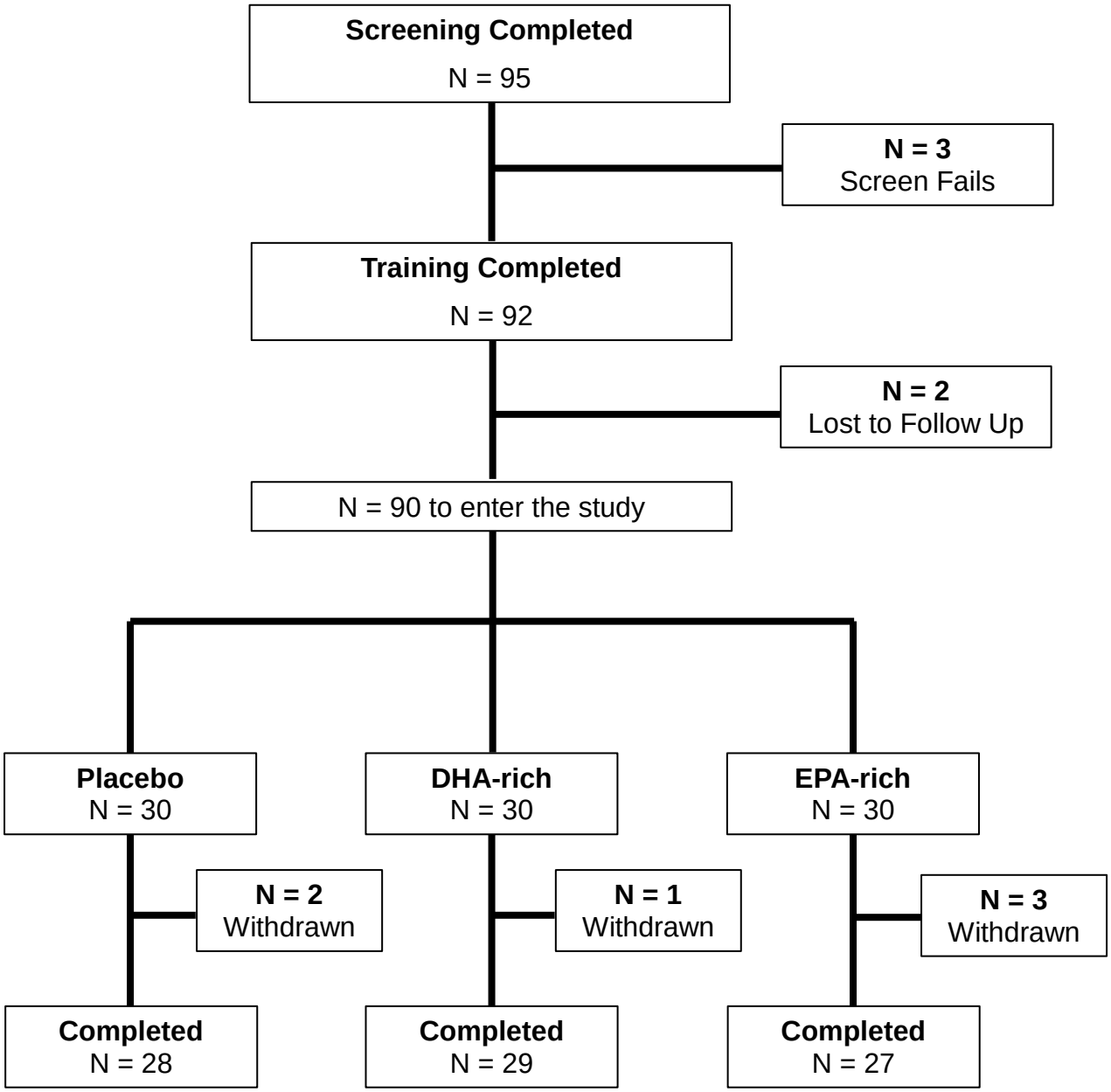

Figure 4.2 Participant disposition through the trial. Figure depicts the disposition of participants throughout the study, culminating in N = 84 of the 90 who were randomised.

Table 4.1. Participant demographic information and baseline characteristics for the 90 subjects enrolled in the study. Means and Std. Deviation (*sd*) are given where appropriate. *F* or χ^2 and *p* values are given for separate one-way ANOVAs or Chi-Square tests that were conducted on this baseline data by treatment group.

		Baseline		Main Effects		
		Mean	Sd		F / χ^2	p
N (Males/Females)	Placebo	8/22	-	Treatment	.433	.805
	DHA-rich	8/22	-			
	EPA-rich	10/20	-			
% of EPA in RBC	Placebo	0.82	0.04	Treatment	1.82	.170
	DHA-rich	0.88	0.05			
	EPA-rich	1.03	0.12			
% of DHA in RBC	Placebo	4.48	0.19	Treatment	.602	.550
	DHA-rich	4.71	0.17			
	EPA-rich	5.04	0.28			
n-3 index	Placebo	5.63	0.21	Treatment	.969	.384
	DHA-rich	5.59	0.20			
	EPA-rich	6.08	0.37			
Age (years)	Placebo	36.33	1.43	Treatment	.219	.804
	DHA-rich	37.13	1.34			
	EPA-rich	35.80	1.52			
Systolic BP	Placebo	123.13	1.99	Treatment	.314	.731
	DHA-rich	120.58	2.37			
	EPA-rich	122.27	2.56			
Diastolic BP	Placebo	81.12	1.43	Treatment	.418	.660
	DHA-rich	79.03	1.78			
	EPA-rich	80.68	1.85			
Heart Rate (BPM)	Placebo	72.30	2.22	Treatment	.884	.417
	DHA-rich	69.15	2.07			
	EPA-rich	72.72	1.92			
Weight (Kg)	Placebo	72.87	2.43	Treatment	.270	.764
	DHA-rich	74.14	3.14			
	EPA-rich	71.36	2.39			
Height (cm)	Placebo	167.62	1.61	Treatment	.417	.661
	DHA-rich	169.17	1.80			
	EPA-rich	167.30	1.16			
BMI (Kg/m^2)	Placebo	26.00	0.86	Treatment	.106	.899
	DHA-rich	25.74	0.82			
	EPA-rich	25.46	0.77			
Years in Education	Placebo	16.03	0.33	Treatment	.859	.427
	DHA-rich	16.23	0.20			
	EPA-rich	15.70	0.17			
Fruit & Vegetable (portions per day)	Placebo	3.83	0.33	Treatment	.664	.517
	DHA-rich	4.13	0.32			
	EPA-rich	4.40	0.39			
Alcohol (Units per day)	Placebo	1.43	0.13	Treatment	2.25	.111
	DHA-rich	1.00	0.13			
	EPA-rich	1.32	0.18			

4.2.3 Actigraphy Measures

Participants were instructed to complete sleep diaries to record time in and out of bed and to wear actigraphy WGT3X-BT watches (Actigraphcorp, FL, USA) on the non-dominant wrist for seven consecutive days and nights both prior to commencing and before completing the 6 month supplementation period. The devices are small and lightweight and have the ability to detect body accelerations in the vertical, horizontal (right to left) and frontal (front and back) planes at varying sample rates. The data from the watches were collected in 1-minute epochs. Using the software Actilife 6.1 the following parameters could then be calculated from the watches:

- Sleep latency *(The difference in minutes between in bed time and sleep onset)*
- Sleep efficiency *(Number of sleep minutes divided by the total number of minutes the subject was in bed; i.e., the difference between the In-Bed and Out Bed time)*
- Total sleep time *(The total number of minutes scored as "asleep")*
- Total minutes in bed *(The total number of minutes in bed both awake and asleep)*
- Wake after sleep onset *(The total number of minutes awake after sleep onset occurred)*
- Number of awakenings *(Total number of awakenings from the time spent in bed)*
- Average awakening length *(The average length, in minutes, of all awakening episodes)*
- Sleep Fragmentation Index *(The sum of the Movement Index – Total of scored awake minutes divided by Total time in bed in hours x 100 and Fragmentation Index - Total of 1 minute scored sleep bouts divided by the total number of sleep bouts of any length x 100)*

4.2.3.1 Actigraphy analysis

Initially, automatically detected sleep/wake times were used to score the actigraphy data using the Cole-Kripke (ActiGraph) algorithm (Cole et al., 1992). This algorithm automatically detects sleep/wake periods based on activity data from the present epoch, the preceding epoch and the following epochs. This algorithm is considered appropriate for use with adult populations as it was developed using participants ranging from 35 to 65 years of age. The Cole-Kripke algorithm was validated in adults wearing a Motionlogger Actigraph (Ambulatory Monitoring, Inc.). In order to match the output of the more sensitive WGT3X-BT device with the Motionlogger Actigraph and AMA-32, ActiGraph adapted the original Cole-Kripke algorithms to the ActiGraph devices by performing a side-by-side test using devices from both companies worn together (Wyatt, 2012; Quante et al., 2013).

However, whilst following the automatic detection of sleep/wake times several issues arose. It became apparent that the automatically identified sleep/wake times did not compare with the data recorded in the participants sleep diaries, appeared to often overestimate or underestimate sleep periods and failed to detect any useable sleep periods in 1/3rd of the overall dataset, resulting in a significantly diminished amount of data. These issues have been reported previously in the literature including inability of sleep algorithms to accurately detect sleep/wake times (Tryon, 2004; Chow et al., 2016) and their tendency to overestimate/underestimate sleep/wake periods (Ancoli-Israel, 2003). As a result, alternative methods can be employed to detect sleep/wake times, including the use of recorded sleep diaries or visually inspecting the data (Lockley, Skene & Arendt, 1999; Paquet, Kawinska & Carrier, 2007; Chow et al., 2016; Tétreault et al., 2018). Consequently, it was decided that to avoid the loss of a significant quantity of data, manual detection of the sleep/wake times was required. This involved visually inspecting the data for decreases and increases in activity, further directed by the participant's recorded in/out of bed times in their sleep diaries. Once the sleep/wake times had been manually entered for each participant during each night the same Cole-Kripke (ActiGraph) algorithm described previously was then used (Cole et al., 1992) to generate the sleep outcomes. A visual comparison between the automatically and manually detected sleep/wake times are presented in Figure 4.3.

Figure 4.3. Differences between the automatically and manually detected sleep/wake times for two separate nights of sleep data from the actiwatches. Pink bars represent the entire period between the identified sleep and wake times; blue spikes represent periods of activity/movement; green bars represent time scored as asleep.

4.2.4 Subjective Sleep Measures

4.2.4.1 Leeds Sleep Evaluation Questionnaire (LSEQ)

The LSEQ is a 10 item scale designed specifically to measure changes in subjective sleep following a pharmacological intervention. The questionnaire measured aspects of sleep including; Getting to Sleep, Quality of Sleep, Awakening from Sleep and Behaviour Following Sleep. The 10 items that make up the four sleep components were presented on a 100mm line with one end representing negative and the other representing a positive answer to the question. Higher scores on these scales represent greater feelings of the respective items.

4.2.4.2 Awakening VAS

These scales measured items related to participant's subjective rating of their awakening state. Participants rated their current subjective state by making a mark on a 100 mm line with the end points labelled "not at all" (left hand end) and "very much so" (right hand end). These scales included; "how rested do you feel?", "how energetic do you feel?", "how relaxed do you feel?", "how irritable do you feel?", "how ready do you feel to perform" and "have you had a good night's sleep?". Higher scores on these scales represent greater feelings of the respective items.

4.2.5 Biological Sleep Measures

4.2.5.1 Urinary 6-Sulfatoxymelatonin (aMT6s)

Urine sampling commenced on the evening prior to the baseline and week 26 testing visits and comprised three separate samples: void at bedtime and the first and second voids of the following day (morning of the testing visit). If a participant needed to urinate during the night, then these voids were also collected in the same manner described below.

Urine was collected in a sterilised measuring cylinder. Void volume, time and date were recorded, before a 10 mL aliquot of urine was retained and refrigerated in a screw cap container pre-labelled with the participants study details. The samples were returned to the laboratory upon the baseline and week 26 testing visits for further labelling and immediate storage at -80^{o}C for later analysis aMT6s by radioimmunoassay (Aldhous and Arendt 1988).

Total excretion of aMT6s (ng) summed from all voids and the bedtime aMT6s (ng) values were calculated. Bedtime excretion of aMT6s specifically was also chosen to be analysed independently from total aMT6s as a measure of melatonin production before sleeping in an attempt to assess the effects of treatment on bedtime melatonin levels, as reduced evening

melatonin production is associated with sleep disturbances (Shekleton et al., 2010) and urinary levels of aMT6s are seen to parallel those of melatonin in the blood, saliva, and urine (De Almeida et al., 2011).

4.2.6 Procedure

All study visits took place at Northumbria University's Brain, Performance and Nutrition Research Centre (BPNRC). Potential participants attended the site for an initial screening visit. The principal investigator or designee discussed with each participant the nature of the trial, its requirements and restrictions in line with the participant information sheet previously given to the participant. No restrictions were placed on the participants prior to this visit. Following informed consent the participant was then eligible to be enrolled and randomised into the trial.

Before the baseline and week 26 assessments participants were required to call into the lab to collect an actiwatch, sleep diary and urine sampling pack. Participants were required to wear the actiwatch and complete the sleep diary for the 7 nights prior to the baseline and week 26 assessments and to provide the urine samples the night before and morning of the baseline and week 26 assessment. Participants were asked to avoid alcohol and refrain from intake of 'over the counter' medications for 24 hours and caffeine for 18 hours before both the baseline and week 26 assessments. Participants were contacted to remind them of the requirements prior to each assessment. On the morning of the baseline testing visit, participants were requested to eat their usual breakfast or no breakfast if they usually skipped breakfast at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products). At the end of the baseline assessments participants were provided with the first batch of capsules (3 bottles of 100 capsules each) and given a diary in which to record their daily consumption of the capsules along with any adverse events and concomitant medications (see Figure 4.4 for schematic depicting the study overview).

Participants also reported to the BPNRC during week 13 to collect the second batch of capsules (3 bottles of 100 capsules each) and to complete the LSEQ and subjective awakening scales. Participants also brought with them their diary, which was replaced with a new diary to complete between week 13-26 and any remaining unused treatment capsules, so that a treatment compliance percentage could be calculated.

The week 26 testing assessment was identical to the baseline assessment in all aspects apart from collecting in the treatment and sleep diaries, all remaining treatments, completion of a treatment guess questionnaire (see Appendix II) and finally a full debrief once all assessments

were completed. During both the baseline and week 26 visits participants were also required to provide a 6 mL venous blood sample to determine blood fatty acid profile.

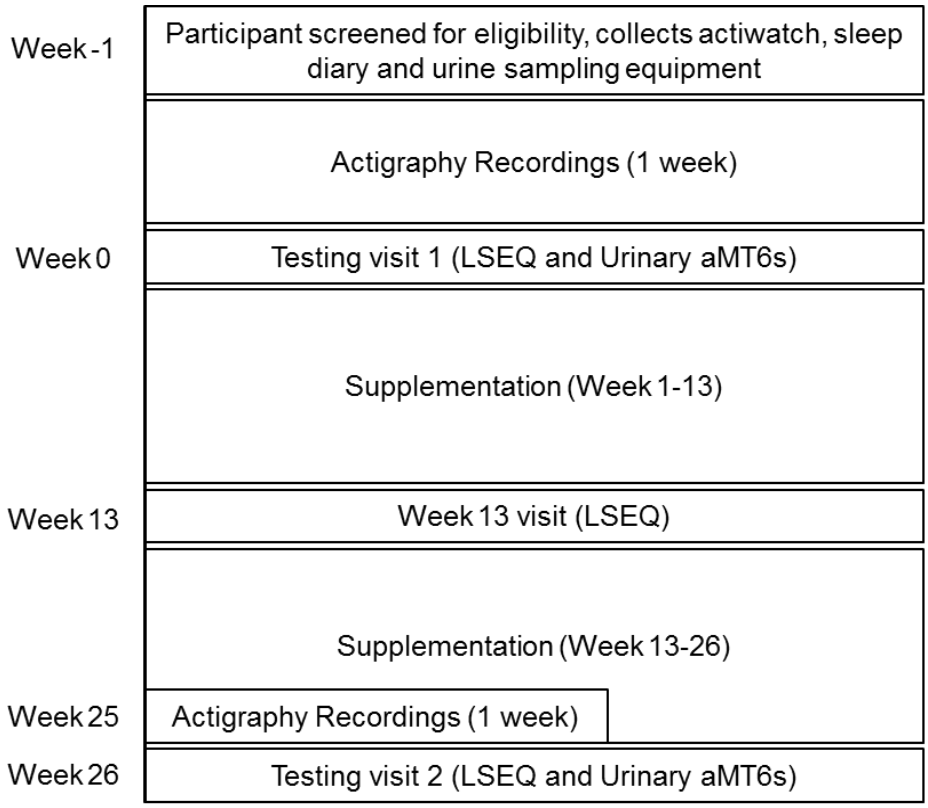

Figure 4.4. Scematic showing the study progression from enrollment to completetion across the 26 weeks. Actigraphy recordings were taken for the seven days and nights prior to the baseline and week 26 testing visits and urinary aMT6s samples were collected the night prior to and morning of the baseline and week 26 testing visits. LSEQ, Leeds Sleep Evaluation Questionnaire.

4.2.7 Statistical Methods

4.2.7.1 Actigraphy

Actigraphy data collected from the Actigraph sleep watches seven night's prior to the week 26 assessment were analysed with the seven night's prior to the baseline assessment acting as a covariate. Actigraphy data included sleep efficiency, sleep latency, total sleep time, total minutes in bed, wake after sleep onset, number of awakenings, average awakening length and sleep fragmentation index. Data were analysed using linear mixed models in SPSS (version 25) with the covariance structure being chosen from the model with the lowest Schwarz's Bayesian Criterion (BIC) indicating the best fitting model for the data. For the sleep latency, total minutes in bed and total sleep time models the identity covariance matrix was used whilst the sleep efficiency, wake after sleep onset, number of awakenings, average awakening length and sleep fragmentation index an autoregressive (first order) covariance matrix was used.

Fixed factors appearing in all actigraph models were; treatment (DHA-rich, EPA-rich, Placebo) and night (1-7). Subject was also added into all models as a random factor and respective baseline values were entered into each model as a covariate.

4.2.7.2 LSEQ

The LSEQ data consisted of the individual summed scores on the items that comprised the quality of sleep, awake following sleep, behaviour following waking and getting to sleep factors. The data were analysed using the same linear mixed model procedure described above with all models using an identity covariance matrix. The fixed factors appearing in all models were; treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13 and week 26). Subject was also added into all models as a random factor and respective baseline values were entered into each model as a covariate.

4.2.7.3 Awakening VAS

The awakening VAS data were analysed using the same linear mixed model procedure described above with all models using an identity covariance matrix. The fixed factors appearing in all models were; treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13 and week 26). Subject was also added into all models as a random factor and respective baseline values were entered into each model as a covariate.

4.2.7.4 Urinary aMT6s

The urinary aMT6s data consisted of the total excretion of aMT6s (ng) summed from all voids and the bedtime aMT6s (ng) values. All urinary aMT6s data was analysed using the linear mixed models procedure outlined above. The only fixed factor appearing in the model was treatment (DHA-rich, EPA-rich, Placebo) with respective baseline values entered as a covariate.

4.3 Results

4.3.1 Compliance

For participants who completed the study, compliance was observed to be very good in all three groups (95.21% Placebo, 96.42% DHA-rich, 95.64% EPA-rich) with a one way ANOVA identifying no significant differences for compliance percentage by treatment group [F (2, 81) = .274, p = .761]. A Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was completed at the end of the final visit and revealed no significant differences in participants' ability to correctly identify whether they had been administered an active or placebo treatment between the three groups [χ^2 (2) = 3.84, p = .147].

4.3.2 Mixed Models Analysis

Due to the number of statistical analyses performed, only those which revealed significant main or interaction effects including treatment are reported. All post-hoc analyses reported are Sidak corrected comparisons.

4.3.2.1 Actigraphy

Analysis identified a significant main effect of treatment for sleep latency [F (2, 322) = 3.68, p =.026] with post hoc comparisons identifying the DHA-rich (3.76; p = .021) but not the EPA-rich (3.98; p = .276) group as showing significantly shorter sleep latency compared to placebo (4.31) (Figure 4.5).

Analysis also identified a significant interaction between treatment and night for sleep latency [F (12, 322) = 2.28, p =.009] with post hoc comparisons identifying the DHA-rich group (3.31) as having a significantly shorter latency period compared to both the placebo (6.43; p = .003) and EPA-rich (5.80; p = .023) on night 1 and both the DHA-rich (3.36, p = .017) and EPA-rich groups (3.34, p = .021) as having a significantly shorter latency period compared to placebo (4.53) on night 6 (Figure 4.6).

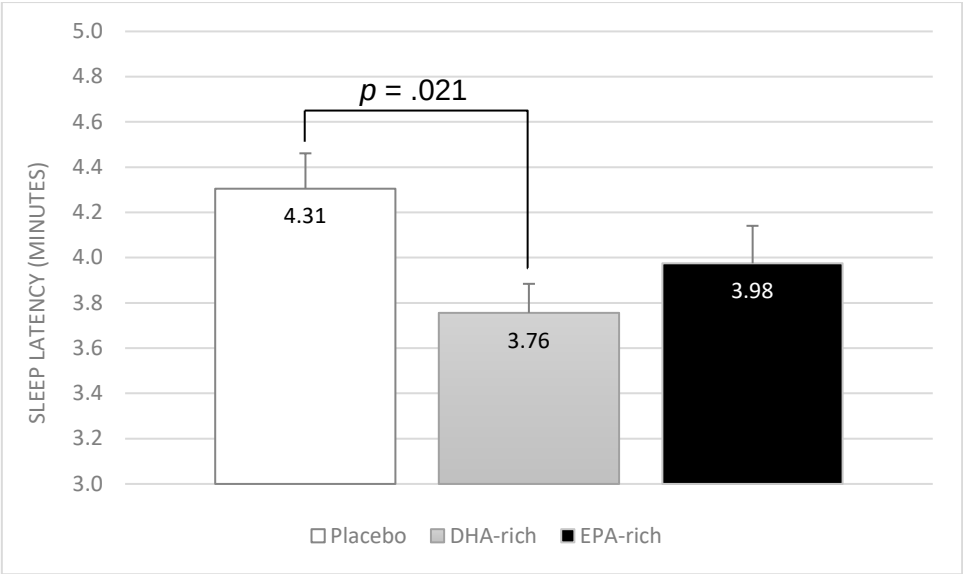

Figure 4.5 Estimated marginal means and standard error (SE) for post-dose values of sleep latency (minutes) by treatment group.

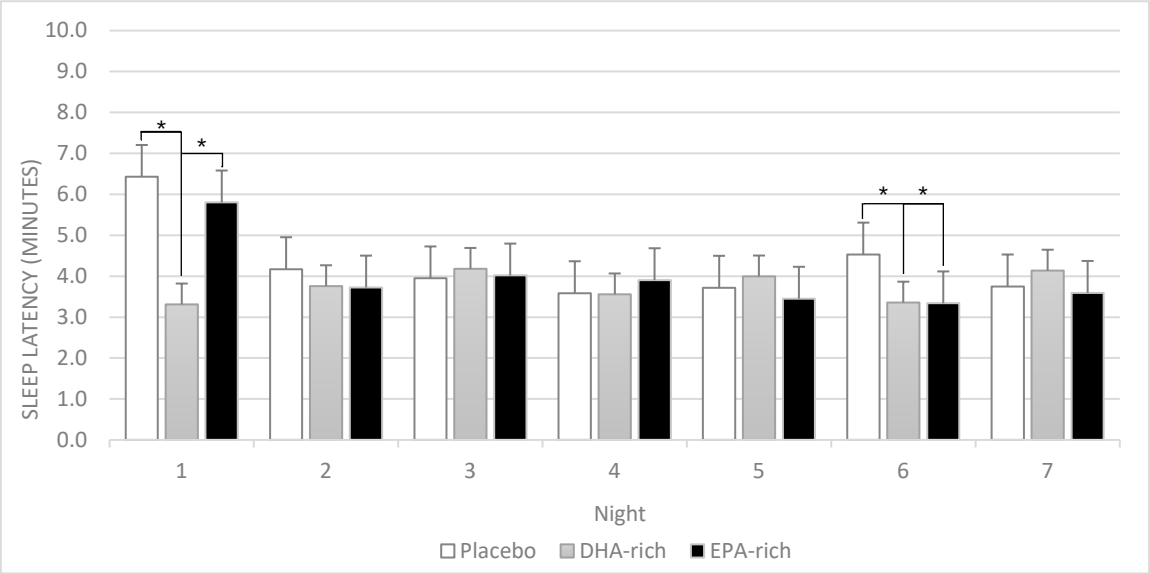

Figure 4.6 Estimated marginal means and standard error (SE) for post-dose values of sleep latency (minutes), split by night and treatment group.

A significant main effect of treatment for sleep efficiency was identified [F (2, 79.79) = 3.68, p =.030] with post hoc comparisons identifying the DHA-rich group (92.02%; p = .037) as having significantly higher sleep efficiency and a trend towards significantly higher sleep efficiency in the EPA-rich group (91.85%; p = .087) compared to placebo (90.30%) (Figure 4.7).

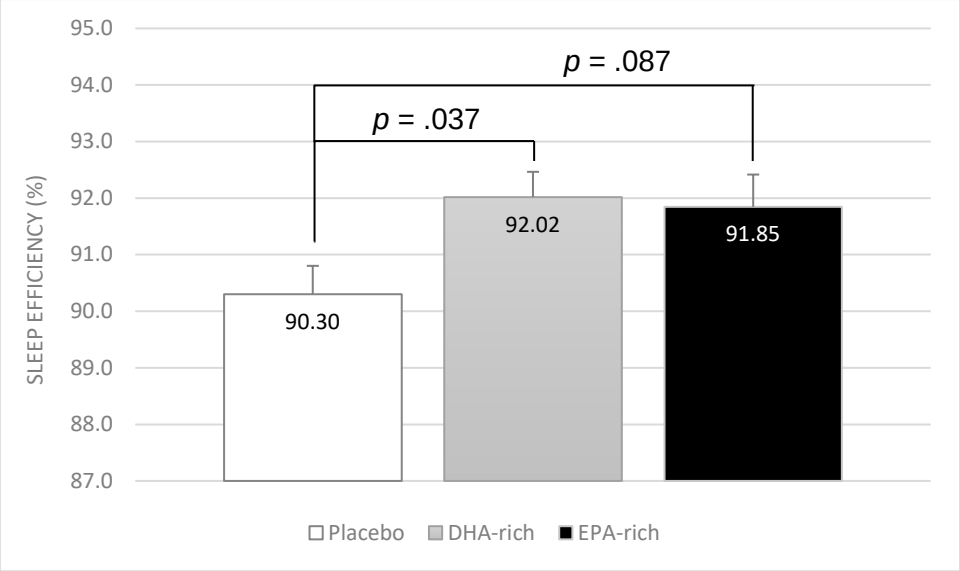

Figure 4.7 Estimated marginal means and standard error (SE) for post-dose values of sleep efficiency (%), by treatment group.

Analysis identified a significant main effect of treatment for total minutes in bed [*F* (2, 328) = 3.29, *p* =.039] with post hoc comparisons identifying no significant differences between the active and placebo groups but the DHA-rich (484.51 minutes) spent significantly more time in bed than the EPA-rich group (467.10; *p* = .032) (Figure 4.8).

A significant main effect of treatment was also identified for total sleep time [*F* (2, 323) = 4.06, *p* =.018] with post hoc comparisons identifying no significant differences between the active and placebo groups but the DHA-rich (455.17 minutes) spent significantly more time asleep than the EPA-rich group (427.28; *p* = .019) (Figure 4.8).

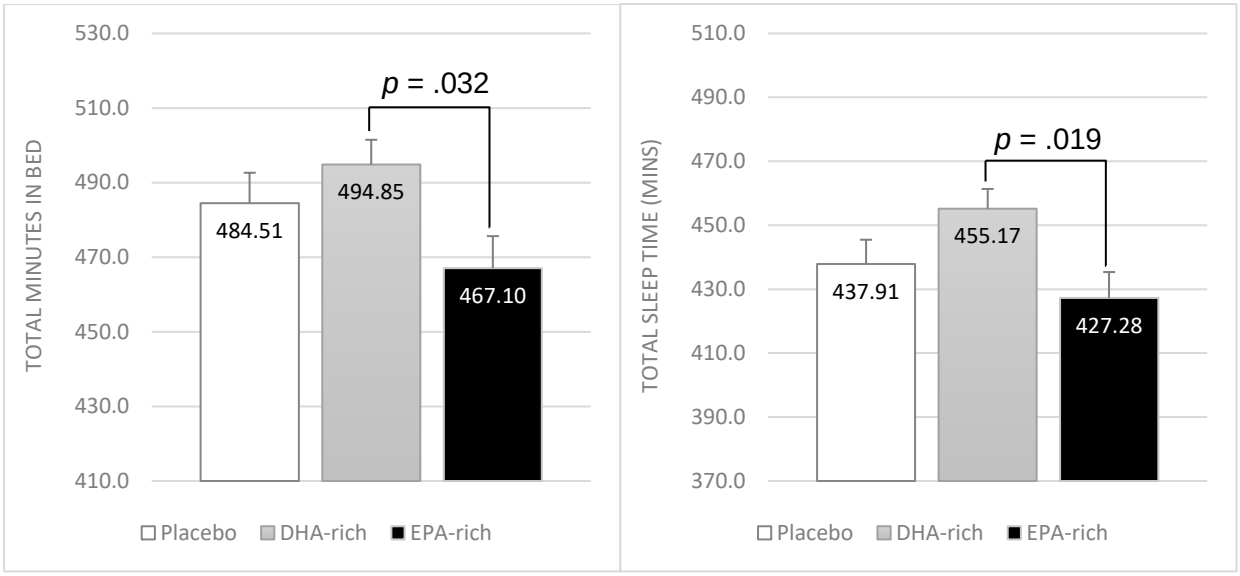

Figure 4.8 Estimated marginal means and standard error (SE) for post-dose values of total minutes in bed **(left)** and total sleep time in minutes **(right)**, by treatment group.

Analysis also identified a significant interaction between treatment and night for sleep fragmentation index [F (12, 227.64) = 1.90, p =.025] with post hoc comparisons identifying the DHA-rich group (15.88; p = .003) as having significantly less sleep fragmentation compared to placebo (26.85) on night 2 only (Figure 4.9).

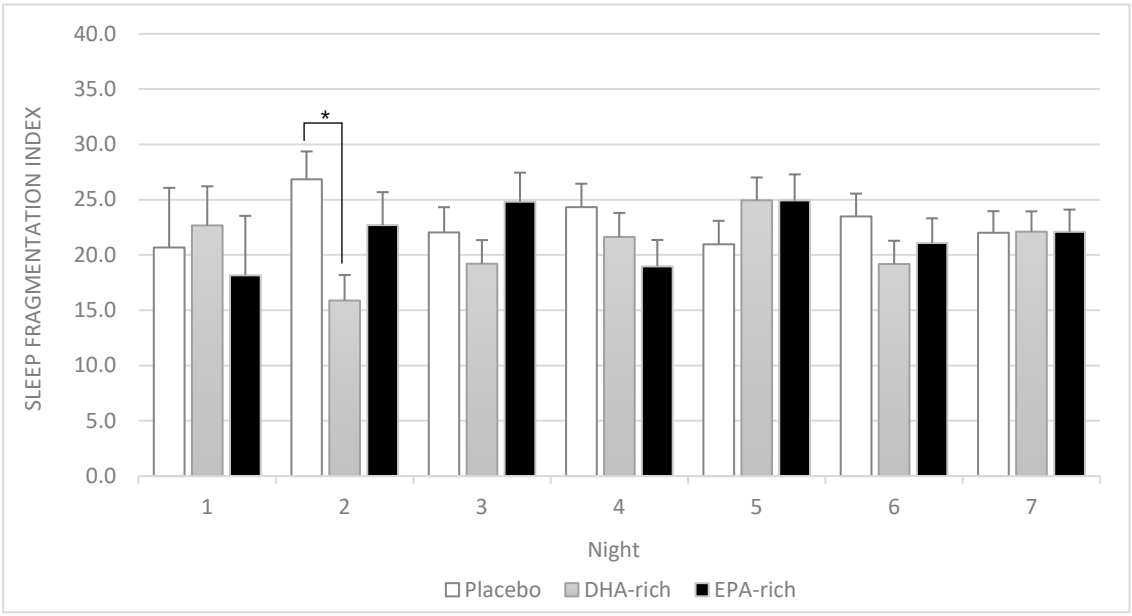

Figure 4.9 Estimated marginal means and standard error (SE) for post-dose values of sleep fragmentation index, by night and treatment group.

Table 4.2. Manually calculated sleep outcomes for Placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Latency (minutes)	Placebo	74	**4.31[a]**	0.21	**Treatment**	**3.68**	**.026**
	DHA-rich		**3.76[a]**	0.26			
	EPA-rich		3.98	0.27	**Treatment*Night**	**2.28**	**.009**
Efficiency (%)	Placebo	72	**90.30[a,T]**	0.50	**Treatment**	**3.68**	**.030**
	DHA-rich		**92.02[a]**	0.49			
	EPA-rich		**91.85[T]**	0.57	Treatment*Night	1.47	.138
Total Minutes in bed (minutes)	Placebo	74	484.51	8.13	**Treatment**	**3.29**	**.039**
	DHA-rich		**494.85[b]**	6.63			
	EPA-rich		**467.10[b]**	8.55	Treatment*Night	.851	.598
Total Sleep Time (Minutes)	Placebo	73	437.91	7.56	**Treatment**	**4.06**	**.018**
	DHA-rich		**455.17[b]**	6.18			
	EPA-rich		**427.28[b]**	8.08	Treatment*Night	1.20	.281
Wake after Sleep Onset (minutes)	Placebo	72	42.02	2.42	Treatment	2.55	.084
	DHA-rich		35.84	2.14			
	EPA-rich		34.77	2.74	Treatment*Night	1.29	.225
Number of Awakenings	Placebo	74	17.50	0.99	Treatment	.813	.446
	DHA-rich		15.87	0.88			
	EPA-rich		16.20	1.08	Treatment*Night	1.19	.289
Average Awakening Length (minutes)	Placebo	74	2.44	0.11	Treatment	.576	.564
	DHA-rich		2.29	0.09			
	EPA-rich		2.38	0.12	Treatment*Night	1.50	.126
Sleep Fragmentation Index	Placebo	74	22.89	1.28	Treatment	.802	.451
	DHA-rich		20.80	1.11			
	EPA-rich		22.22	1.38	**Treatment*Night**	**1.90**	**.036**

[a] = significant difference between active and placebo groups below p <.050; **[b]** = significant difference between the active treatment groups below p <.050; [T] = trend towards a significant difference between active and placebo groups below p < .10

4.3.2.2 Subjective Measures

No significant main effects of Treatment were found in the LSEQ dataset. Though, there was a significant treatment by visit interaction effect for behaviour following waking, [*F* (2, 77.01) = 5.03, *p* =.009], however, post hoc comparisons identified no significant differences between any of the groups at either week 13 or 26.

A significant effect of treatment for feeling energetic was also identified [*F* (2, 79.35) = 3.545, *p* =.034], with post hoc comparisons identifying the DHA-rich (53.79; *p* = .041) but not the EPA-rich (64.94; *p* = .970) group as feeling significantly less energetic compared to placebo (62.47) (Figure 4.10).

A significant effect of treatment for feeling rested was identified [F (2, 76.42) = 4.71, p =.017], with post hoc comparisons identifying no significant difference between the active and placebo groups, but the DHA-rich group (53.55) ratings were significantly less rested than the EPA-rich group (64.94; p = .017) (Figure 4.10).

Finally, a significant effect of treatment for feeling ready to perform was identified [F (2, 84.12) = 3.211, p =.045], with post hoc comparisons identifying no significant difference between the active and placebo groups but the DHA-rich group (59.12) showed trends towards being significantly less ready to perform than the EPA-rich group (66.65; p = .075) (Figure 4.10).

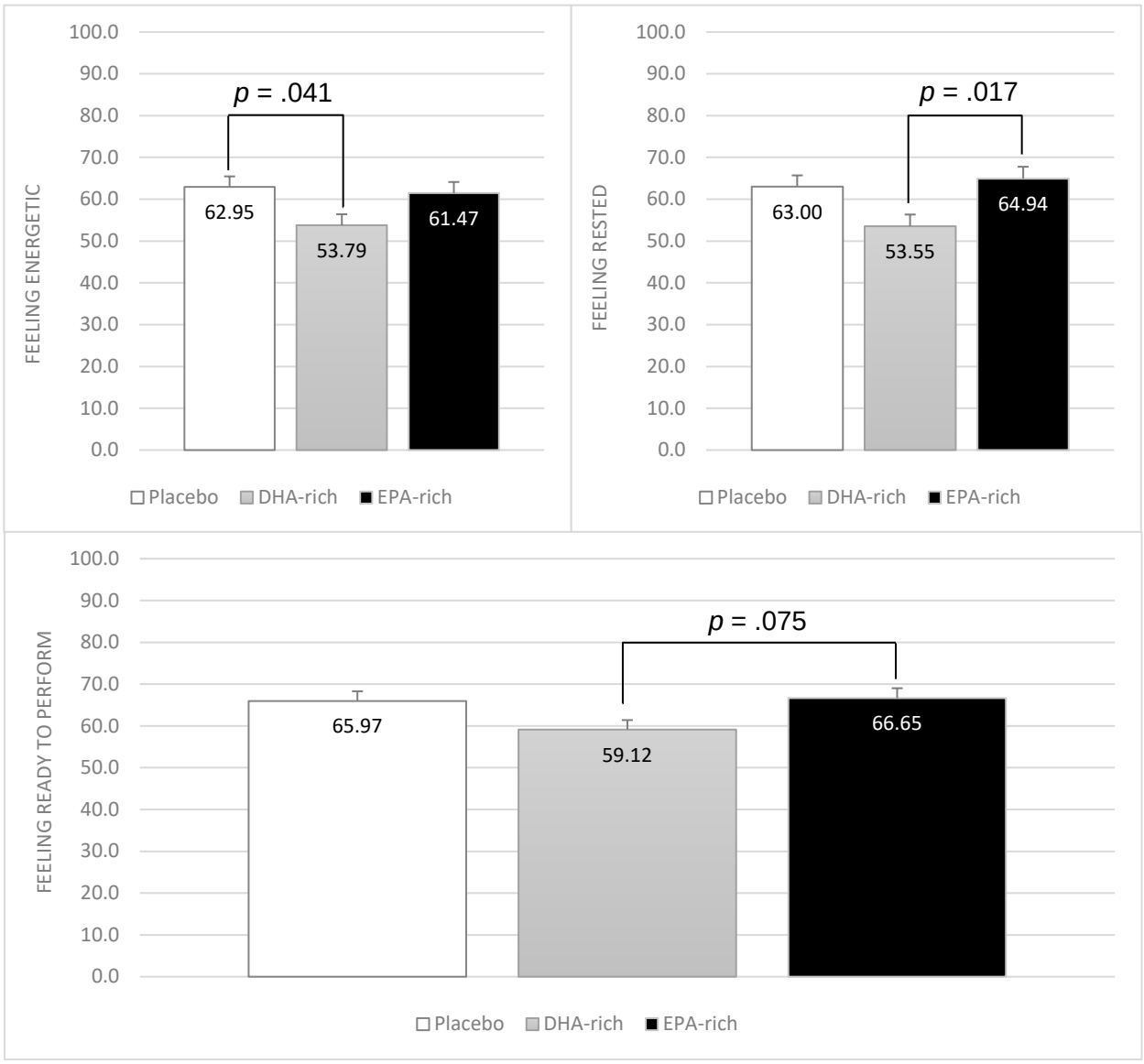

Figure 4.10. Estimated marginal means and standard error (SE) for post-dose ratings for feeling energetic **(Top Left),** feeling rested **(Top right)** and feeling ready to perform (**Bottom**), by treatment group.

Table 4.3. Subjective sleep outcomes for placebo, DHA-rich and EPA-rich treatment groups. Week 13 and week 26 estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

			Week 13		Week 26		Main Effects		
		n	Mean	SE	Mean	SE		*F*	*p*
Getting to Sleep (0 – 300)	Placebo	86	182.49	6.69	170.63	6.69	Treatment	.243	.785
	DHA-rich		177.13	7.28	177.05	6.56			
	EPA-rich		176.04	7.09	167.88	6.98	Treatment*Visit	.557	.575
Quality of Sleep (0 - 200)	Placebo	86	118.12	6.65	112.38	6.74	Treatment	.438	.647
	DHA-rich		118.47	7.19	118.64	6.53			
	EPA-rich		109.22	7.02	112.26	6.92	Treatment*Visit	.392	.677
Awake Following Sleep (0 – 200)	Placebo	86	107.23	6.01	113.52	6.12	Treatment	.518	.598
	DHA-rich		118.80	6.62	115.34	5.91			
	EPA-rich		112.80	6.37	113.05	6.27	Treatment*Visit	.379	.686
Behaviour Following Wakening (0 – 300)	Placebo	86	191.09	7.68	180.02	7.79	Treatment	.814	.447
	DHA-rich		181.98	8.38	165.39	7.57			
	EPA-rich		169.66	8.20	188.93	8.09	**Treatment*Visit**	**5.03**	**.009**
Rested (%)	Placebo	86	**66.21[a]**	3.67	59.80	3.74	**Treatment**	**4.71**	**.012**
	DHA-rich		**56.44[a]**	4.06	50.65	3.60			
	EPA-rich		68.79	3.91	61.09	3.82	Treatment*Visit	.034	.966
Energetic (%)	Placebo	86	**65.69[a]**	3.21	60.20	3.26	**Treatment**	**3.55**	**.034**
	DHA-rich		**56.35[a]**	3.56	51.23	3.16			
	EPA-rich		60.42	3.42	62.51	3.37	Treatment*Visit	1.05	.354
Relaxed (%)	Placebo	86	64.87	3.21	65.82	3.26	Treatment	1.37	.260
	DHA-rich		61.12	3.49	58.81	3.14			
	EPA-rich		65.60	3.40	65.47	3.35	Treatment*Visit	.191	.827
Irritable (%)	Placebo	86	26.74	3.70	27.70	3.77	Treatment	1.46	.238
	DHA-rich		31.95	4.09	35.10	3.64			
	EPA-rich		28.70	3.92	27.25	3.85	Treatment*Visit	.196	.822
Ready to Perform (%)	Placebo	86	65.70	2.81	66.23	2.86	**Treatment**	**3.21**	**.045**
	DHA-rich		**61.56[b]**	3.08	56.68	2.76			
	EPA-rich		**66.88[b]**	2.98	66.43	2.92	Treatment*Visit	.668	.515
Good Night's Sleep (%)	Placebo	86	65.88	4.30	59.53	4.38	Treatment	1.61	.205
	DHA-rich		63.06	4.72	50.85	4.23			
	EPA-rich		68.37	4.54	62.69	4.45	Treatment*Visit	.392	.677

[a] = significant difference between active and placebo groups below p= .050; **[b]** = significant difference between the active treatment groups below p= .050.

4.3.2.3 Urinary aMT6s

No significant main effects of treatment were found in the aMT6s dataset.

Table 4.4. aMT6s outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Total aMT6s (ng)	Placebo	67	15,289.27	1,267.50	Treatment	.558	.575
	DHA-rich		15,335.89	1,267.88			
	EPA-rich		13,585.56	1,346.06			
Bedtime aMT6s (ng)	Placebo	60	563.98	120.67	Treatment	2.12	.130
	DHA-rich		468.62	123.42			
	EPA-rich		805.34	117.08			

4.4 Discussion

Overall the results from the current study show that supplementation with the DHA-rich treatment resulted in a significant decrease in sleep latency and a significant increase in sleep efficiency compared to the placebo. Interestingly, despite these improvements in certain parameters of sleep in the DHA-rich group, it was also found that this group rated their state of feeling rested and feeling energetic significantly worse than the placebo and EPA-rich groups respectively. Additionally, a trend towards a significant increase in sleep efficiency was identified within the EPA-rich group compared to placebo and a significant decrease in sleep fragmentation index was observed in the DHA-rich group compared to placebo. Although, this effect was found to only be evident during the second night of the seven nights recorded and should therefore not be over interpreted. Additionally, the EPA-rich treatment resulted in a significant decrease in both total time in bed and total sleep time compared to the DHA-rich group, although no significant differences between the active and placebo groups were identified for these measures. Finally, no significant effects of treatment were identified for urinary aMT6s secretion.

The beneficial effects of DHA in reducing sleep latency and increasing sleep efficiency are consistent with previous animal models. For example, Yehuda (2003) investigated the effects of a DHA injection of 40 mg/kg and 10mg/kg AA for 4 weeks in 36 Sprague-Dawley rats and reported a significantly improved sleep profile measured via EEG and motor activity compared to the controls which included improved sleep efficiency, duration and reduced latency. The findings also provide further support to those of Montgomery et al., (2014) who found that 16 weeks' supplementation with DHA resulted in positive increases in several sleep parameters in children. These consistent findings across animal and human trials and within samples containing both children and healthy, young adults potentially provides further evidence in support of the physiological role of DHA in sleep processes. Indeed, in the underlying processes of sleep, DHA in particular has been found to positively influence the binding of serotonin to its receptors via its effects on increasing membrane fluidity (Heron et al., 1980; Paila, Ganguly & Chattopadhyay, 2010), increases serotonin levels in the prefrontal cortex (Owens & Innis, 1999; Chalon, 2006) and appears crucial for AA-NAT, one of the enzymes which transforms serotonin into melatonin (Catala, 2010; Peuhkuri et al., 2012). Additionally, DHA has also been found to induce neurogenesis (Nascimento et al., 2016; García-Cáceres & Tschöp, 2016), reduce inflammation (Pimentel et al., 2013) and regulate gene expression (Shaikh, Shaver & Shewchuk, 2018) within the hypothalamus, the brain region containing the suprachiasmatic nucleus (SCN), which is a well-known regulator of circadian rhythms (Buijs et al., 2019; Hastings, Maywood & Brancaccio et al., 2019). Taken together with the previous

findings within the literature, the current findings appear to provide further support for the efficacy of dietary supplementation with DHA in improving sleep efficiency and reducing sleep latency within healthy populations.

Within the current study, urinary aMT6s samples were collected in an attempt to measure one aspect of the biological regulatory processes of sleep and to provide a more holistic measurement of sleep. The null effects of treatment identified on urinary aMT6s appears to contradict the idea that the positive effects of DHA on sleep are underpinned by the effects DHA has on the melatonin/serotonin synthesis pathway. It would be reasonable to expect to observe an effect of treatment within the secretion of aMT6s in the DHA-rich groups as longer sleep times have previously been associated with increased secretion of melatonin throughout the night (Wehr, 1992; Aeschbach et al., 2003). However, the null findings concerning urinary aMT6s are consistent with those reported in other RCTs. For instance, Cornu et al., (2010) has previously identified no significant effects of 4 weeks' supplementation with a PUFA treatment on both urinary melatonin and aMT6s, collected overnight, compared to an olive oil placebo. Interestingly, Cornu et al., (2010) also identified no differences between the groups for both LSEQ scores and actiwatch scores which contradicts the current study's findings. Possible explanations for these inconsistent findings may be due to the relatively short study duration of only 4 weeks and composition of the PUFA supplement employed by Cornu et al., (2010). Additionally, the consistent null findings in both studies concerning aMT6s could potentially be due to the short overnight urine sample collection method that was employed in both studies. It may be that overnight collection of aMT6s provides too short a window to observe changes in production and diurnal variation of melatonin. Indeed, Benloucif et al., (2008) have described a method of collecting urine samples every 2 to 8 hours over a 24- to 48-hour period as the most practical method, to estimate the global timing and amount of melatonin production. Additionally, they also note that the higher melatonin levels present in blood plasma may actually allow for greater resolution and sensitivity than sampling by urine or saliva. Therefore, it may be that the current study's methodology of collecting overnight urinary samples was not adequate or sensitive enough to detect changes in the production or diurnal variation of melatonin.

Due to the null findings of treatment on aMT6s it could also be that DHA impacted sleep parameters via other mechanisms than the melatonin synthesis pathway. For example, insufficient sleep and poor sleep quality has previously been seen to invoke a pro-inflammatory response via increased cytokine secretion (Vgontzas et al.,1999; Irwin, Olmstead & Carroll, 2016; Milrad et al., 2017) with a range of cytokines previously being found to be associated with sleep, including IL-1, tumour necrosis factor (TNF) and IL-6 (Opp, 2005;

Moltivala & Irwin, 2007). Additionally, Shear et al., (2001) have shown that total sleep deprivation for 4 days is associated with significant increases in IL-6 and TNF-α and sleep deprivation has been shown to increase IL-6 and TNF-α the morning following sleep deprivation compared to controls as well as the transcription of IL-6 and TNF-α messenger RNA (Irwin et al., 2006). These findings indicate that sleep deprivation increases blood levels of several pro-inflammatory cytokines and that changes in cytokine levels can affect both sleep and subjective ratings of sleep (Simpson & Dinges, 2007). Due to the anti-inflammatory actions of n-3 PUFAs (see section 1.4.3), it could be that the observed effects of supplementation with the DHA-rich oil on sleep in the current study are a result of lowered levels of inflammatory cytokines previously associated with poor sleep quality (Milrad et al., 2017). Indeed, Ferrucci et al., (2006) has identified total n-3 PUFA status to be associated with lower levels of the pro-inflammatory markers (IL-6, IL-1ra, TNFα, CRP) seen to be increased in sleep deprived individuals and individuals with poor sleep quality. Taken together, this evidence may help to explain the null findings concerning aMT6s within the current study as the observed effects on sleep following supplementation with the DHA-rich oil may have been a result of decreased levels of pro-inflammatory cytokines rather than via impacting upon the melatonin synthesis pathway. If this is true, then this may also help to explain the trend towards increased sleep efficiency observed following supplementation with the EPA-rich treatment.

Concerning the negative subjective ratings identified in the DHA-rich group, the findings are inconsistent with the actigraphy data which identified improvements across several sleep parameters following supplementation with the DHA-rich oil. One potential explanation of this may come from research into populations suffering from insomnia. For instance, Feige et al., (2013) explains how a major enigma of insomnia research constitutes the frequently noted discrepancy between the subjective experience of sleep (measured by sleep questionnaires) and the “objective” polysomnographic (PSG) findings. PSG studies often demonstrate that patients suffering from insomnia tend to underestimate their nocturnal sleep time (Carskadon et al., 1976; Frankel et al., 1976; Adam et al., 1986; Feige et al., 2008; Manconi et al., 2010) leading to terms such as ‘sleep state misperception’ for patients with a relatively normal sleep continuity and architecture (Edinger & Krystal, 2003), in spite of large subjective complaints of disturbed sleep. Harvey and Tang (2012) highlight the possible role of both psychological and physiological factors within sleep and even suggest that attempting to define sleep parameters, such as sleep latency, may not actually be useful in capturing the subjective experience of falling asleep, because the parameter is operationalised in a very crude way. Additionally, focusing more specifically on sleep architecture may offer further explanations into these findings with Feige et al., (2008) previously showing that differences between

subjective and objectively measured wake times were correlated with the amount of REM sleep in insomnia patients i.e. patients with higher amounts of REM sleep tended to report more minutes of subjective wakefulness. Overall, these findings may offer an explanation into why discrepancies between 'objective' and subjective measures may occur within sleep research, consequently, rendering it difficult to interpret the findings as either positive or negative due to these discrepancies. It could be that more in depth PSG studies are required to accurately observe changes in sleep architecture that may occur from supplementation with DHA- and EPA-rich oils to help to further explain any discrepancies between objective and subjective measures. These studies could also help to identify what aspects of sleep may contribute to the discrepancy or indeed identify if there are particular stages of sleep that are more or less liable to manipulation with dietary intake of n-3 PUFAs.

Similarly, new data emerging regarding the natural diurnal variations of EPA and DHA within the body may provide further evidence for some potential impacts on sleep architecture, especially when supplements are taken at bedtime. For instance, Jackson et al., (unpublished data; Appendix X) has observed that, night time dosing (22:00hr) with the same SMEDS formulated oils resulted in significant increase in DHA in blood plasma for 6 hours post dose compared to placebo, but in contrast, concentrations of EPA in plasma had a similar peak but were also significantly greater for the entire 24 hour period compared to placebo. These increases in circulating levels of DHA and EPA overnight following night time supplementation may potentially affect the natural sleep/wake cycle in some way, especially when a substantial amount of research supports the influence of both DHA and EPA on the serotonin/melatonin synthesis pathway, which in turn is linked to SWS. Perhaps a natural tendency for circulating levels of EPA and DHA to decrease overnight may actually be synchronised with the sleep/wake cycle and altering this rhythm results in changes to the sleep/wake cycle in some way. If this is true and changes in EPA and DHA levels through the night are related to changes in sleep architecture then this may potentially explain why there were differences identified between the groups in relation to the subjective experience of sleep.

The findings from Jackson et al., (unpublished data; Appendix X) in relation to the significant increase in circulating EPA in plasma for the entire 24hour period compared to placebo may also potentially offer insights into the observed decrease in total time in bed and total sleep time observed in the current study for the EPA-rich group. Very little focus has been applied to the effects of EPA supplementation on sleep parameters previously and to our knowledge no study has ever assessed the effects of supplementation with an EPA-rich treatment on sleep in healthy samples. However, the findings from Jackson et al., (unpublished data; Appendix X) suggest that night time supplementation with EPA results in increased circulating

plasma levels of EPA throughout the night, which may then further effect serotonin levels, as EPA is known to impede the formation of E_2 series prostaglandins, which inhibit the release of serotonin (Schlicker, Fink & Göthert, 1987; Günther et al., 2010). In line with this, Monti (2011) describes how the results obtained from genetic, neurochemical, electrophysiological and neuropharmacological studies conducted over the past three decades propose that serotonin functions predominantly to promote wakefulness and to inhibit REM sleep. Based on this information it could potentially be that increased levels of circulating EPA overnight leads to an increased release of serotonin via inhibition of the formation of E_2 series prostaglandins and therefore an upregulated promotion of wakefulness that results in a decrease in total sleep time. However, it should be noted that although the EPA-rich group reported the shortest sleep times, this did not appear to lead to a reduction in the quality of sleep. In fact, a trend towards a significant increase in sleep efficiency, compared to placebo, was observed along with no increase in the time spent awake, number of awakenings and no decreased ratings of subjective sleep quality. This may suggest that although the total amount of sleep was lowest in the EPA-rich group, it did not result in the need for additional sleep and therefore could potentially suggest that the 'refreshing' effects of sleep were actually achieved in a shorter amount of time after supplementation with EPA. Overall, this once again supports the need for measurements of sleep architecture to explain more thoroughly the effects of EPA on sleep and to potentially highlight where sleep is being reduced throughout the night, as the averaged in and out of bed times available from the current study appears to show that the EPA group arose from bed earliest but did not go to bed earliest (Appendix VI). Additionally, the observed significant increases in EPA levels following supplementation with the DHA-rich oil (Appendix XI) further complicates this effect, as although EPA levels rose within the DHA group they slept for the longest duration. Therefore, more detail into the effects of EPA and DHA on the specific stages of sleep is still warranted to explain these findings in greater detail.

The current study is one of the first to investigate the separate effects of DHA and EPA, in a sample of healthy, young adults, with a rigorous study protocol that allowed for both objective and subjective measurements of sleep. Additionally, the measurement of aMT6s offered the potential to gain insight into biological processes that may underpin the effects of n-3 PUFAs on sleep parameters. However, there were several issues that arose within the study in relation to the collection of actigraphy data as well as with the subjective recording of sleep/wake times. Issues with incomplete and even unusable actigraph data, as a result of improper use of the equipment by participants, resulted in a reduced sample size in the actigraphy datasets, although this is in line with missing data in previous actigraphy studies (Price et al., 2012; Montgomery et al., 2014). Furthermore, there were issues concerning

incomplete subjective sleep diaries (see Appendix III) and that the use of only 'approximate bedtime' and 'approximate waking time the following morning' as measures were not precise enough to determine the time in/out of bed as well as time asleep/waking. Upon reflection, these sleep diaries should have also asked 'time getting into bed' and 'time getting out of bed' as these factors are separate from the time the participant fell asleep/woke up. Additionally, and as stated previously, it can be argued that the overnight collection of urinary aMT6s was not precise enough to detect changes in the production of melatonin. Therefore, collecting samples over a longer period of time may have been more appropriate.

Future research in this area should consider either the use of 24/48 hour collection periods of urinary aMT6s or the collection of blood samples to allow for greater resolution and sensitivity than sampling via urine or saliva (Benloucif et al., 2008). This longer collection period was considered within the current study but would have placed further constraints on participants and would have increased the financial demands of the study. Furthermore, it was decided that overnight collection would be adequate to allow the effects of n-3 PUFA supplementation to be evident. However, as no effects of treatment on urinary aMT6s were identified it may be advisable that future studies measuring urinary aMT6s employ longer collection periods. Similarly, future research may be benefited from the inclusion of measurements of inflammatory markers such as pro-inflammatory cytokines. As increases in pro-inflammatory cytokines are related to poor sleep quality (Milrad et al., 2017), it may be useful to measure the effects of increased dietary intake of EPA and DHA on these markers in relation to sleep parameters to allow for a more comprehensive measurement of the regulatory processes of sleep. Finally, the employment of PSG in future studies to more accurately investigate the 'objective' measures of sleep would allow for more specific insights into the potential effects of n-3 PUFAs on the architecture of sleep in relation to the amounts of REM and NREM sleep and would allow for further investigation of the specific stages of sleep that are effected by supplementation with EPA and DHA.

The findings from the current study appear to provide additional support for n-3 PUFAs, particularly DHA, at improving parameters of sleep. These include the reduction of sleep latency and an overall increase in sleep efficiency although these positive actigraphy measures of increased sleep quality were not consistent with subjective ratings following supplementation with DHA. Investigations into the relationship between n-3 PUFAs and the serotonin/melatonin synthesis pathway and how this specifically effects the architecture of sleep is required to progress the research area further. Nonetheless, as beneficial effects of sleep were identified following supplementation with n-3 PUFAs in healthy, young adults these

data help to provide additional evidence towards the role of n-3 PUFAs in facilitating healthy regulation of crucial behaviours and brain functioning.

CHAPTER 5: SUPPLEMENTATION WITH DHA- AND EPA-RICH OILS HAS NO EFFECT ON MEASURES OF MEMORY CONSOLIDATION AND MORNING ALERTNESS IN HEALTHY, YOUNG ADULTS

5.1 Introduction

Dietary DHA and EPA have been previously linked to a number of positive measures of cognitive function and brain health, such as increased neural efficiency (Bauer et al., 2011; 2014a), reductions in reaction times of complex cognitive tasks (Bauer et al., 2011; 2014a; Stonehouse et al., 2013) and reduced grey matter atrophy in certain brain regions (Samieri et al., 2012). Recent evidence from Aryal et al., (2019) has begun to show that the relationship between intake of n-3 PUFAs and positive cognitive outcomes may be underpinned, at least in part, by the role n-3 PUFAs might play in the processes of memory consolidation including synaptic plasticity and LTP (Beck et al., 2000; Cao et al., 2009). Memory consolidation relates to the conversion of memory representations from being heavily dependent on the hippocampus to being rooted in cortical networks (Rothschild, Eban & Frank, 2017). According to the 'two-stage model' (Buzsáki, 1996; Buzsáki, 2015) of consolidation, the first stage occurs during behaviour, when the hippocampus quickly encodes numerous aspects of an experience via changes in synaptic strengths, while the second stage occurs during SWS with the newly acquired hippocampal information being repeatedly replayed, driving plasticity in the neocortex resulting in the longer-term storage of the memory (Rothschild, Eban & Frank, 2017). Previously, increased dietary intake and supplementation with n-3 PUFAs has shown positive learning and memory consolidation effects in a number of animal studies. Dietary DHA has been seen to ameliorate learning-related spatial memory deficits in DHA-deficient rats (Bazan, Molina & Gordon, 2011) and 12 weeks' dietary EPA enhances spatial cognition learning ability in rats (Hashimoto et al., 2009). Additionally, Pan et al., (2010) has identified that appropriate doses of DHA resulted in improved spatial learning performance as well as retention during the Morris water maze task (Morris, 1984) in two-month old rats. Similarly, Lim and Suzuki (2000) have found that n-3 supplemented mice performed better in a maze compared to mice fed a control diet. Recently, Alquraan et al., (2019) identified that n-3 PUFA supplementation prevented memory impairments induced by single-prolonged stress whilst also normalising antioxidant mechanisms in the hippocampus. DHA-derived mediators have also been found to impact upon learning and memory with Hashimoto et al., (2015) showing that DHA-derived mediators demonstrated a significant negative correlation with the number of reference memory errors, in rats, during a radial maze task.

Previously, Beck et al., (2000) have provided evidence to suggest that the positive effects of DHA and EPA on plasticity and LTP observed previously in animal studies may also occur in humans. However, studies that do measure the effects of EPA and DHA supplementation on synaptic plasticity have not yet been conducted in humans, as this is often measured via post-mortem examination of the brain in animal studies. As a result, most of the current knowledge of the effects of n-3 PUFAs on neural plasticity and LTP comes from animal studies with little evidence existing from human samples. Furthermore, although a large number of studies assessing the relationship between n-3 PUFA intake and cognition have been conducted, only a small number have employed learning-memory tasks likely to induce LTP and memory consolidation. A randomised, placebo-controlled study by Dalton et al., (2009) identified that children aged 7-9 years performed better on the Hopkins verbal learning test battery, a reading test and a spelling test following 6 months daily consumption of a fish flour bread spread (~892mg of DHA/week) compared to an equivalent spread without fish flour. Additionally, Yurko-Mauro et al., (2010) conducted a randomised, double-blind, placebo-controlled, clinical study with a sample of 485 healthy participants, aged over 55 years with age-related cognitive decline and found that 24 weeks supplementation with 900mg/d DHA improved learning and memory function, measured via the CANTAB paired associate learning task. Furthermore, Kulzow et al., (2016) measured the effects of 26 weeks' supplementation with 2,200mg/d n-3 PUFAs (1,320mg/d EPA + 880 mg/d DHA) on object-location memory and performance on the Rey auditory verbal learning test, in a sample of 44 cognitively healthy older adults aged 50-75 years. Recall of object locations was found to be significantly better following supplementation with the n-3 PUFAs but no significant effect was seen for performance on the verbal learning test, compared to placebo. However, the effects of n-3 PUFAs on measures of learning-memory are not unequivocal as Dangour et al., (2010) reported no significant performance effects on the California Verbal Learning Test, in a sample of 867 cognitively healthy older adults aged 70-79 years, following 24 months supplementation with 200mg/d EPA + 500mg/d DHA compared to an olive oil placebo.

One potential way in which DHA and EPA could support the processes involved in memory consolidation is via the effects they have on serotonin, as studies have shown these n-3 PUFAs to effect serotonin receptor functioning (Patrick & Ames, 2015), increase serotonergic neurotransmission and concentrations within the frontal cortex (Owens & Innis, 1999; Chalon, 2006) and via reducing the production of E_2 series prostaglandins that inhibit serotonin release (Rees et al, 2006; Vedin et al., 2010). These effects may be important with regards to the consolidation of memories as serotonin is known to play a major role in sleep processes during both waking and sleeping periods (Cespuglio, 2018), with Monti and Jantos (2003) demonstrating that systemic injection of flesinoxan, a potent agonist of the serotonin receptor

5-HT_{1A}, increases wakefulness and reduces SWS and REMS in rats. Therefore, as there is evidence to support the influence of serotonin on amounts of SWS, which has previously been linked to memory consolidation (Rothschild, Eban & Frank, 2017), these mechanisms potentially represent one pathway in which n-3 PUFAs could influence memory consolidation. Moreover, night time dosing with n-3 PUFAs may further influence the effects of EPA and DHA on serotonin as findings from Jackson et al., (unpublished data; Appendix X) suggest that night time supplementation with EPA and DHA results in increased circulating plasma levels of EPA and DHA throughout the night. This increase in the circulating plasma levels of EPA and DHA within the body could therefore allow for the effects of EPA and DHA on serotonin to be enhanced throughout the night, due to the increased bioavailability of the n-3 PUFAs in circulation. Although the precise timings of the effects of EPA and DHA on serotonin are currently not well documented within the literature, previous literature has indeed identified associations between higher plasma concentrations of n-3 PUFAs, particularly DHA, and higher concentrations of serotonin metabolites (Hibbeln et al., 1998).

As both DHA and EPA have been seen to influence serotonin the majority of the studies that have been conducted in humans thus far have been limited by exclusively focussing on DHA-rich supplements, with only Kulzow et al., (2016) placing a focus on the effects of an EPA-rich supplement and with no study employing both a DHA-rich and EPA-rich supplement within the same trial. As well as influencing serotonin, previous research has begun to highlight the importance of not only DHA, but EPA as well, in the mechanisms that underpin memory consolidation. For example, both EPA and DHA have been seen to increase neurite outgrowth in adult and aged rats (Robson et al., 2010) and both appear to enhance differentiation of neural stem cells at similar amounts in cultured rat neural stem cells. Additionally, Katakura et al., (2013) has shown that EPA can increase the expression of transcription factor Hes6 leading to decreased activity of the neuronal differentiation inhibitor Hes1 therefore promoting neuronal differentiation, a key component of neurogenesis (Balu & Lucki, 2009). Both Dyall et al., (2016) and Katakura et al., (2013) describe how DHA and EPA have been seen to have differing effects on proliferation of neural stem cells and that these effects appear to be mediated via different cell signalling pathways. Furthermore, Kawashima et al., (2010) has found that dietary EPA modulates LTP of the CA1 hippocampal region and protects against neurodegeneration by modulating synaptic plasticity and activating the PI3-kinase/Akt pathway, which is important for regulating the cell cycle, possibly via its direct effects on neurons, glial cells and its ability to increase brain DHA levels. Overall, it appears that evidence does exist that supports the role of EPA on mechanisms that underpin learning and memory consolidation. However, there is currently a clear lack of trials in the research area that investigate the potentially separate effects of both dietary DHA and EPA on the processes

that underpin learning and memory consolidation in human samples with Kulzow et al., (2016) being the only study thus far to supplement with an EPA-rich oil in humans. Therefore, it appears that human RCTs that measure the effects of both DHA-rich and EPA-rich supplements on aspects of learning and memory are still warranted.

In addition to this limitation of previous research, the human RCTs that have been conducted thus far which employ learning-memory tasks have been conducted in either children or older adults. No study thus far has focussed on the learning and memory effects of n-3 PUFAs in healthy, young adults. As healthy, young adults appear to require both improvements in n-3 PUFA levels and sleep, whilst being free from any other health complaints and intake of concomitant medications. It could be that this population are able to provide a clear insight into the effects of supplementation with n-3 PUFAs on learning and memory consolidation. Overall, the studies conducted thus far in humans do appear to suggest that there is a positive effect of DHA, specifically on learning and memory, in both children and older-adult populations. However, the studies to date have not employed methodological paradigms that allow for SWS memory consolidation to occur between the learning and recall phases. A methodological paradigm that allowed for memory consolidation to occur overnight between the learning and recall phase of the tasks may allow for underlying memory consolidation processes to be measured via the level of success during the recall phase the following day, potentially acting as a proxy measure of memory consolidation. Indeed, Holz et al., (2012) has previously employed a similar overnight study design to investigate the effects of sleep architecture on memory consolidation, identifying that both EEG sigma power and slow-wave activity were positively correlated with the pre–post-sleep consolidation of declarative (word list) memories. Therefore, it appears that a similar overnight study design may also be appropriate to investigate the effects of increased dietary intake of n-3 PUFAs on learning and memory consolidation.

Collectively, the distinct lack of human data regarding the individual, potentially separate, effects of dietary DHA and EPA on learning and memory consolidation in healthy, young adult populations requires investigation. Therefore, the present study will aim to investigate the effects of 26 weeks' supplementation with 1.2g/d of either DHA-rich, EPA-rich or an olive oil placebo, delivered at bedtime, on both verbal and visual learning and memory retention in healthy, young adults, consuming low amounts of oily fish. Performance was assessed using verbal and visual learning and recall tasks performed prior to going to sleep and upon waking, respectively, in an attempt to allow the mechanisms that underpin memory consolidation to occur (Holz et al., 2012). Additionally, the current study's methodology will allow for investigation into the effects of both the DHA-rich and EPA-rich supplements on early morning

performance on certain cognitive tasks and measures of morning alertness. Therefore, completion of both attention and executive function tasks, as well as subjective ratings of morning alertness will be administered along with the recall phase of the learning tasks completed upon waking. Measurements of morning alertness and attention upon waking may also provide further insights into the effects n-3 PUFAs have on the sleep-wake cycle as n-3 PUFAs are known to influence serotonin which has been previously associated with wakefulness (Monti and Jantos 2003; Monti, Torterolo & Pandi-Perumal, 2016; Saito et al., 2018) and therefore it is hypothesised that supplementation with the EPA-rich and DHA-rich oils may result in improved ratings of morning alertness and performance on attention tasks upon waking. These measurements also offer the opportunity to provide further insights into the unexpected findings identified in the study described in Chapter 4 concerning the negative VAS ratings of feeling rested, energetic and ready to perform in the morning, following supplementation with the DHA-rich treatment.

5.2 Materials and Methods

5.2.1 Design

This study employed a randomised, placebo-controlled, double-blind, parallel groups design. With participants being randomly assigned to one of three treatment groups (placebo, DHA-rich, EPA-rich; see section 2.2). A similar study design to Holz et al., (2012a; 2012b) was employed to measure overnight memory consolidation and was adapted within the current study to be administered via COMPASS. This involved completion of a number of learning tasks prior to going to sleep and completion of attention and executive function tasks, morning alertness ratings and recall tasks upon waking.

5.2.2 Participants

One hundred and eighty-three males and females aged 25-49 years were recruited and of the one hundred and eighty-three participants screened one hundred and sixty-nine were enrolled into the study and one hundred and fifty-one completed all requirements. Of the eighteen participants who withdrew: eight were lost to follow up; four withdrew for personal reasons; two were withdrawn after randomisation due to having a BMI above 35; two withdrew due to gastrointestinal upset; one participant withdrew due to an unrelated illness; and one withdrew due to becoming pregnant. This sample size was calculated based on a small effect size (d = 0.20). Given this effect size, an a priori calculation of the size of sample required in order to detect a significant difference between the groups given 80% power and an alpha level of 0.05, is fifty-one participants per treatment arm. Additionally, in order to account for any potential dropouts the total sample size was increased by 10%. This resulted in fifty-six participants per treatment arm or one hundred and sixty-eight participants overall. Power calculations were made using GPower 3.1.3. Participant disposition through the trial is displayed in Figure 5.1 and their demographic data in Table 5.1.

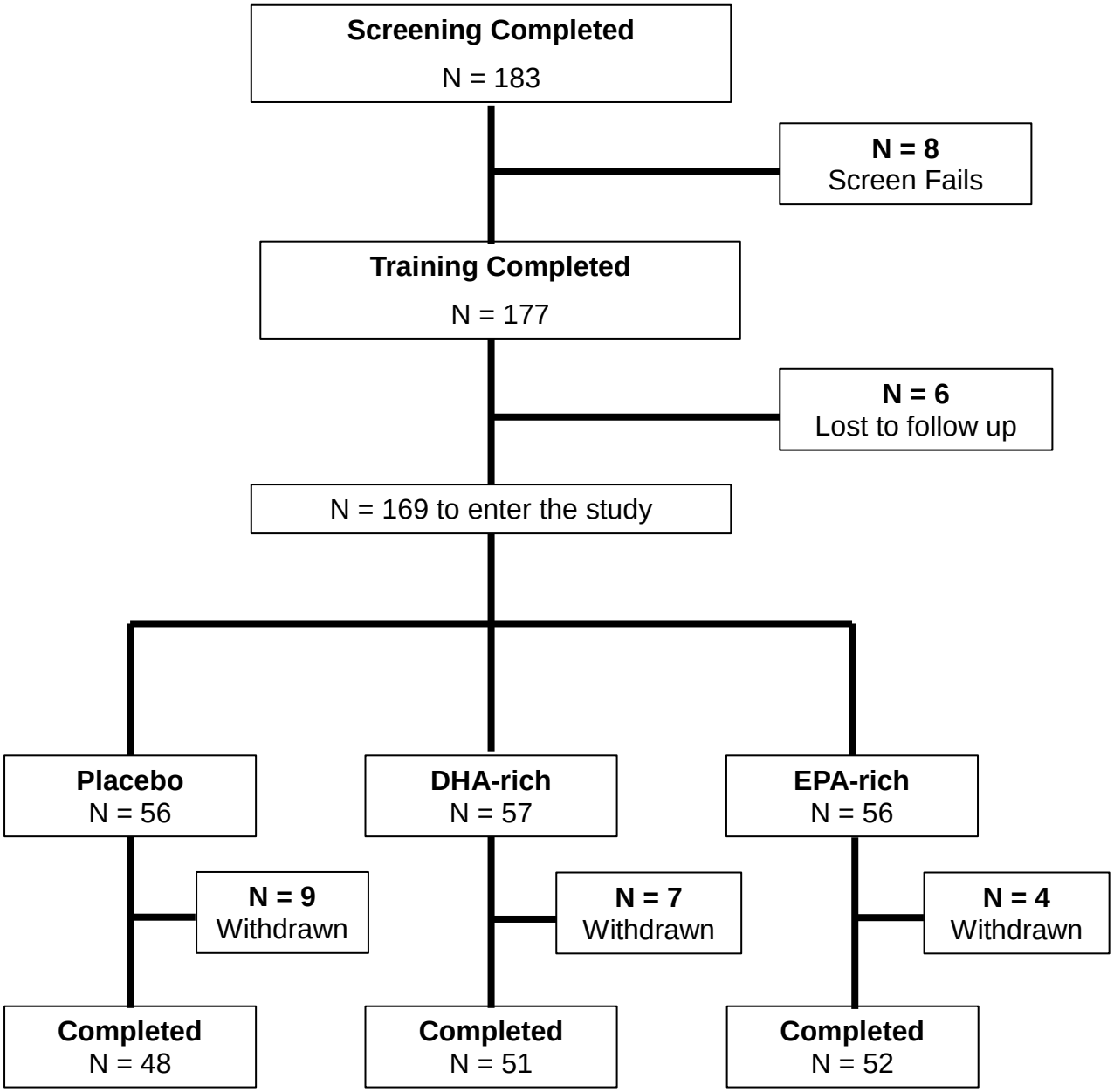

Figure 5.1 Participant disposition through the trial. Figure depicts the disposition of participants throughout the study, culminating in N = 151 of the 169 who were randomised.

Table 5.1. Participant demographic information and baseline characteristics. Means and Std. Deviation (*sd*) are given where appropriate. *F* or χ^2 and *p* values are given for separate one-way ANOVAs or Chi-Square tests that were conducted on this baseline data by treatment group.

		Baseline		Main Effects		
		Mean	Sd		*F* / χ^2	*p*
N (M/F)	Placebo	16/40	-	Treatment	1.78	.410
	DHA-rich	11/46	-			
	EPA-rich	11/45	-			
% of EPA in RBC	Placebo	0.83	0.04	Treatment	.383	.682
	DHA-rich	0.78	0.04			
	EPA-rich	0.81	0.04			
% of DHA in RBC	Placebo	4.72	0.13	Treatment	1.55	.215
	DHA-rich	4.48	0.16			
	EPA-rich	4.86	0.16			
n-3 index	Placebo	5.54	0.16	Treatment	1.41	.248
	DHA-rich	5.25	0.18			
	EPA-rich	5.67	0.19			
Age (Years)	**Placebo**	**37.15**	0.87	**Treatment**	**3.20**	**.043**
	DHA-rich	**33.71**	1.05			
	EPA-rich	**36.20**	10.4			
Systolic BP	Placebo	122.20	1.73	Treatment	.347	.708
	DHA-rich	123.82	1.72			
	EPA-rich	122.01	1.63			
Diastolic BP	Placebo	80.82	1.42	Treatment	.597	.552
	DHA-rich	82.43	1.52			
	EPA-rich	80.25	1.46			
Heart Rate (BPM)	Placebo	71.89	1.43	Treatment	.074	.929
	DHA-rich	72.28	1.60			
	EPA-rich	72.74	1.66			
Weight (Kg)	Placebo	73.14	1.76	Treatment	.329	.720
	DHA-rich	75.08	2.13			
	EPA-rich	73.13	1.98			
Height (cm)	Placebo	168.37	1.18	Treatment	.064	.938
	DHA-rich	167.81	1.16			
	EPA-rich	168.03	1.02			
BMI (Kg/m^2)	Placebo	25.73	0.50	Treatment	.607	.546
	DHA-rich	26.54	0.58			
	EPA-rich	25.83	0.61			
Years in Education	Placebo	16.78	0.36	Treatment	.193	.825
	DHA-rich	16.46	0.38			
	EPA-rich	16.55	0.37			
Fruit & Vegetable (portions per day)	Placebo	4.04	0.25	Treatment	.261	.771
	DHA-rich	4.00	0.30			
	EPA-rich	3.94	0.20			
Alcohol (Units per day)	Placebo	1.09	0.13	Treatment	1.39	.251
	DHA-rich	1.11	0.11			
	EPA-rich	1.36	0.13			

5.2.3 Cognitive Tasks

5.2.3.1 Word List Learning & Recall

Participants were presented sequentially with 15 words selected at random from a large bank of words derived from the MRC Psycholinguistic Database and matched for word length, frequency, familiarity and concreteness. Stimulus duration was one second, with an inter-stimulus duration of one second. Following this, 30 words, comprising the 15 words presented during the stimuli presentation period plus 15 distractor words were presented, with the participant making a yes/no response indicating whether the word was in the original set or not. Task outcomes were accuracy (% correct) and reaction time for correct responses (msec). This sequence was repeated 5 times with the same 15 'target words' but different 15 'decoy words' every sequence to ensure maximum retention of the 15 'words to be remembered'.

A total displacement score was calculated as the sum of the percentage of errors on the five learning trials and a learning index was calculated as the average relative difference in performance between trials. This was calculated following the procedure adapted from Kessels et al., (2006) by subtracting the error percentage of word recognition during learning trial 1 (A) from the error percentage of word recognition during learning trial 2 (B) and then dividing this value by the error percentage of learning trial 1 (A). This same calculation was then made for; trial 2 (B) subtract trial 3 (C) divide error percentage of trial 2 (B); trial 3 (C) subtract trial 4 (D) divide error percentage of trial 3 (C) and trial 4 (D) subtract trial 5 (E) divide error percentage of trial 4 (D). The summed values of these calculations were then divided by 4 to generate a learning score for each participant. These calculations were completed for each of the testing visits. These calculations are visualised below:

$$\frac{\left(\frac{A-B}{A}+\frac{B-C}{B}+\frac{C-D}{C}+\frac{D-E}{D}\right)}{4}$$

Figure 5.2. Formula used to create the learning index scores where the letters A-E indicate one of the five learning trials.

During the recall phase participants were given 60 seconds to write down as many of the 15 words that they were presented with during the learning phase the night prior to their study visit. Outcomes are number of words correct and number of errors.

5.2.3.2 Computerised Location Learning & Recall

Participants were shown a 5x5 grid containing 10 pictures of objects and asked to remember the location of the objects as accurately as possible. The presentation duration was 15 seconds. They were then shown an empty grid and asked to relocate the objects to the correct location shown to them previously. There was no time limit for responding. This was repeated five times during the learning phase. For each of the five learning trials, a displacement score was calculated as the sum of the errors made for each object (calculated by counting the number of cells the object had to be moved both horizontally and vertically in order to be in the correct location). A total displacement score was calculated as the sum of the displacement scores on the five learning trials. A learning index was also calculated using the same formula outlined in Figure 5.2 (Kessels et al., 2006) as the average relative difference in performance between trials.

During the recall phase, participants were again asked to place the objects in the correct location on the empty grid as presented during the learning phase with no further prompting. The delayed trial was scored for displacement, and a delayed displacement score was then calculated as the difference between displacement score on the final learning trial and the delayed trial.

5.2.3.3 Simple Reaction Time

An upwards pointing arrow was displayed on the screen at irregular intervals. Participants responded as quickly as they could as soon as they saw the arrow appear. Task outcome was reaction time for correct responses (msec). Fifty stimuli were presented.

5.2.3.4 Digit Vigilance

A fixed number appeared on the right of the screen and a series of changing numbers appeared on the left side of the screen. Participants were required to respond when the number on the left matched the number on the right. Task outcomes were accuracy (%), reaction time to correct responses (msec) and number of false alarms. This timed task lasted for five minutes.

5.2.3.5 Peg & Ball

Two configurations were shown on the screen. In each there was three coloured balls (blue, green, red) on one of 3 pegs. The configuration at the top of the screen was the goal configuration and participants needed to arrange the balls on the starting configuration (shown

in the centre of the screen) to match the position of balls in the goal configuration. They needed to do this in the least number of moves possible with difficulty increasing as the task progressed. Task outcomes were number of errors, average thinking time (msec) and speed of performance (msec). Five stimuli at each of the three levels (3, 4 and 5 moves) were completed.

5.2.3.6 Alertness VAS

Participants rated how alert they felt by making a mark on a line representing 0-100% with the end points labelled “not at all” (left hand end; 0) and “extremely” (right hand end; 100)

5.2.4 Procedure

All study visits took place at Northumbria University’s Brain, Performance and Nutrition Research Centre (BPNRC). Potential participants attended the site for an initial screening visit. The principal investigator or designee discussed with each participant the nature of the trial, before providing the participant information sheet previously given to the participant. No restrictions were placed on the participants prior to this visit. Following informed consent eligible participants underwent training on the computerised cognitive tasks. The training session followed standard operating procedures to decrease the chance of learning effects during the main trials. This entailed the participants completing one full length version of the tasks on a tablet computer. Once this session was completed to the required standard the participant was then eligible to be enrolled and randomised into the trial.

The day before the baseline, week 13 and week 26 assessments participants collected tablet computers from the BPNRC to take home with them. At bedtime, the night before the respective testing visit, participants were instructed to complete the learning phase of the word list learning and computerised location learning tasks. Once the learning tasks had been completed, on-screen instructions informed participants to lock the tablet computer until the morning. In the morning, within 5 minutes of waking, participants completed the simple reaction time, digit vigilance and peg and ball tasks along with the alertness VAS. Participants were then instructed to return the tablet computer back to the BPNRC. Once the tablet computer was returned to the research centre (either 7am, 8.30am or 10am), the participant was then instructed to complete the recall phase of the word list and computerised location learning tasks.

Before completing the morning cognitive tasks participants were asked to avoid alcohol and to refrain from intake of ‘over the counter’ medication for 24 hours and caffeine for 18 hours

prior to each respective assessment. Participants were contacted to remind them of the requirements prior to each assessment. On the morning of the baseline assessment, participants were requested to eat their usual breakfast or no breakfast if they usually skipped breakfast at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products). At the end of the baseline assessments participants were provided with the first batch of capsules (3 bottles of 100 capsules each) and given a diary in which to record their daily consumption of the capsules along with any adverse events and concomitant medications (see Figure 5.3 for schematic depicting the study overview).

Participants also reported to the BPNRC during week 13 to collect the tablet computer to take home for the night and return the following morning. Once the tablet computer was returned and the morning cognitive tasks and recall phases had been completed, participants were provided with the second batch of capsules (3 bottles of 100 capsules each). Participants also brought with them their diary, which was replaced with a new diary to complete between week 13-26 and any remaining unused treatment capsules, so that treatment compliance could be calculated.

The week 26 testing assessment was identical to the baseline assessment in all aspects apart from collecting in the subject diaries, all remaining treatments, completion of a treatment guess questionnaire (see Appendix II) and finally a full debrief once all assessments were completed. During both the baseline and week 26 visits participants were also required to provide a 6 mL venous blood sample to determine blood fatty acid profile.

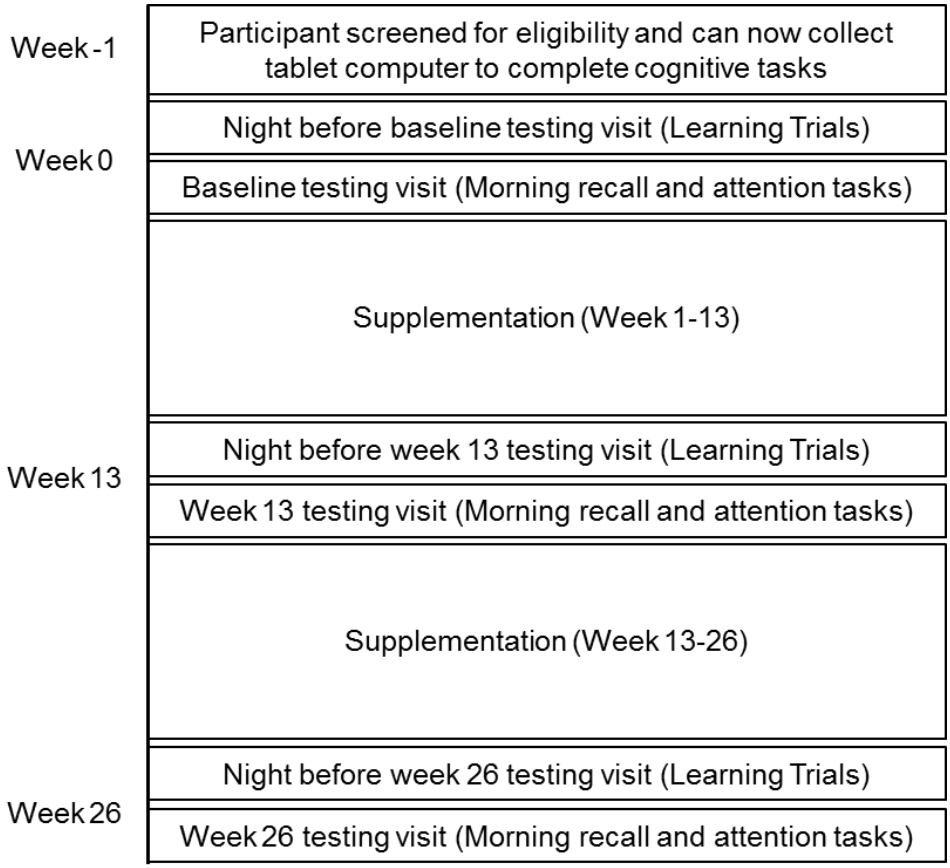

Figure 5.3. Scematic showing the study progression from enrollment to completetion across the 26 weeks. Learning tasks were completed the night before the baseline, week 13 and week 26 and morning attention, executive functioning and recall tasks and alertness ratings were completed upon waking the following morning.

5.2.5 Statistical Methods

As age was identified in the analysis of the demographics data as a factor that differed significantly between the three treatment groups, participants' age was entered as a covariate into each model to account for this difference. Additionally, all post-hoc analyses were conducted using Sidak controlled group comparisons.

5.2.5.1 Learning, Recall and Morning Cognitive Performance

The data were analysed using the same linear mixed model procedure described previously with all models using an identity covariance matrix. The fixed factors appearing in all models were treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13 or week 26). Respective pre-dose values and age were entered into each model as a covariate. Subject was also entered as a random factor in the model for delayed word recall, computerised location learning, computerised location recall, simple reaction time, digit vigilance and peg & ball.

5.3 Results

5.3.1 Compliance

For participants who completed the study compliance was observed to be very good in all three groups (96.43% Placebo, 98.33% DHA-rich, 96.41% EPA-rich) with a one way ANOVA identifying no significant differences for compliance by treatment group [F (2, 148) = 2.12, p = .123]. A Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was completed at the end of the final visit and revealed no significant differences in participant's ability to correctly identify whether they had been administered an active or placebo treatment between the three groups [χ^2 (2) = 3.56, p = .169].

5.3.2 Learning and Recall

A trend towards a significant main effect of treatment for total location learning displacement was identified [F (2, 131.20) = 2.94, p =.056]; with post hoc comparisons identifying the DHA-rich (16.80; p = .049) but not the EPA-rich (14.25; p = .539) group as showing significantly higher displacement scores and therefore more errors during the learning trials compared to placebo (11.74). Although, no significant differences were identified between the two active treatments (p = .523) (Figure 5.4).

A significant main effect of treatment for word recognition was identified [F (2, 1306.00) = 4.42, p =.012]; with post hoc comparisons identifying the DHA-rich (851.67; p = .009) but not the EPA-rich (869.23; p = .281) group as having significantly faster reaction times during the word learning trials compared to placebo (885.18). Although, no significant differences were identified between the two active treatments (p = .373) (Figure 5.4).

No other significant effects of treatment or significant interaction effects between treatment and visit were identified for any of the other outcomes from the learning or recall tasks.

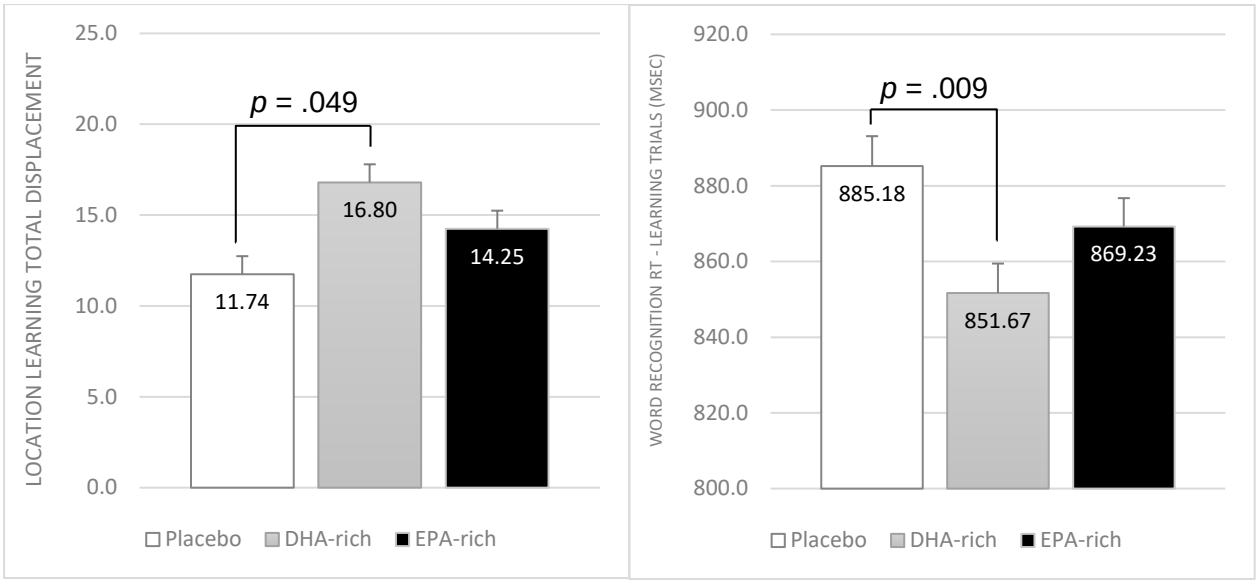

Figure 5.4. Estimated marginal means and standard error (SE) for week 26 values of (**left**) location learning total displacement and (**right**) word recognition RT during the learning trials, by treatment group.

Table 5.2 Computerised location learning analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Total Location Displacement	Placebo	151	**11.74***	1.47	**Treatment**	**2.94**	**.056**
	DHA-rich		**16.80***	1.45			
	EPA-rich		14.25	1.46	Treatment*Visit	.501	.607
Location Learning Index	Placebo	152	0.86	0.03	Treatment	2.23	.112
	DHA-rich		0.79	0.03			
	EPA-rich		0.80	0.03	Treatment*Visit	2.32	.102
Delayed Location Recall	Placebo	151	-2.08	0.52	Treatment	.148	.863
	DHA-rich		-2.31	0.52			
	EPA-rich		-2.48	0.52	Treatment*Visit	1.59	.206
Total Word Displacement	Placebo	146	5.80	0.48	Treatment	.432	.650
	DHA-rich		6.40	0.50			
	EPA-rich		5.89	0.49	Treatment*Visit	1.07	.346
Word Learning Index	Placebo	152	0.62	0.03	Treatment	.658	.519
	DHA-rich		0.58	0.03			
	EPA-rich		0.63	0.03	Treatment*Visit	1.18	.310
Word Recognition RT (msec)	Placebo	144	**885.18***	7.92	**Treatment**	**4.42**	**.012**
	DHA-rich		**851.67***	7.78			
	EPA-rich		869.23	7.50	Treatment*Visit	.595	.552
Delayed Word Recall (Correct)	Placebo	150	4.09	0.29	Treatment	.239	.788
	DHA-rich		4.33	0.28			
	EPA-rich		4.35	0.28	Treatment*Visit	.437	.647
Delayed Word Recall (Inorrect)	Placebo	150	1.36	0.16	Treatment	.257	.774
	DHA-rich		1.42	0.15			
	EPA-rich		1.51	0.15	Treatment*Visit	.505	.605

***** = significant difference between the placebo and an active treatment group below p< .050.

5.3.3 Morning Cognitive Performance

Analysis identified no significant main effects of treatment or significant interactions between treatment and visit for any of the cognitive tasks.

Table 5.3 Cognitive task analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Simple Reaction Time (msec)	Placebo	147	405.60	7.37	Treatment	1.52	.222
	DHA-rich		416.20	7.38			
	EPA-rich		398.13	7.24	Treatment*Visit	.136	.873
Digit Vigilance Accuracy (%)	Placebo	151	83.40	1.54	Treatment	1.52	.220
	DHA-rich		79.89	1.55			
	EPA-rich		80.34	1.51	Treatment*Visit	.070	.932
Digit Vigilance Correct Reaction Time (msec)	Placebo	151	514.74	4.39	Treatment	.341	.712
	DHA-rich		519.58	4.41			
	EPA-rich		515.55	4.36	Treatment*Visit	.876	.418
Digit Vigilance False Alarms	Placebo	151	11.02	1.00	Treatment	1.62	.202
	DHA-rich		13.55	1.00			
	EPA-rich		11.89	0.99	Treatment*Visit	.597	.552
Peg & Ball Thinking Reaction Time (msec)	Placebo	152	2926.11	218.38	Treatment	1.08	.341
	DHA-rich		3325.51	216.68			
	EPA-rich		2934.77	217.70	Treatment*Visit	.180	.836
Peg & Ball Time to Complete (msec)	Placebo	152	8363.12	274.09	Treatment	.368	.693
	DHA-rich		8595.21	271.49			
	EPA-rich		8685.02	272.78	Treatment*Visit	1.12	.329
Peg & Ball Number of Errors	Placebo	152	4.45	0.50	Treatment	1.58	.210
	DHA-rich		5.39	0.50			
	EPA-rich		4.19	0.50	Treatment*Visit	2.39	.096
Alertness VAS (%)	Placebo	152	52.79	2.01	Treatment	.452	.637
	DHA-rich		54.58	1.98			
	EPA-rich		51.94	2.00	Treatment*Visit	1.17	.312

5.4 Discussion

The current study was the first to investigate the potentially separate effects of dietary supplementation with DHA and EPA, in a sample of healthy, young adults, on learning and overnight memory consolidation. Overall, the findings from the current study showed that there was a significant effect of treatment for total location learning displacement scores during the five learning trials, with the DHA-rich group making more errors across the learning trials compared to the placebo group. Additionally, a significant effect of treatment was identified for word recognition reaction time during the five learning trials, with the DHA-rich group performing significantly faster compared to placebo. Both the DHA-rich and EPA-rich treatments significantly increased levels of DHA and EPA in RBC compared to placebo (Appendix XI). However, no significant effects of treatment were identified during completion of the simple reaction time, digit vigilance and peg and ball tasks or ratings of morning alertness.

Concerning the significant effect of supplementation with the DHA-rich treatment on word recognition reaction times during the learning trials, the finding is consistent with previous studies. For instance, Yurko-Mauro et al., (2010) have previously identified a beneficial effect of 24 weeks' supplementation with 900 mg/d of DHA on both immediate and delayed verbal recognition memory scores. However, this was in a sample of 485 healthy, older adults. Additionally, although the word recognition task employed within the current study was immediate rather than delayed, the findings can still be considered consistent with Stonehouse et al., (2013) who identified a reduction in episodic memory reaction times. This composite episodic memory measure comprised of Zscores from delayed word and picture recognition reaction times, following 26 weeks' supplementation with a DHA-rich treatment (1.16g/d DHA + 0.1g/d EPA). Both the current study and Stonehouse et al., (2013) employed 26 week supplementation periods in samples of healthy, young adults with similar dosages for the DHA-rich treatment. However, it should be noted that it was delayed versions of the recognition tasks that comprised Stonehouse et al., (2013) episodic memory domain in comparison to the immediate recognition employed within the current studies learning trials. Nonetheless, these data appear to provide evidence for the effectiveness of supplementation with DHA at improving reaction times during episodic memory tasks in healthy adults.

Conversely, a negative effect of DHA supplementation on total location displacement scores was also identified. This finding is inconsistent with findings from Kulzow et al., (2016) who identified significantly better recall of object locations and Yurko-Mauro et al., (2010) who identified a beneficial effect of DHA on visuospatial learning and episodic memory tasks.

However, it should be noted that both Kulzow et al., (2016) and Yurko-Mauro et al., (2010) samples were aged 50 years and over which may potentially explain these inconsistencies. Additionally, Dalton et al., (2009) has previously identified positive effects of DHA on measures of verbal learning, reading and spelling when compared to placebo in children aged 7-9 years. Overall, it could be that the inconsistencies between the current studies negative findings and previous positive findings in relation to DHA and learning-recall tasks could be a result of the different samples used. However, this finding is difficult to interpret due to the fact that no effects of treatment were identified for the location learning index scores, which represents the improvement in performance as the learning trials progress. As there was no effect of treatment for location learning index scores, it can be concluded that all three treatment groups appeared to learn the locations of the pictures at the same rate as the trials progressed. However, the increased displacement score identified within the DHA-rich group suggests that this group made more errors on average per learning trial, even though their performance still improved at the same rate as the other groups as the trials progressed. Furthermore, this negative effect was not present on the recall phase the following morning. One potential explanation of this effect could be due to the significantly faster reaction times observed for word recognition reaction times during the learning trials in the DHA-rich group compared to placebo. Although there was no measure of reaction times for the location learning trials, the faster reaction times in the DHA-rich group during the word recognition learning trials may suggest that there was a speed-accuracy trade off in this group when completing the learning trials. The speed-accuracy trade off refers to a phenomenon by which a decision maker speeds up their response at the expense of accuracy during completion of a task. This may potentially offer an explanation as to why the DHA-group had the worst location learning displacement scores but the fastest word recognition reaction times during the learning trials. Additionally, it could also simply be that the significant finding concerning location learning displacement scores is a chance finding, with the significance value of this main effect being equivalent to a 1 in 20 chance of finding this specific effect. As a result, this finding is difficult to interpret and should be taken with caution.

Overall, the null effects of supplementation with both the EPA-rich and DHA-rich treatments during completion of the recall tasks, used as a proxy measure of memory consolidation, seem to contradict previous findings. Previously, positive effects of n-3 PUFAs have been observed on LTP and synaptic plasticity, processes that underpin memory consolidation (Rao et al., 2007; Cao et al., 2009; Kawashima et al., 2010; Vetrivel et al., 2012; Ferreira et al., 2013; Dyall et al., 2016; Abdel-Maksoud et al., 2017; Milovanovic & De Camili, 2017; Aryal et al., 2019), as well as in learning memory tasks (Lim and Suzuki, 2000; Hashimoto et al., 2009; Pan et al., 2010; Bazan, Molina & Gordon, 2011 Hashimoto et al., 2015; Alquraan et al., 2019).

However, it should be noted that the current literature is heavily based on animal models and not human trials. Nonetheless, there seems to be an inconsistency between the findings in animal models and the current study. In addition to the previously discussed issues of sample ages in n-3 PUFA RCTs, a potential factor that may have also influenced the null findings in the current study could be the methodological paradigm that was employed. To our knowledge, this is the first RCT investigating the effects of both DHA-rich and EPA-rich supplements on verbal and spatial learning and memory consolidation overnight. As a result, the current study's methodological paradigm and the use of tablet computers to measure aspects of learning and memory, outside of the laboratory, has not previously been used and may not be sensitive enough to detect the desired effects of treatment in a non-controlled environment. For example, Holz et al., (2012a) employed a similar five trial word-list learning-recall task during their study investigating the effects of sleep architecture on memory consolidation and identified that both EEG sigma power and slow-wave activity were positively correlated with the pre–post-sleep consolidation of declarative (word list) memories. However, the researchers in this study did not include any distractor words, as the current study did, which may not have been necessary in the learning of the correct words and may have potentially made the word-list learning task too difficult. Indeed, the current study reported an average delayed word recognition score of four words whilst Holz et al., (2012a) report almost twelve correct words being recalled the following morning, suggesting that the distractor words within the current study did make the word recognition task more difficult than in previous studies. Furthermore, the original location learning test was developed to display the recall phase of the tasks only 30 minutes post learning trials (Bucks & Willison, 1997). However, the current study did not ask participants to recall the location of the objects until the following morning, potentially making it too difficult to achieve a satisfactory performance on the tasks. Certainly, Haskell-Ramsay et al., (2012) has previously reported mean delayed location recall scores down to -1.3 in healthy, young adults 30 minutes after the learning trials were administered, whereas the current study reported scores almost double this down to -2.5. Although, both Haskell-Ramsay et al., (2018) and the current study reported similar location learning displacement and location learning index scores, the larger decrease in delayed location scores in the current study does suggest that the location learning task was made more difficult with the extended delay in recall times in the current study that may have impacted upon participants performance on the task.

Another interesting finding from the current study is the null effect of treatment on the measures of morning alertness, particularly, the scores on the alertness visual analogue scale. The findings from the current study are inconsistent with the results identified in Chapter 4, which found that supplementation with the same DHA-rich treatment resulted in significantly

lower ratings of feeling rested and energetic in the morning compared to placebo and ratings of feeling ready to perform compared the EPA-rich group. This appears to suggest that, although participants rated that they felt less rested and energetic following the DHA-rich treatment in Chapter 4, they do not appear to feel less alert. Furthermore, these previous negative ratings are not paired with poorer performance on attention and executive functioning tasks upon waking. Similarly, as there was no effect of treatment observed for ratings for feeling awake following sleep in Chapter 4 the current findings concerning morning alertness appear to corroborate this even though the current VAS was completed upon waking rather than retrospectively on the LSEQ. These findings may again call for the measurement of sleep architecture through PSG or EEG to help to further interpret these findings. It could be that structural changes in sleep architecture occur following supplementation with DHA-rich treatments resulting in feelings related to how refreshed you feel following sleep but not on how alert or attentive you feel. Indeed, previous research has identified the impacts of sleep fragmentation on daytime functioning (Stepanski et al., 1987; Roehrs et al., 1994; Philip, Stoohs & Guilleminault, 1994; Martin et al., 1996; Martin et al., 1999), which may suggest that any structural changes related to intake of DHA in the architecture of sleep may then also impact upon the restorative feelings of sleep during the day time.

Although the current study was the first to investigate the effects of both a DHA-rich and EPA-rich supplement on learning and memory consolidation, it did have a number of drawbacks that should be discussed. One of the limitations with the current study could be the way in which the learning-memory tasks were employed, as comparisons to previous studies appears to show that including distractor words during the word learning trials and extending the delayed recall phase of both the word and location learning tasks increased the difficulty of the tasks and impacted upon participant's performance (Holz et al., 2012a; Haskell-Ramsay et al., 2018). Furthermore, the use of the recall phase of the learning-memory tasks as a proxy measure of memory consolidation may not have been the most appropriate measure. For example, tDCS could potentially have been used instead as a more direct measurement of LTP and the mechanisms underpinning learning and memory. A growing body of evidence suggests that tDCS can have beneficial effects on long-term memory, synaptic plasticity and adult neurogenesis (Di Lazzaro et al., 2013; Coffman et al., 2014; Leone et al., 2014, 2015; Podda et al., 2014), as well as a consistent body of evidence that indicates that tDCS exerts modulatory effects of LTP (Ranieri et al., 2012; Rohan et al., 2015; Podda et al., 2016; Kronberg et al., 2017). The measurement of tDCS-induced LTP and synaptic plasticity pre and post supplementation with DHA and EPA may potentially provide a more comprehensive methodological paradigm.

Future research in this area should focus on developing a methodological paradigm that can be proven to be capable of measuring learning and memory consolidation overnight. As there are several practical issues regarding participants staying within the laboratory overnight, the current study's paradigm, which allows participants to complete cognitive tasks outside of the laboratory, would be the most ideal if it can be proven to be reliable. Future research should aim to identify whether COMPASS tasks performed on tablet computers outside of the laboratory setting are consistent with outcomes of the same tasks on the COMPASS software conducted within the laboratory under the supervision of the researcher. Secondly the use of the location learning test performed overnight needs to be found to be consistent with the same location learning test performed over the shorter periods of time that the test was designed for. Additionally, other measures such as tDCS could be employed in future studies to more directly and objectively measure the mechanisms that underpin memory consolidation or perhaps biomarkers, such as BDNF, could be used as a biological measure of synaptic plasticity. BDNF levels are known to have potent effects on synapses (Leal et al., 2015) and neurogenesis (Hearing et al., 2016), are seen to be readily detectable in human serum and can be measured in serum, plasma or whole blood (Polacchini et al., 2015).

Despite these limitations, the present study is the first to investigate the potentially separate effects of DHA-rich and EPA-rich supplements, dosed at bed time, on learning, memory consolidation and morning alertness, in a sample of healthy, young adults. The study identified a negative finding in relation to the ability to identify the correct locations of objects paralleled with improved reaction times during the learning trials following 26 weeks' supplementation with DHA-rich treatments. These findings may suggest that there was a speed-accuracy trade off within the group supplemented with DHA. It is also important to note that this study was experimental and the first of its kind within the research area. As a result, it seems appropriate not to over emphasise the null findings identified within the current study concerning memory consolidation outcomes. Instead, it seems logical to conclude that further investigation is required, with more rigorous methodological paradigms capable of measuring learning and memory consolidation in greater detail, in order to advance the research area further.

CHAPTER 6: SUPPLEMENTATION WITH EPA-RICH, BUT NOT DHA-RICH, OILS IMPROVES GLOBAL COGNITIVE FUNCTION AND REACTION TIMES ON EPISODIC MEMORY AND EXECUTIVE FUNCTION TASKS IN HEALTHY, YOUNG ADULTS.

6.1 Introduction

Fifty percent of the dry weight in an adult brain is known to be composed of lipids (Hamilton et al., 2007; Bruce, Zsombok & Eckel, 2017), 35% of which are n-3 and n-6 PUFAs (Haag, 2003). This accretion of lipids within the brain signifies that they play an important role in maintaining both the structure and function of the brain. Indeed, McNamara et al., (2010) has previously identified the role of n-3 PUFAs, particularly DHA, in the functioning of the dorsolateral prefrontal cortex in children, a brain region widely associated with higher cognitive functions such as working memory, executive functions and cognitive flexibility (Kaplan, Gimbel & Harris, 2016). Furthermore, other studies have shown the importance of adequate consumption of n-3 PUFAs with n-3 deficiencies depleting brain fatty acid composition in rhesus monkeys (Neuringer et al., 1986) resulting in serious behavioural derangements (Reisbick et al., 1994) and neuronal atrophy in the dorsolateral and dorsomedial prefrontal cortex of mice (Larrieu et al., 2014). There is also evidence to support the importance of n-3 PUFAs in a number of other aspects of brain functioning, such as neurotransmission (Song et al., 2007; Forster et al., 2008; Grosso et al., 2014; Patrick & Ames, 2015; Sugasini & Lokesh, 2015), neuro-inflammation and neuroprotection (Farooqui & Farooqui, 2016; Satyanarayanan et al., 2018), hippocampal neurogenesis (Kawakita, Hashimoto & Shido, 2006; Beltz et al., 2007) and synaptic plasticity (Cao et al., 2009; Robson et al., 2010; Aryal et al., 2019). Due to the crucial role of n-3 PUFAs in maintaining brain structure and functioning, increased consumption throughout the lifespan has been linked to improved cognitive outcomes in older adults in several epidemiological studies (Kalmijn et al., 2004; Morris et al., 2005; Morris et al., 2009). However, the findings from human RCTs thus far that have assessed the effects of n-3 PUFAs on cognitive function have varied widely in their methodology and have yielded inconclusive results. Despite the now large number of trials that have investigated this area, three reviews have reported limited effects of n-3 PUFA supplementation on cognitive function. The first by Rangel-Huerta & Gil (2017) concluded that the current evidence indicates that n-3 PUFA supplementation during pregnancy or breastfeeding has no effect on the skills or cognitive development of children in later stages of development and it is still unclear if n-3 PUFAs can improve cognitive development or prevent cognitive decline in young or older adults. The second review found that supplementation with n-3 PUFAs has a beneficial effect on episodic memory in samples characterised as suffering from MCI (Yurko-Mauro, Alexander

& Van Elswyk, 2015). Finally, a marginal effect of supplementation on working memory in samples who have low baseline n-3 PUFA status was reported by the third review (Cooper et al., 2015). Several methodological factors that have been identified as potentially contributing to these weak conclusions include dose, duration of treatment, sample population and appropriate selection of outcome measures.

One of the largest n-3 PUFA RCTs conducted to date by Chew et al., (2015) supplemented 3,501 participants at risk for developing late age-related macular degeneration with 1g/d n-3 (350mg/d DHA + 650mg/d EPA) for 5 years, assessing a range of cognitive functions found no significant effects of treatment on measures of cognitive function. Likewise, Dangour et al., (2010) supplemented 867 cognitively healthy, older adults with either 700mg/d of n-3 (500mg/d DHA + 200mg/d EPA) or an olive oil placebo for 2 years. However, administering a battery of cognitive tests revealed no effects of treatment on cognitive function or cognitive decline. To date, no RCT in adult populations free from neurological disease, administering a dose of less than 720mg/d DHA has reported beneficial effects on measures of cognitive function regardless of the intervention duration (Freund-Levi, et al., 2006; Chiu et al., 2008; Johnson et al., 2008, Yurko-Mauro et al., 2010; Rondanelli et al., 2012; Scheltens et al., 2012; Sinn et al., 2012; Lee, Shahar, Chin & Yusoff, 2013; de Waal et al., 2014; Witte et al., 2014; Pardini et al., 2015). Furthermore, both Rogers et al., (2008) and van de Rest et al., (2008) have also reported null effects on cognition following 850mg/d DHA suggesting that even higher dosages should be administered in RCTs measuring cognitive function.

Concerning the effects of DHA in particular and due to the fact that several previous n-3 PUFA RCTs have identified null effects on cognitive function whilst supplementing with doses lower than 850mg/d DHA, one study stands out within the research area. Stonehouse et al., (2013) has previously reported positive effects on cognitive function in 176 healthy, young adults aged 18-45 years following 26 weeks supplementation with a DHA-rich treatment (1.16g/d DHA + 0.17g/d EPA) compared to a high-oleic acid sunflower oil placebo. Performance on individual cognitive tasks, administered via COMPASS, were standardised and grouped into specific memory domains for the analysis and both performance and reaction times in the episodic memory and working memory domains were seen to improve in the DHA group. This is one of the most convincing studies conducted thus far to demonstrate cognitive effects of dietary supplementation with DHA in otherwise healthy, young adults. As this study employed a higher dosage of DHA than the previously identified studies reporting null effects and due to the rigorous study design, sufficient supplementation duration and appropriate selection of outcome measures future RCTs should also aim to employ similar study protocols.

Although the study by Stonehouse et al., (2013) has several strengths, there remains an opportunity to expand on the findings via inclusion of an EPA-rich treatment in future trials. For instance, Luchtman and Song (2013) have previously reported that EPA specifically may be of interest in regards to cognitive function as it has been observed previously to help modulate brain neurochemistry and long term potentiation (Robson et al., 2010; Kawashima et al., 2010; Katakura et al., 2013; Dyall et al., 2016), suggesting that EPA, despite its trace contents in the brain can still modulate neuronal function. However, there has been a lack of RCTs thus far that have aimed to investigate the effects of dietary EPA on cognitive outcomes, despite the recent evidence from short term trials that indicate treatment with EPA in healthy adults may be relevant to cognitive function in this population. There is evidence that dietary intake of EPA, in particular, may be beneficial for increasing neural efficiency (Bauer et al., 2014b). Previously, Bauer et al., (2011; 2014a) have conducted two intervention studies comparing the effects of 4 weeks' supplementation with either a DHA-rich (420mg/d DHA + 160mg/d EPA) or EPA-rich fish oil (590mg/d EPA + 140mg/d DHA). The authors reported improved reaction times during completion of a Stroop and choice reaction time task as well as enhanced neural recovery of the magnocellular visual system (Bauer et al., 2011) and reduced activation in the anterior cingulate cortex (Bauer et al., 2014a) following supplementation with the EPA-rich fish oil supplement. Overall, the authors concluded that their data comprises evidence that increased dietary intake of EPA may result in enhanced neural efficiency. There is also initial evidence that EPA may be effective for reducing mental fatigue during times of high cognitive demand with Jackson et al., (2012c) identifying reduced ratings of mental fatigue during a cognitive demand battery, administered via COMPASS, following 12 weeks' supplementation with an EPA-rich oil (300mg/d EPA + 200mg/d DHA) in 159 healthy, young adults aged 18-35 years. However, Jackson et al., (2012c) reported no effects of supplementation on cognitive function or mood outcomes. The lack of cognitive findings by Jackson et al., (2012c) may have been influenced by both a relatively small sample size, low dosage and short supplementation period. In comparison, Stonehouse et al., (2013) identified several effects of supplementation with DHA on cognitive function in their sample of healthy, young adults, whilst also employing a larger sample, higher doses of n-3 and a 26 week supplementation period. This suggests that future RCTs need to ensure employment of an adequate sample size, n-3 dose and supplementation period. Together, both Bauer et al., (2011; 2014a) and Jackson et al., (2012c) provide evidence to support the potential beneficial effects of EPA supplementation on aspects of cognition in healthy, young samples. However, the relatively short study durations employed only provides evidence for the transient effects of increased dietary supplementation with EPA on cognitive function. Therefore, further investigation into the effects of supplementation over longer time periods is still warranted to

help to provide a more comprehensive understanding of the chronic effects of EPA on cognition.

In addition to improvements in cognition, intake of n-3 PUFAs has also been associated with improvements in mood. A meta-analysis conducted by Martins (2009) including 28 RCT's, identified that 13 studies using supplements containing more than 50% EPA and 8 studies using pure ethyl-EPA identified significant reductions in depression. In contrast, 3 studies using pure DHA and 4 studies using supplements containing more than 50% DHA did not report significant reductions in depression. The authors concluded that EPA may therefore be more efficacious than DHA in treating depression. Although evidence does exist for the role of n-3 PUFA intake being beneficial for reducing depression (Martins, 2009; Panagiotakos et al., 2010; Larrieu & Layé, 2018; Othman et al., 2018), RCTs addressing the causal nature of the relationship between n-3 PUFAs and mood in healthy adults are less clear (Giles et al., 2015). Only a few published empirical studies have assessed the effects of n-3 PUFA supplementation on mood in young, healthy populations. For instance, Fontani et al., (2005a; 2005b) assessed the effects of 35 days supplementation with 2.8 g/day n-3 PUFAs (1.6g/d EPA + 0.8g/d DHA) in 33 healthy, young adults aged 22-51 years in a parallel groups design. Effects of supplementation on mood were measured using the POMS and found increased feelings of vigour and reduced feelings of anger, anxiety, fear, depression and confusion in the n-3 PUFA group. It should be noted however, that although these studies did administer an olive oil placebo to a parallel group and the results for this group were null after the trial period, the results from the active group were not actually ever compared to the placebo group during the analysis and therefore should be interpreted cautiously. Similarly, Antypa et al., (2009) has also found that 2.3 g/day n-3 PUFA (1.74g/d EPA + 0.25g/d DHA) for 4 weeks reduced feelings of POMS fatigue in 56 healthy individuals and Giles et al., (2015) has employed a double-blind, placebo-controlled design in 72 young adults who were randomised to receive 2800 mg/day fish oil (1680 mg EPA +1120 mg DHA) or olive oil control for 35 days. The researchers identified that ratings of anger and confusion were increased in the olive oil group but remained stable in the fish oil group following a trier social stress test. However, fish oil had no further effects on mood or cognitive function. Additionally, Watanabe et al., (2018) have identified that there was a significant difference between total depression and anxiety scores on the hospital anxiety and depression scale (HADS) at 52 weeks in a sample of 80 healthy, young adults following 13 weeks' supplementation with 1.2g/day EPA and 0.6 g/day DHA compared to placebo. Finally, Kiecolt-Glaser et al., (2011) have also identified a reduction in anxiety symptoms after 12 weeks' supplementation with 2500 mg/day (2085mg EPA, 348mg DHA) in a sample of 68 healthy, young adults, compared to placebo. However, Kiecolt-Glaser et al., (2012) found no effect of 16 weeks' supplementation of either 1.25 g/day

or 2.5 g/day on outcomes of the Centre for Epidemiological Studies Depression Scale in 68 overweight, otherwise healthy, middle-aged and older adults. Overall, the RCTs that have been conducted in healthy individuals thus far have yielded inconclusive results whilst again employing relatively short intervention periods, small sample sizes, or they report within treatment effects rather than placebo comparisons. As a result, it may be that larger scale placebo controlled RCTs supplementing over longer periods may be necessary to reveal enduring effects of increased dietary intake of n-3 PUFAs on measures of mood in healthy adults.

Largely, a more direct comparison between the effects of increased dietary intake of EPA and DHA within studies is missing within the RCTs that have been conducted thus far in relation to cognitive function and mood in healthy individuals. It would be valuable to be able to draw more definite conclusions about the potential cognitive enhancing effects of increased consumption of EPA compared to DHA (Luchtman & Song, 2013) or the effects of increased DHA compared to EPA in relation to measures of mood, potentially due to the effects DHA has been seen to have on certain neurotransmitters related to mood (Chalon, 2006; Patrick & Ames, 2015). Indeed, Drouin et al., (2019) have recently described how the full extent of both the separate and similar effects of DHA and EPA are still poorly understood and Dyall, (2011) describes how these fatty acids can no longer be considered to have mechanistic equivalence and should be viewed as distinct, albeit related, chemical species. Additionally, a greater understanding of the individual roles of the n-3 PUFAs on brain health and function are also still required (Dyall, 2015). Overall, there appears to be a lack of consistency in the findings related to both cognition and mood in RCTs that employ healthy, young adult samples, implying that there is still a need for further investigation of the effects of n-3 PUFAs in this population via large scale RCTs. The present study therefore aims to build upon the findings of Stonehouse et al., (2013) by comparing the effects of both EPA-rich and DHA-rich treatments following a similar rigorous study protocol, whilst also adding measures of mood to the design. For this reason, the present study will investigate the effects of 26 weeks' supplementation with 1.2g/d of either DHA-rich, EPA-rich or an olive oil placebo on a range of cognitive tasks measuring aspects of episodic memory, working memory, information processing speed, attention and global cognition as well as ratings of subjective mood, alertness and mental fatigue.

6.2 Materials and Methods

6.2.1 Design

This study employed a randomised, placebo-controlled, double-blind, parallel groups design. With participants being randomly assigned to one of three treatment groups (placebo, DHA-rich fish oil, EPA-rich fish oil; see section 2.2).

6.2.2 Participants

Three hundred and sixty-six males and females aged 25-49 years were recruited and of the three hundred and sixty-six participants screened three hundred and thirty-seven were enrolled into the study and three hundred and ten completed all requirements. Of the twenty-seven participants who were withdrawn from the study; fifteen participants were lost to follow up; five withdrew for personal reasons; two were withdrawn after randomisation due to having a BMI above 35; two withdrew due to gastrointestinal upset; two withdrew due to an unrelated illness; and one withdrew due to becoming pregnant. This sample size was calculated based on a medium effect size (d = 0.42) identified by Stonehouse et al., (2013) for performance on the episodic memory domain following 26 weeks supplementation with a DHA-rich oil. Given this effect size, an a priori calculation of the size of sample required in order to detect a significant difference between the groups given 80% power and an alpha level of 0.05, is one hundred and two participants per treatment arm. Additionally, in order to account for any potential dropouts the total sample size was increased by 10%. This resulted in one hundred and twelve participants per treatment arm or three-hundred and thirty-six participants overall. Power calculations were made using GPower 3.1.3. Participant disposition through the trial is displayed in Figure 6.1 and their demographic data in Table 6.1.

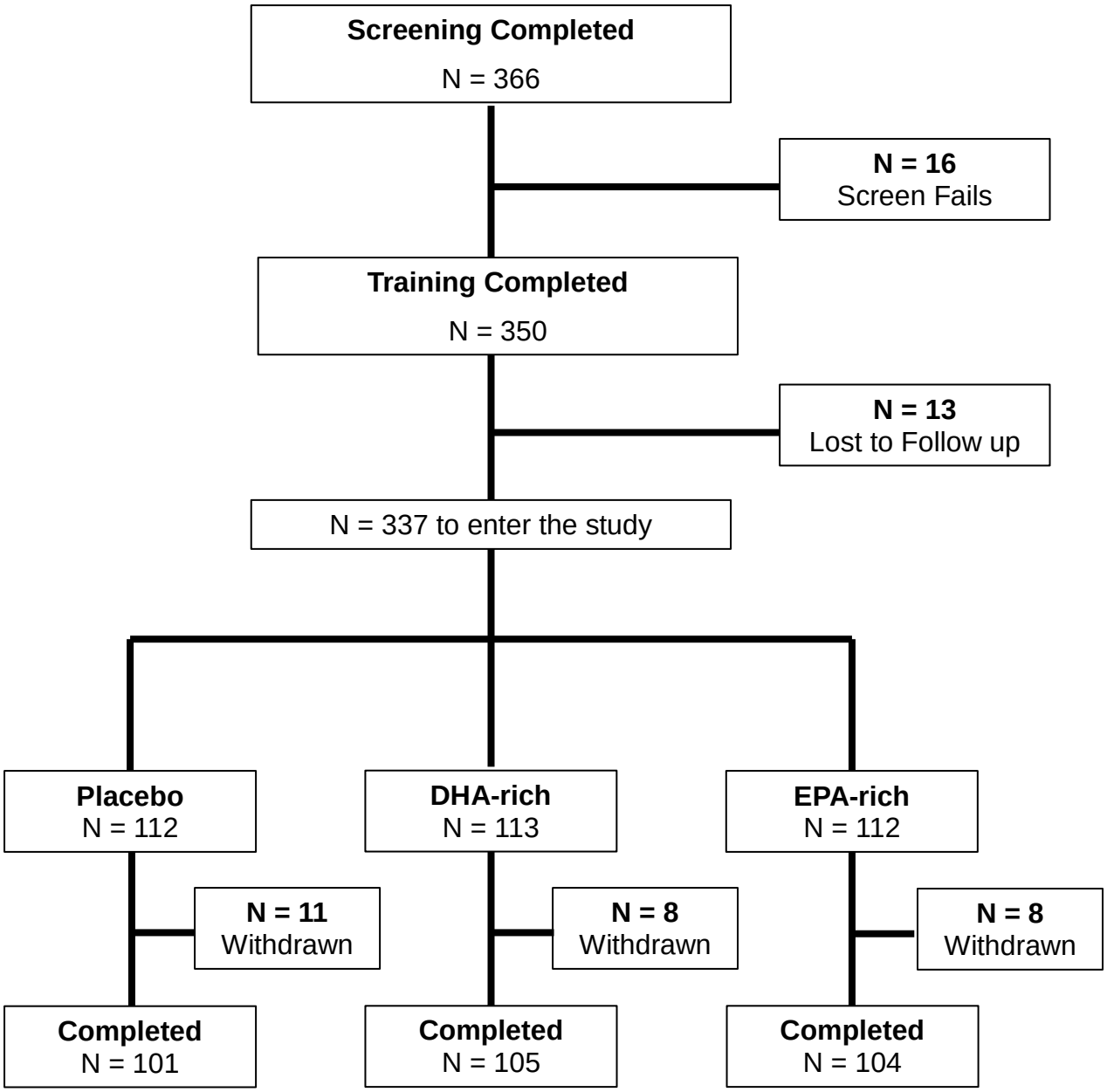

Figure 6.1 Participant disposition through the trial. Figure depicts the disposition of participants throughout the study, culminating in N = 310 of the 337 who were randomised.

Table 6.1. Participant demographic information and baseline characteristics. Means and Std. Deviation (*sd*) are given where appropriate. *F* or χ^2 and *p* values are given for separate one-way ANOVAs or Chi-Square tests that were conducted on this baseline data by treatment group.

		Baseline		Main Effects		
		Mean	Sd		*F* / χ^2	*p*
N (M/F)	Placebo	38/74	-	Treatment	2.01	.366
	DHA-rich	29/84	-			
	EPA-rich	36/76	-			
% of EPA in RBC	Placebo	0.86	0.03	Treatment	.019	.981
	DHA-rich	0.87	0.03			
	EPA-rich	0.87	0.04			
% of DHA in RBC	Placebo	4.95	0.10	Treatment	.721	.487
	DHA-rich	4.79	0.12			
	EPA-rich	4.98	0.13			
n-3 Index	Placebo	5.81	0.12	Treatment	.513	.599
	DHA-rich	5.66	0.14			
	EPA-rich	5.85	0.15			
Age (Years)	Placebo	36.15	0.69	Treatment	.717	.489
	DHA-rich	34.95	0.76			
	EPA-rich	35.33	0.07			
Systolic BP	Placebo	123.40	1.21	Treatment	.222	.801
	DHA-rich	122.75	1.18			
	EPA-rich	122.29	1.17			
Diastolic BP	Placebo	81.10	0.90	Treatment	.440	.644
	DHA-rich	80.99	0.98			
	EPA-rich	79.96	0.95			
Heart Rate (BPM)	Placebo	72.08	1.03	Treatment	.324	.723
	DHA-rich	71.05	1.07			
	EPA-rich	72.16	1.15			
Weight (Kg)	Placebo	73.84	1.33	Treatment	.107	.898
	DHA-rich	74.26	1.51			
	EPA-rich	73.34	1.36			
Height (cm)	Placebo	169.18	0.92	Treatment	.125	.883
	DHA-rich	168.98	0.85			
	EPA-rich	169.59	0.84			
BMI (Kg/m^2)	Placebo	25.74	0.38	Treatment	.334	.716
	DHA-rich	25.86	0.40			
	EPA-rich	25.43	0.40			
Years in Education	Placebo	16.88	0.24	Treatment	.877	.417
	DHA-rich	16.85	0.24			
	EPA-rich	16.49	0.22			
Fruit & Vegetable (portions per day)	Placebo	3.83	0.17	Treatment	.077	.926
	DHA-rich	3.89	0.19			
	EPA-rich	3.93	0.16			
Alcohol (Units per day)	Placebo	1.24	0.08	Treatment	2.01	.135
	DHA-rich	1.12	0.08			
	EPA-rich	1.35	0.09			

6.2.3 Cognitive Tasks

The cognitive tasks were selected to measure a range of cognitive domains including episodic memory, information processing speed, working memory, sustained attention, and executive function.

6.2.3.1 Stimuli Presentation

Prior to the start of the Cognitive Demand Battery participants were presented with fifteen randomly selected photographic images to remember. Presentation was at a rate of 1 picture every 3 seconds, with a stimulus duration of one second. Following this participants were presented sequentially with 15 words selected at random from a large bank of words derived from the MRC Psycholinguistic Database (Coltheart, 1981) and matched for word length, frequency, familiarity and concreteness. Stimulus duration was one second, with an inter-stimulus duration of one second.

6.2.3.2 Immediate Word Recall

Immediately after the presentation of the words participants were given 60 seconds to write down as many of the 15 words that they were presented with during the stimulus presentation period. Outcomes are accuracy (number correct), errors (number incorrect).

6.2.3.3 Delayed Word Recall

After completing all other tasks participants were once again given 60 seconds to write down as many of the 15 words that they were presented with during the stimulus presentation period. Outcomes are accuracy (% correct), errors (number).

6.2.3.4 Delayed Picture Recognition

Thirty pictures, comprising the 15 pictures presented during the stimuli presentation period plus 15 distractor pictures were presented, with the participant making a yes/no response indicating whether the picture was in the original set. Outcomes are accuracy (% correct), reaction time of correct responses (msecs).

6.2.3.5 Delayed Word Recognition

Thirty words, comprising the 15 words presented during the stimuli presentation period plus 15 distractor words were presented, with the participant making a yes/no response indicating

whether the word was in the original set. Outcomes are accuracy (% correct), reaction time of correct responses (msecs).

6.2.3.6 Verbal Fluency

Participants were presented with a letter on a sheet of paper (F, A or S) and were given 60 seconds to write down as many words as they could, beginning with that letter. Outcomes are total number of permitted words, with names (proper nouns) and perseverations (e.g. ask, asked, asks) discounted from the total score.

6.2.3.7 Simple Reaction Time

An upwards pointing arrow was displayed on the screen with a randomly varying inter-stimulus interval of between 1 and 3 seconds. Participants responded with a single button press as quickly as they could as soon as they saw the arrow appear. Outcomes are overall mean reaction time (msec).

6.2.3.8 Stroop Task

In this computerised version of the classic task 50 words describing one of four colours ('RED', 'YELLOW', 'GREEN', 'BLUE') were presented in different coloured fonts in the centre of a computer screen. The participant needed to press one of four coloured response buttons in order to identify the font colour (e.g. if the word 'GREEN' was presented in a blue font, the correct response would be to respond with the blue button). The presented words were either 'congruent' (word and font are the same colour) or 'incongruent' (word and font are different colours) and were presented in a random order. Outcomes are reaction time of correct responses (msec), and for accuracy (% correct).

6.2.3.9 Numeric Working Memory (NWM)

Five random digits from 1-9 were presented sequentially for the participant to hold in their memories. This was followed by a series of 30 probe digits (15 targets and 15 distractors) for each of which the participant indicated whether or not it was in the original series by pressing 'yes' or 'no'. The task consisted of 3 separate trials and outcomes are overall accuracy (% correct) and mean reaction time for correct responses (msec).

6.2.4 Cognitive Demand Battery

The following tasks were repeated four times in the order of: Serial three subtraction, Serial seven subtraction, Rapid Visual Information Task, 'Mental Fatigue' Visual Analogue Scale and 'Alertness' Visual Analogue Scale. Previously, this battery has been successfully used to investigate the effects of various nutritional interventions on cognitive and mental fatigue during periods of sustained cognitive processing (Kennedy & Scholey, 2004; Reay, Kennedy & Scholey, 2005; 2006; Kennedy et al., 2008; Scholey et al., 2010).

6.2.4.1 Serial Threes Subtraction Task

Two minutes of the same task as outlined in section 3.2.5.1

6.2.4.2 Serial Sevens Subtraction Task

Two minutes of the same task as outlined in section 3.2.5.1

6.2.4.3 Rapid Visual Information Task (RVIP)

The participant was required to monitor a continuous series of digits for targets of three consecutive odd or three consecutive even digits. The digits were presented at the rate of 100 per minute and the participant responded to the detection of a target string by pressing the response button as quickly as possible. The task was continuous and lasted for 5 minutes, with 8 correct target strings being presented each minute. Outcomes are percentage of target strings correctly detected (% correct), average reaction time for correct detections (msec) and number of false alarms.

6.2.4.4 'Mental Fatigue' Visual Analogue Scale

Participants rated their current subjective 'mental fatigue' state by making a mark on a 100 mm line with the end points labelled "not at all" (left hand end) and "very much so" (right hand end). Higher scores represented higher levels of mental fatigue.

6.2.4.5 'Alertness' Visual Analogue Scale

Participants rated their current subjective 'Alertness' by making a mark on a 100 mm line with the end points labelled "not at all" (left hand end) and "very much so" (right hand end). Higher scores represented higher levels of alertness.

6.2.5 Mood Assessments

6.2.5.1 Profile of Mood States (POMS; McNair et al., 1992)

The 65 item POMS provided scales of tension-anxiety, depression-dejection, anger-hostility, vigour-activity, fatigue-inertia and confusion-bewilderment. A total “mood disturbance” score was also computed via subtracting the vigour-activity score from the sum of tension-anxiety, depression-dejection, anger-hostility, fatigue-inertia and confusion-bewilderment.

6.2.6 Procedure

All study visits took place at Northumbria University’s Brain, Performance and Nutrition Research Centre (BPNRC). Potential participants attended the site for an initial screening visit. The principal investigator or designee discussed with each participant the nature of the trial, its requirements and restrictions in line with the participant information sheet previously given to the participant. Following informed consent eligible participants underwent training on the computerised cognitive tasks. The training session followed standard operating procedures to decrease the chance of learning effects during the main trials. This entailed the participants completing three shortened versions of the tasks to gain familiarity, followed by two full length versions of the tasks. This results in participants achieving their highest scores in one of the full length versions of the tasks once they are completely familiar with said task. Once this session was completed to the required standard the participant was then eligible to be enrolled and randomised into the trial.

Before the baseline and week 26 assessments participants were asked to avoid alcohol and refrain from intake of ‘over the counter’ medications for 24 hours and caffeine for 18 hours. Participants were contacted to remind them of the requirements prior to each visit. On the morning of the baseline testing visit, participants were requested to eat their usual breakfast or no breakfast if they usually skipped breakfast at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products). Adherence to this abstinence was ensured via completion of the case report form (CRF) prior to the participant completing the cognitive tasks, mood measures and blood samples. After completion of the CRF participants then completed all cognitive tasks outlined in section 6.2.3 and 6.2.4 as well as completing the POMS questionnaire which took approximately 60 minutes to complete (see Figure 6.2 for schematic depicting the baseline and week 26 assessment schedule). Participants were then provided with the first batch of capsules (3 bottles of 100 capsules each) and given a diary in which to record their daily consumption of the capsules along with any adverse events and concomitant medications, should there be any throughout the supplementation period.

Participants also reported to BPNRC during Week 13 to collect the second batch of capsules (3 bottles of 100 capsules each). Participants also brought with them their diary, which was replaced with a new diary to complete between week 13-26 and any remaining unused treatment capsules, so that treatment compliance could be calculated.

The week 26 assessment was identical to the baseline assessment in all aspects apart from collecting in the subject diaries, all remaining treatments, completion of a treatment guess questionnaire (see Appendix II) and finally a full debrief once all assessments were completed. During both the baseline and week 26 visits participants were also required to provide a 6 mL venous blood sample to determine blood fatty acid profile. Finally a full debrief was given once all assessments had been completed. An outline of the baseline and week 26 study assessments is given in Figure 6.2.

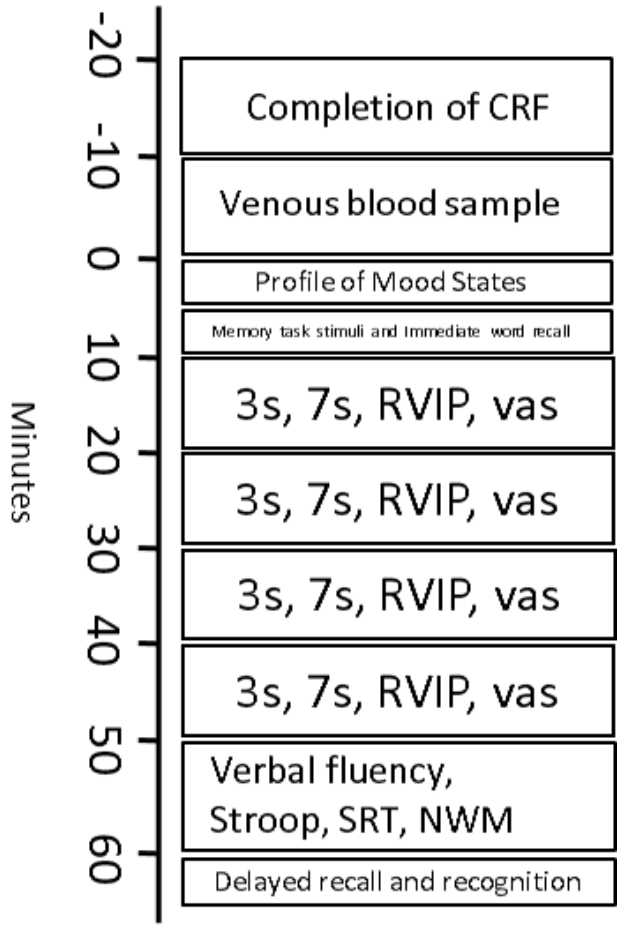

Figure 6.2. Schematic showing the procedures for the study testing visits. CRF, Case Report Form; 3s, Serial 3 subtractions; 7s, Serial 7 subtractions; RVIP, Rapid visual information processing; vas, visual analogue scales; SRT, Simple reaction time; NWM, Numeric working memory.

6.2.7 Statistical Methods

For each of the linear mixed models conducted throughout the analysis: age, years in education and baseline n-3 index were also added as covariates only if these respective variables were identified as having a significant effect on the dependent variable. If any of these variables were found to have a significant effect on the dependent variable ($p < .05$) then it was decided to include the factor as a covariate to control for its effects within the model. If any of these covariates were added to the respective models then they will be named when describing each model below.

6.2.7.1 COMPASS Tasks

The COMPASS task data were analysed using the same linear mixed model procedure described previously with treatment (DHA-rich, EPA-rich, Placebo) appearing as a fixed factor in the models and respective pre-dose values were entered into each model as a covariate. Age was also added as a covariate in the model for Stroop accuracy and years spent in education was also added as a covariate for the word recognition task accuracy model.

6.2.7.2 Cognitive Demand Battery Data

The data were analysed using the same linear mixed model procedure described previously with all models using an identity covariance matrix. The fixed factors appearing in all models were; treatment (DHA-rich, EPA-rich, Placebo) and repetition (1-4). Subject was also added into all models as a random factor and respective pre-dose values were entered into each model as a covariate. Age was also added as a covariate in the model for; serial 3 subtraction, serial 7 subtraction and VAS ratings. Years spent in education was added as a covariate in the model for serial 3 subtraction, serial 7 subtraction and RVIP.

6.2.7.3 Cognitive Domain Data

As the current study aimed to build upon the findings of Stonehouse et al., (2013) the same cognitive domains that were calculated and analysed by the researchers previously were also analysed within the current study wherever possible. This included measures of memory and attention. Additionally, measures of global cognition were also included representing both global speed and global accuracy in an attempt to capture the overall performance on all tasks. Calculation of these cognitive domains involved transforming outcomes from the individual tasks into z scores and clustering these z scores into their respective cognitive domains. The calculations for each cognitive domain are outlined below.

6.2.7.3.1 Attention Domains

The attention domains consisted of both accuracy and speed of attention. For the accuracy of attention the domain consisted of the standardised values from the accuracy outcomes on the stroop and RVIP tasks. For the speed of attention the domain consisted of the standardised values from the RT on the stroop, RVIP and SRT tasks. It should be noted that the lowest outcomes for the speed of attention represent the fastest reaction times. The calculations for both attention domains are outlined below:

> **Accuracy of Attention** = (ZStroop accuracy + ZRVIP average accuracy) ÷ 2
>
> **Speed of Attention** = (ZStroop RT + ZRVIP average RT + ZSRT) ÷ 3

The data were analysed using the same linear mixed model procedure described previously. The only fixed factor appearing in both models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into both models as a covariate.

6.2.7.3.2 Memory Domains

The memory data consisted of both accuracy of memory and speed of memory. For the accuracy of memory the domain consisted of the standardised values from the number of words recalled correctly during both the immediate and delayed word recall tasks, as well as accuracy % from the word and picture recognition tasks (Stonehouse et al., 2013). The speed of memory domain consisted of the standardised values from the RT during the word and picture recognition tasks. It should be noted that the lowest outcomes for the speed of memory represent the fastest reaction times. The calculations for both memory domains are outlined below:

> **Accuracy of memory** = (Zimmediate word recall accuracy + Zdelayed word recall accuracy + Zword recognition accuracy + Zpicture recognition accuracy) ÷ 4
>
> **Speed of Memory** = (Zword recognition RT + Zpicture recognition RT) ÷ 2

The data were analysed using the same linear mixed model procedure described previously. The only fixed factor appearing in the models were treatment (DHA-rich, EPA-rich, Placebo).

Respective pre-dose values were entered into each model as a covariate and age was entered as a covariate for both speed and accuracy of memory models.

6.2.7.3.3 Global Cognition Domains

The global cognition data consisted of both global accuracy and global speed. These domains represented total accuracy and speed across all tasks. It should be noted that the lowest outcomes for global speed represent the fastest reaction times. The calculations for these domains are outlined below:

> **Global Accuracy** = (Zimmediate word recall accuracy + Zdelayed word recall accuracy + Zword recognition accuracy + Zpicture recognition accuracy + Zverbal fluency accuracy + ZStroop accuracy + ZNWM accuracy + ZRVIP average accuracy) ÷ 8
>
> **Global Speed** = (Zword recognition RT + Zpicture recognition RT + ZStroop RT + ZSRT + ZNMW RT + ZRVIP average RT) ÷ 6

The data were analysed using the same linear mixed model procedure described previously. The only fixed factor appearing in both models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into both models as a covariate.

6.2.7.4 Subjective Mood (POMS)

The subjective mood data consisted of scores for tension-anxiety, depression-dejection, anger-hostility, vigour-activity, fatigue-inertia, confusion-bewilderment and total mood disturbance. The data were analysed using the same linear mixed model procedure described previously. The only fixed factor appearing in the models was treatment (DHA-rich, EPA-rich, Placebo). Respective pre-dose values were also entered into the model as a covariate.

6.2.7.5 Blood Fatty Acid Profile

Blood fatty acid data was presented as a percentage of total fatty acid content for EPA, DHA and the sum of EPA + DHA (n-3 index). All fatty acid data were analysed using the Mixed Models procedure outlined previously. The only fixed factor appearing in the model was treatment (DHA-rich, EPA-rich, Placebo) with respective pre-dose values entered as a covariate.

6.3 Results

6.3.1 Compliance

For participants who completed the study, compliance was observed to be very good in all three groups (96.06% Placebo, 97.43% DHA-rich, 96.57% EPA-rich) with a one way ANOVA identifying no significant differences for compliance percentage by treatment group [F (2, 306) = 1.39, p = .250]. A Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was completed at the end of the final visit and revealed no significant differences in participants' ability to correctly identify whether they had been administered an active or placebo treatment between the three groups [χ^2 (2) = 1.52, p = .467].

6.3.2 COMPASS Task Analysis

Analysis revealed a significant effect of treatment for Stroop correct reaction time [F (2, 303) = 3.29, p = .039] with post hoc comparisons identifying the EPA-rich group (768.22; p = .065), but not the DHA-rich group (801.43; p = .997) as showing a trend towards significantly faster reaction times compared to the placebo group (804.48). Additionally, no significant differences were identified between the two active treatment groups (p = .099) (Figure 6.3).

Analysis also revealed a significant effect of treatment for word recognition correct reaction time [F (2, 303) = 3.34, p = .037] with post hoc comparisons identifying the EPA-rich group (936.98; p = .032), but not the DHA-rich group (964.28; p = .319) as having significantly faster reaction times compared to the placebo group (1006.21). Additionally, no significant differences were identified between the two active treatment groups (p = .671) (Figure 6.3).

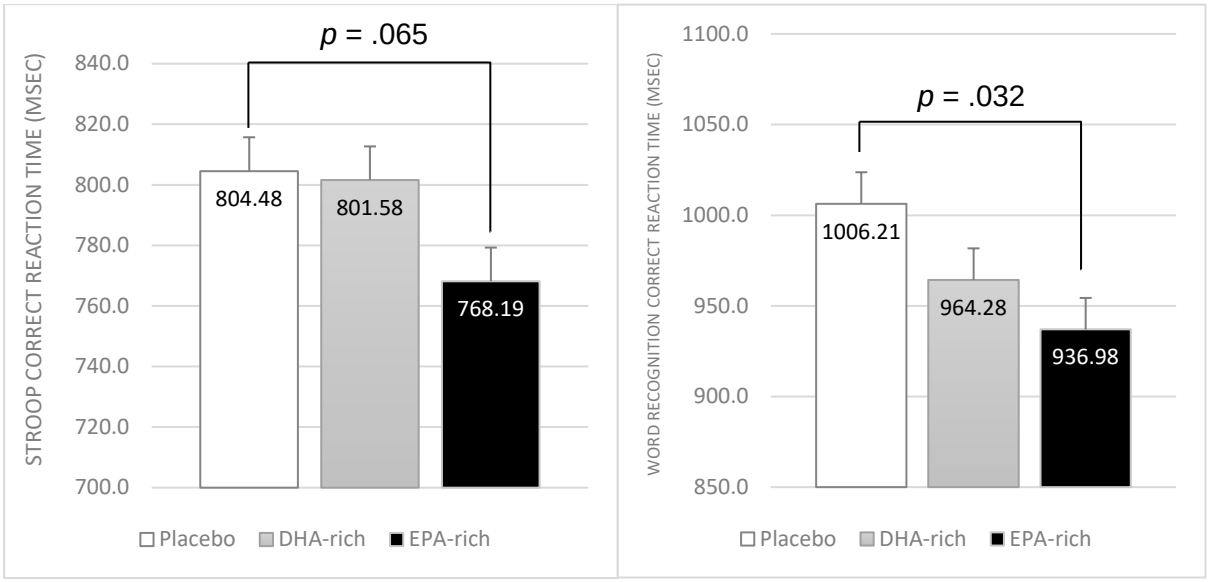

Figure 6.3. Estimated marginal means and standard error (SE) for post-dose values of Stroop task correct reaction time (**left**) and word recognition correct reaction time (**right**) in msec, by treatment group.

Analysis also revealed a significant effect of treatment for delayed word recall, [F (2, 303) = 3.30, p = .037] with post hoc comparisons showing no significant differences for both the EPA-rich (3.41; p = .482) and DHA-rich (2.69; p = .503) groups compared to the placebo group (3.05). However, post hoc comparisons identified a significant difference between the active groups with the EPA-rich group correctly remembering more words than the DHA-group (p = .031) (Figure 6.4).

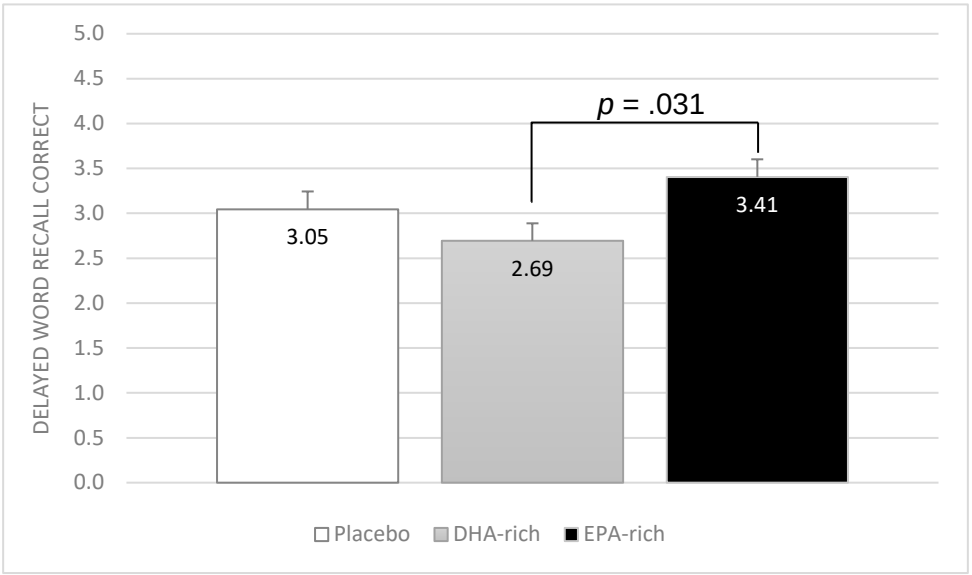

Figure 6.4. Estimated marginal means and standard error (SE) for post-dose values of delayed word recall, by treatment group.

Table 6.2. Cognitive task analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Immediate Word Recall (Correct)	Placebo	309	5.94	0.20	Treatment	1.08	.342
	DHA-rich		5.83	0.20			
	EPA-rich		6.23	0.20			
Delayed Word Recall (Correct)	Placebo	307	3.05	0.20	**Treatment**	**3.33**	**.037**
	DHA-rich		**2.69[b]**	0.20			
	EPA-rich		**3.41[b]**	0.20			
Picture Recognition Accuracy %	Placebo	297	94.16	0.62	Treatment	2.88	.058
	DHA-rich		92.61	0.62			
	EPA-rich		92.17	0.61			
Picture recognition Correct Reaction Time (msec)	Placebo	300	845.35	10.19	Treatment	.193	.825
	DHA-rich		852.28	9.96			
	EPA-rich		844.19	9.91			
Verbal Fluency Total Correct	Placebo	287	38.74	0.50	Treatment	.420	.657
	DHA-rich		38.17	0.49			
	EPA-rich		38.71	0.50			
Simple Reaction Time (msec)	Placebo	300	320.26	4.51	Treatment	.749	.474
	DHA-rich		312.64	4.49			
	EPA-rich		315.05	4.47			
NWM Accuracy %	Placebo	302	95.42	0.39	Treatment	.886	.413
	DHA-rich		96.09	0.39			
	EPA-rich		96.01	0.39			
NWM Correct Reaction Time (msec)	Placebo	306	907.32	10.71	Treatment	1.33	.267
	DHA-rich		890.29	10.48			
	EPA-rich		883.41	10.59			
Stroop Accuracy %	Placebo	283	98.21	0.18	Treatment	2.15	.118
	DHA-rich		98.24	0.17			
	EPA-rich		98.66	0.17			
Stroop Correct Reaction Time (msec)	**Placebo**	307	**804.48[T]**	11.22	**Treatment**	**3.29**	**.039**
	DHA-rich		801.58	11.11			
	EPA-rich		**768.19[T]**	11.10			
Word Recognition Accuracy %	Placebo	308	75.15	1.05	Treatment	2.72	.068
	DHA-rich		73.29	1.04			
	EPA-rich		76.70	1.03			
Word Recognition Correct RT (msec)	**Placebo**	297	**1006.21***	18.95	**Treatment**	**3.34**	**.037**
	DHA-rich		964.28	18.64			
	EPA-rich		**936.98***	18.63			

***** = significant difference between active and placebo groups below p< .050; [b] = significant difference between the active treatment groups below p< .050; [T] = Trend towards a significant difference between active and placebo groups.

6.3.3 Cognitive Demand Battery Analysis

Analysis identified no significant effects of treatment or interaction effects between treatment and repetition for any of the CDB tasks or VAS.

Table 6.3. Cognitive demand battery task analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
S3's Total Responses	Placebo	307	36.53	0.41	Treatment	.188	.829
	DHA-rich		36.86	0.41			
	EPA-rich		36.80	0.41	Treatment*Rep	.963	.449
S3's Errors	Placebo	306	2.42	0.13	Treatment	.571	.566
	DHA-rich		2.29	0.13			
	EPA-rich		2.22	0.13	Treatment*Rep	.365	.901
S7's Total Responses	Placebo	307	23.01	0.35	Treatment	.267	.766
	DHA-rich		22.80	0.34			
	EPA-rich		23.15	0.35	Treatment*Rep	.971	.444
S7's Errors	Placebo	305	2.77	0.14	Treatment	.015	.985
	DHA-rich		2.80	0.14			
	EPA-rich		2.80	0.14	Treatment*Rep	1.60	.144
RVIP Accuracy %	Placebo	286	54.75	1.31	Treatment	.823	.441
	DHA-rich		56.30	1.29			
	EPA-rich		57.09	1.32	Treatment*Rep	.913	.485
RVIP Correct Reaction Time (msec)	Placebo	292	507.24	4.03	Treatment	.765	.765
	DHA-rich		505.20	3.99			
	EPA-rich		503.06	4.04	Treatment*Rep	1.46	.188
RVIP False Alarms	Placebo	278	3.79	0.21	Treatment	2.29	.104
	DHA-rich		3.42	0.21			
	EPA-rich		4.06	0.21	Treatment*Rep	1.55	.160
Mental Fatigue	Placebo	310	59.92	1.68	Treatment	2.28	.104
	DHA-rich		64.75	1.65			
	EPA-rich		61.20	1.65	Treatment*Rep	1.27	.271
Alertness	Placebo	310	42.24	1.72	Treatment	1.69	.186
	DHA-rich		41.45	1.67			
	EPA-rich		45.58	1.69	Treatment*Rep	.589	.740

6.3.4 Cognitive Domain Analysis

A significant effect of treatment for accuracy of memory was identified [F (2, 290) = 3.28, p = .039], with post hoc comparisons showing no significant differences for both the EPA-rich (0.66; p = .339) and DHA-rich (-0.08; p = .670) groups compared to the placebo group (0.22). However, post hoc comparisons revealed a significant difference between the EPA-rich and DHA-rich groups (p = .034) (Figure 6.5).

A significant effect of treatment for global accuracy was identified [F (2, 215) = 4.82, p = .009], with post hoc comparisons identifying significantly more accurate scores in the EPA-rich group (0.17) compared to both the placebo (0.03; p = .044) and DHA-rich groups (0.01; p = .013) (Figure 6.6).

Additionally, a significant effect of treatment for global speed was identified [F (2, 255) = 5.74, p = .004], with post hoc comparisons identifying significantly faster reaction times in the EPA-rich group (-0.15) compared to the placebo group (0.03; p = .003) and a trend towards significantly faster reaction times compared to the DHA-rich groups (-0.03; p = .062) (Figure 6.6).

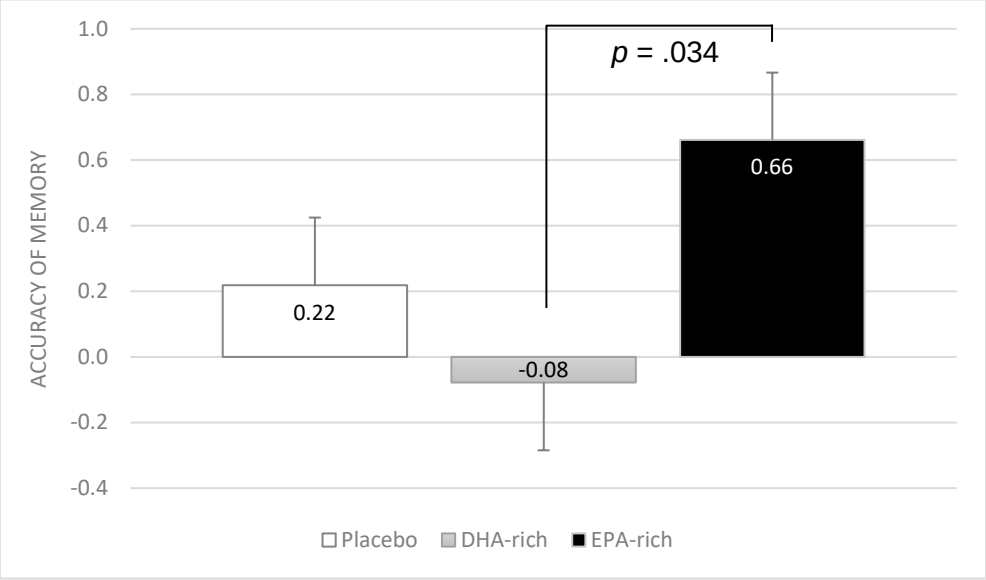

Figure 6.5. Estimated marginal means and standard error (SE) for post-dose values of accuracy of memory by treatment group.

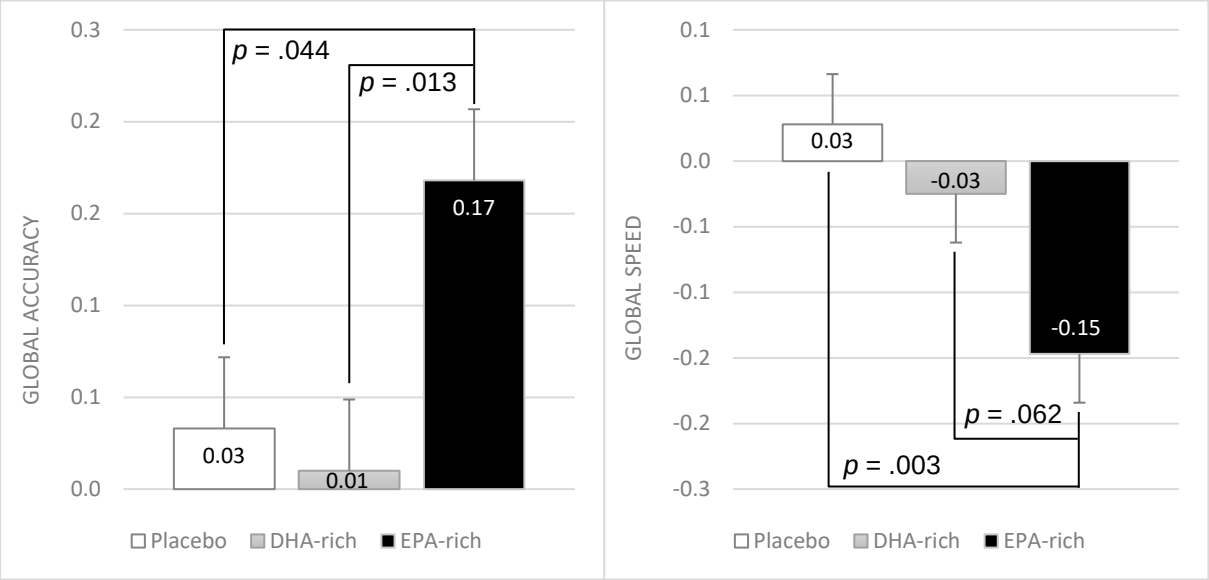

Figure 6.6. Estimated marginal means and standard error (SE) for post-dose values of global accuracy **(left)** and global speed **(right)** by treatment group.

Table 6.4 Cognitive domain analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Accuracy of Attention	Placebo	252	-0.03	0.60	Treatment	2.36	.096
	DHA-rich		0.04	0.58			
	EPA-rich		0.15	0.58			
Speed of Attention	Placebo	267	0.02	0.05	Treatment	2.01	.137
	DHA-rich		-0.03	0.05			
	EPA-rich		-0.11	0.05			
Accuracy of Memory	Placebo	290	0.22	0.21	**Treatment**	**3.28**	**.039**
	DHA-rich		**-0.08[b]**	0.21			
	EPA-rich		**0.66[b]**	0.21			
Speed of Memory	Placebo	290	-.014	0.06	Treatment	.594	.553
	DHA-rich		-.072	0.06			
	EPA-rich		-.097	0.06			
Global Accuracy	**Placebo**	219	**0.03***	0.04	**Treatment**	**2.77**	**.009**
	DHA-rich		**0.01[b]**	0.04			
	EPA-rich		**0.17*[b]**	0.04			
Global Speed	**Placebo**	259	**0.03***	0.04	**Treatment**	**5.74**	**.004**
	DHA-rich		**-0.03[T]**	0.04			
	EPA-rich		**-0.15*[T]**	0.04			

*= significant difference between the placebo and an active treatment group below p< .05; [b] = significant difference between the active treatment groups below p< .05; [T] = Trend towards a significant difference between active and placebo groups p< .10.

6.3.5 Subjective Mood (POMS) Analysis

Analysis identified no significant effects of treatment for subjective mood.

Table 6.5. Subjective mood analysis outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated marginal means and standard error (SE) are presented with F and *p* values of the main effects from the linear mixed models.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Tension-Anxiety	Placebo	289	8.21	0.49	Treatment	.141	.869
	DHA-rich		8.27	0.48			
	EPA-rich		7.93	0.48			
Depression-Dejection	Placebo	290	3.90	0.48	Treatment	.650	.523
	DHA-rich		4.50	0.48			
	EPA-rich		4.62	0.48			
Anger-Hostility	Placebo	289	4.94	0.39	Treatment	.717	.489
	DHA-rich		4.31	0.38			
	EPA-rich		4.74	0.38			
Vigour-Activity	Placebo	300	18.24	0.56	Treatment	1.77	.173
	DHA-rich		16.92	0.55			
	EPA-rich		18.15	0.56			
Fatigue-Inertia	Placebo	297	7.06	0.46	Treatment	.634	.531
	DHA-rich		6.88	0.46			
	EPA-rich		6.36	0.46			
Confusion-Bewilderment	Placebo	294	8.75	0.41	Treatment	.276	.759
	DHA-rich		9.01	0.41			
	EPA-rich		8.58	0.41			
Total Mood Disturbance	Placebo	277	14.67	2.07	Treatment	.840	.433
	DHA-rich		16.00	2.03			
	EPA-rich		12.30	2.05			

6.3.6 Blood Fatty Acid Profile

Analysis revealed a significant effect of treatment for RBC EPA [F (2, 259) = 154.64, p <.001] with both the EPA-rich (2.75%; p <.001) and DHA-rich (2.09%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (0.85%). A significant effect of treatment for RBC DHA [F (2, 259) = 140.39, p <.001] with both the EPA-rich (6.01%; p <.001) and DHA-rich (7.49%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (4.74%). A significant effect of treatment for RBC n-3 index [F (2, 259) = 153.57, p <.001] with both the EPA-rich (8.75%; p <.001) and DHA-rich (9.58%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (5.59%) (Figure 6.7).

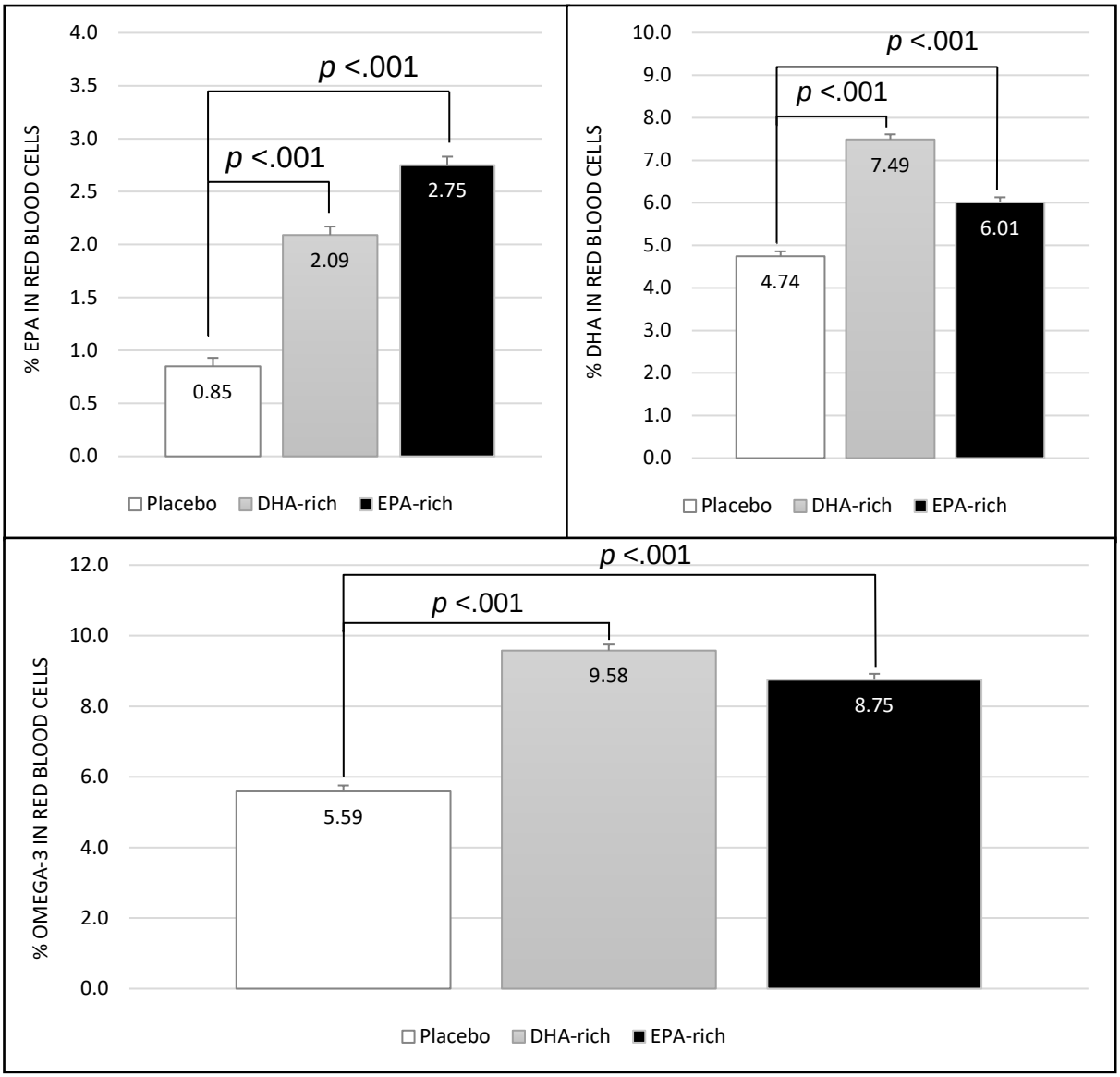

Figure 6.7. Estimated marginal means and standard errors (SE) for the post-dose percentage of EPA **(top left)** percentage of DHA **(top right)** and total percentage of EPA+DHA **(bottom)**, out of total fatty acids, in red blood cells by treatment group.

6.4 Discussion

Overall, it was found that supplementation with the EPA-rich treatment resulted in a significant increase in global accuracy scores compared to placebo and DHA-rich treatments. Additionally, supplementation with the EPA-rich treatment also resulted in a significant improvement in global speed scores compared to placebo and a trend towards significance compared to the DHA-rich treatment. Furthermore, the EPA-rich treatment also resulted in significant improvements in reaction times during the word recognition task, as well as a trend towards a significant improvement in reaction times during the Stroop task, compared to the placebo group. Moreover, supplementation with the EPA-rich treatment resulted in significant increases for both the accuracy of memory domain and the number of words recalled correctly during the delayed word recall task, compared to the DHA-rich group. No significant effects of treatment were identified for the subjective mood scales or during the cognitive demand battery tasks. However, both the DHA-rich and EPA-rich treatments were found to significantly increase levels of DHA and EPA in RBC compared to placebo.

The current study identified significant improvements to both global accuracy and global speed following supplementation with the EPA-rich treatment compared to placebo. Previous findings from older samples have also identified that higher proportions of EPA in RBC membranes were associated with better cognitive outcome in samples with MCI and AD (Chiu et al., 2008). Furthermore, Nishihira et al., (2016) have found that higher serum EPA and n-3 index, but not DHA, were associated with better scores on global cognitive function in a sample of 185 relatively healthy participants aged 80 years and over, measured via the Japanese version of the Mini-Mental State Examination. To our knowledge, the current study is the first to observe an effect on both global accuracy and global speed in a sample of healthy, young adults following supplementation with an EPA-rich treatment. The current study's findings are in line with the data regarding group performance on the individual cognitive tasks as the EPA-rich group are often the most accurate and quickest group on the tasks. These findings are interesting as it suggests that although EPA is stored in the brain in low amounts (Chen et al., 2005; 2009; 2011), it may still play an important role during high order cognitive functions (Bauer et al., 2014b). The findings from the current study could once again provide further support for the role of EPA in relation to increased neural efficiency during higher order cognitive tasks, described previously in work by Baeur et al., (2011; 2014a; 2014b). The current study is the largest RCT to date measuring the effects of supplementation with an EPA-rich oil on cognitive function in healthy, young adults, and as a result this is the first study to identify a positive impact of increased intake of EPA on global cognitive function in healthy,

young adults free from MCI or AD. Overall, these findings provide support for the role of supplementation with EPA on cognitive functioning, potentially via increased neural efficiency.

The observed reduction in reaction times during completion of the Stroop task is consistent with the findings from Bauer et al., (2014a) who also identified a reduction in reaction times during the same Stroop task after only 30 days' supplementation with an EPA-rich treatment (590mg EPA and 137mg DHA), as well as Bauer et al., (2011) who identified a significant reduction in reaction times during a choice reaction time task following 30 days' supplementation with the same EPA-rich treatment (590mg EPA and 137mg DHA). The current study employed a far longer supplementation period as well as a far larger sample size than Bauer et al., (2011; 2014a) which both supplemented for only 30 days and had small sample sizes of only 13 and 22 participants respectively. Additionally, both Bauer et al., (2011; 2014a) lacked a placebo control treatment with Bauer et al., (2014a) also reporting no significant differences in the percentage of EPA, DHA or total n-3 between baseline and after supplementation in blood plasma, most likely a result of the small supplementation period employed. As the present study's findings are still consistent with Bauer et al., (2011; 2014a) after supplementing for a longer duration, it provides evidence for an enduring effect of dietary supplementation with EPA on improvements in speed during executive functioning tasks rather than transient effects. Additionally, the use of a placebo comparison within the current study helps to further support the previous findings of Bauer et al., (2011; 2014a), as this was an aspect missing from their studies. Overall, the findings suggest that supplementation with EPA appears to have beneficial effects on measures of executive functioning in healthy, young adults.

The findings from the current study are also consistent with those from Stonehouse et al., (2013) who identified a reduction in episodic memory reaction times following 26 weeks' supplementation with a DHA-rich treatment (1.16 g/d DHA + 0.17 g/d EPA). Both the current study and Stonehouse et al., (2013) employed 26 week supplementation periods in a sample of healthy, young adults with similar dosages for the DHA-rich treatment. The current study observed a significant reduction in reaction times during the word recognition task, a component of Stonehouse et al., (2013) memory domain, following supplementation with the EPA-rich treatment compared to placebo. These findings are interesting as the current study's DHA-rich supplementation was seen to increase overall n-3 index to a greater extent than the EPA-rich treatment. However, it was the EPA-rich supplement that was observed to improve episodic memory reaction times. This could potentially suggest that the ratio of DHA and EPA that is delivered during supplementation is important. For example, although the DHA-rich treatment increases overall n-3 index to a greater extent than the EPA-rich treatment, it could

be that this results in a surplus of DHA without increasing levels of EPA sufficiently. Whereas, the EPA-rich treatment effectively increases both EPA and DHA at a more equal rate, even though this results in a lower n-3 index overall. Benefits of increased amounts of EPA could potentially be a result of competing with n-6 fatty acids, such as AA, to a greater extent than DHA. For instance, Bauer et al., (2014a) has previously identified that an EPA-rich treatment, but not the DHA-rich treatment, reduced total n-6 levels. Indeed, EPA has been seen to compete with AA in production of a number of eicosanoids that regulate inflammation (Simpoulos, 2011; Schunck, 2016), promoting the release of anti-thrombotic anti-aggregatory eicosanoids such as thromboxanes and prostaglandins (Calder, 2006, Raz & Gabis, 2009), which then helps to regulate vascular tone via increased NO production that may lead to a rise in cerebral blood perfusion (reviewed in Sinn & Howe, 2008). If the ratios of dietary intake of EPA and DHA are indeed important with regards to episodic memory function, then this may explain why the current study identified a significant effect in the EPA-rich group only and highlights that the EPA:DHA ratio of supplements and their administration should be considered more carefully in future trials.

Additionally, the current study identified a significant increase in the number of words recalled correctly during the delayed word recall task and overall accuracy of memory scores, following supplementation with the EPA-rich treatment compared to the DHA-rich group. Although this finding is between the two active treatment groups and not the placebo group, the finding is actually somewhat contradictory with those of Stonehouse et al., (2013) who identified a significant increase in episodic memory scores following a DHA-rich treatment when compared to placebo, as no such increase was observed in the current study. In a meta-analysis, Mauro, Alexander and Elswyk (2015) concluded that currently there is a lack of an apparent relationship between DHA/EPA supplementation and episodic memory among adults with no cognitive complaints at baseline. This may potentially be due to the lack of large scale RCTs that supplement with an EPA-rich oil, as most RCTs focus on the relationship between DHA and cognitive function. This is usually based off the fact that the percentage of DHA, compared to EPA, stored in the brain is far greater. However, it may actually be EPA that has the greatest impact upon episodic memory as Samieri et al., (2012) has identified that higher plasma EPA, but not DHA, was associated with lower grey matter atrophy of the right hippocampal/parahippocampal area and amygdala, brain regions extensively linked to episodic memory (Moscovitch et al., 2016; Barker et al., 2017; Eichenbaum, 2017; Wixted et al., 2018). Though, it should be noted that the findings from Samieri et al., (2012) were observed in a sample of 281 older adults aged 65 years and over and not healthy, young adults. These findings are again interesting given that brain EPA levels are typically 250-300 times lower than DHA (Chen et al., 2013), suggesting that EPA may exert its effects on

episodic memory via different mechanism than brain accretion. As EPA has been seen to be rapidly oxidised by the brain into further derivatives such as eicosanoid lipid mediators (Chen & Bazinet, 2015), it could be these EPA derivatives that then impact upon cognitive function and signalling in the brain (Serhan et al., 2000). Additionally, the previously reported effects of EPA on neural efficiency may also help to explain these findings. EPA specifically may have direct actions on mitochondrial enzymes that produce ATP (Bauer et al., 2014b), which then supply energy in neuronal cells and acts as an extracellular signalling molecule within the brain (Illes, Nieber, & Nörenberg, 1995; Devine & Kittler, 2018). It may be via these mechanisms that EPA is able to exert effects on episodic memory as well as increase neural efficiency.

Concerning the effects on mood, the current study's null findings are mostly inconsistent with the small number of RCTs that have employed healthy, adult samples previously (Fontani et al., 2005a; 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011). The most likely reason for the inconsistent findings between the current study's findings and previous RCT's is due to the differences in sample size. For instance, Fontani et al., (2005a, 2005b) employed a sample of 33 participants, Antypa et al., (2009) a sample of 56 participants and Kiecolt-Glaser et al., (2011) a sample of 68 participants. As the current study employed a far larger sample size, it would appear that the null effects on mood identified are likely to be more representative of the effects of n-3 PUFA supplementation in healthy, young adult populations. Previous research does appear to support the effects of n-3 PUFA supplementation on reducing depression in clinical populations (Martins, 2009; Panagiotakos et al., 2010; Larrieu & Layé, 2018; Othman et al., 2018) rather than increasing mood within healthy samples (Giles et al., 2015). However, the previous RCTs that have identified positive effects of n-3 PUFAs on mood in healthy, adult samples have supplemented with higher doses for shorter periods. This could potentially suggest that if effects of n-3 PUFAs on mood do exist in healthy, young adults these effects may be transient rather than enduring effects. It could be that effects of n-3 PUFAs on mood occur quickly in healthy samples, potentially as a result of addressing deficiencies in the low n-3 PUFA status of low consumers. Indeed, rapid changes in mood have previously been identified following supplementation with just a single multivitamin several hours after intake in healthy older people (Macpherson et al., 2015), and research has also identified rapid negative changes in mood following injection of *Salmonella typhi* (ST) to increase inflammation (Wright et al., 2005). Negative changes in mood, measured via POMS, following injection with ST were seen to be significantly correlated with increases in IL-6 production after just 3 hours. These findings may potentially provide evidence for the acute effects of supplementation with n-3 PUFAs on mood via reduction of pro-inflammatory cytokines. If the effects of n-3 PUFAs on mood in healthy adults are indeed transient, then future RCTs should include measures of

mood at increased intervals across the supplementation period in an attempt to identify the length of the effects of n-3 PUFAs on mood within healthy populations. However, it should be clearly stated that the findings from the current study do suggest that there may be little or no effects of n-3 PUFA supplementation on mood in healthy, young adults.

In relation to the observed increase in EPA and DHA in RBC the current study's findings are consistent with those reported previously by Stonehouse et al., (2013) following supplementation with a DHA-rich treatment. The authors reported a change in the percentage of fatty acids that were DHA of 2.61 compared to the current study's change of 2.62 and a change in n-3 index of 2.82 compared to 3.81 in the current study, between baseline and week 26 (see Appendix VII). Therefore, the current study reported a similar change in DHA percentage of total fatty acids and a greater change in n-3 index following supplementation with a DHA-rich treatment (0.90 g/d DHA + 0.27 g/d EPA). This comparable change in DHA and greater n-3 index percentages observed in the current study following supplementation with a lower dosage of DHA and overall n-3 PUFA content, compared to Stonehouse et al., (2013), suggests that the current study's use of SMEDS formulated oils appears to have been beneficial in regards to the absorption of n-3 PUFAs across the supplementation period. However, it should be noted that the greater change in n-3 index in the current study is most likely due to an increase in EPA levels due to the higher EPA content of the current studies DHA-rich treatment (see Appendix VII).

Overall, the current study addressed a number of limitations that have been present in previous RCTs regarding sample sizes, supplementation periods and lack of large scale RCTs investigating the effects of an EPA-rich supplement on cognitive function. As a result the current study is one of the largest RCTs conducted thus far to employ an EPA-rich treatment that allows for the comparison of the potentially separate effects of increased dietary intake of DHA or EPA on cognitive function. Additionally, the results from the bloods analysis seem to suggest that the employed methodological paradigm, in regards to night time supplementation and SMEDS formulation, appears to have been successful in increasing the absorption of the n-3 PUFAs when compared to a similar study that also supplemented for 26 weeks with similar dosages.

Conversely, a potential limitation of the current study was solely relying on the fish consumption questionnaire (Benisek et al., 2002) as a screening tool to ensure participants were low consumers of fish. It can be argued that baseline n-3 blood index should have also been used as a screening tool, to ensure low fish consumption. Indeed, McLennan and Pepe (2017) have discussed how there is a failure in most RCTs in recruiting samples with the

lowest baseline levels of n-3 index. Cohort studies comparing those eating little or no fish and regular consumers of fish have revealed consistent differences (Nestle et al., 2015; Alexander et al., 2017) and consequently it is not surprising that RCTs, with a sample that is not equivalent to the lowest quintile according to fish intake, do not replicate the same strong effects (McLennan & Pepe, 2017). Appropriately designed RCTs rely on the clear separation of the active groups to the control groups and it is not acceptable for the placebo group to be exposed to significant amounts of the test product (McLennan & Pepe, 2015). However, in RCTs supplementing with n-3 PUFAs, every subject within the placebo group will have a baseline whole body n-3 index that is significantly different to zero (McLennan & Pepe, 2015) due to the essential nature of n-3 PUFAs in human populations. No clear "lower quintile" or "low n-3 index" is offered within the literature thus far with Rice et al., (2016) ambiguously concluding that future intervention trials should recruit participants with low baseline n-3 fatty acid levels following a workshop held at the 11th International Society for the Study of Fatty Acids and Lipids (ISSFAL) congress. However, Harris, Del Gobbo and Tintle (2017) did conclude that typical n-3 index status in low-fish intake individuals was around 4% total fatty acid content in RBC across 10 cardiovascular cohort studies.

Future research in this area should consider using blood samples as a screening tool to determine baseline n-3 PUFA status to support subjective reporting of low-fish intake. Based on some previous recommendations it may also be reasonable to exclude participants with an n-3 index greater than 4% total fatty acid content in RBC (Block et al., 2008; Harris, 2010; Harris, Del Gobbo & Tintle, 2017) to ensure a sample that truly represents the lowest n-3 PUFA intake. Within the current study, baseline n-3 index was seen to range between 3-12% which suggests there was wide range of dietary intakes of n-3 PUFAs between the participants prior to enrolment that should have been controlled for to a greater extent. However, it should also be noted that within the current study only 7% of participants were found to have an n-3 index below 4% which could suggest that this recommendation may actually be extremely difficult to achieve within RCTs with regards recruitment of participants. Additionally, for future studies measuring mood outcomes it may be beneficial to include measures of certain lifestyle factors such as dietary habits and physical activity. Intake of fruits and vegetables and exercise habits may potentially act as proxy measures for 'healthiness'. Exercise has been extensively found to ameliorate negative moods in a wide range of populations (Powers, Asmundson & Smits, 2015; Hearing et al., 2016; Crush, Frith & Loprinzi, 2018; Yang, Ko & Roh, 2018) and can be measured via the International Physical Activity Questionnaire (IPAQ; Craig et al., 2003) and baseline dietary habits can be measured via a food frequency questionnaire (FFQ). Collecting, additional data on baseline healthiness would allow for greater control over the selection of participants and analysis of data.

In regards to samples, future research should consider the role of several factors. The ApoE4 genotype has previously been identified as an influencing factor on n-3 PUFA status, with a number of human studies retrospectively reporting that the cognitive benefits associated with fish intake were lower or absent in ApoE4 carriers (Huang et al., 2005; Whalley et al., 2008; Quinn et al., 2010), potentially suggesting that ApoE4 carriers should be analysed separately from non ApoE4 carriers, as they were in Stonehouse et al., (2013). Additionally, a recent systematic review by Groot et al., (2019) identified sex as an influencing factor on human n-3 PUFA levels and Howe et al., (2018) has also suggested that it is important for future studies to take account of the sex differences in responses to n-3 supplementation, potentially providing evidence for the need to analyse outcomes by sex in future studies.

The findings from the current study provide support for the beneficial effects of increased consumption of n-3 PUFAs, specifically EPA, for improving global cognitive function and reaction times during executive functioning and episodic memory tasks. Additionally, the observed findings for the total percentage of n-3 PUFAs in RBC seem to be favourable with regards to the employment of night time dosing with SMEDS formulations. However, further validation of this methodology would be needed to confirm this. Future RCTs measuring cognitive function and mood should consider the effects of EPA-rich oils, sex, genotype, time of dosage and formulation of the treatments. Overall, the findings suggest that 26 weeks supplementation with n-3 PUFAs, particularly EPA, may offer some benefits with regards to cognitive function and short term brain health in healthy, young adults.

CHAPTER 7: GENERAL DISCUSSION

7.1 Summary of Objectives

The aim of this thesis was to investigate the effects of both DHA-rich and EPA-rich SMEDS formulated supplements, dosed at bedtime, on brain function, sleep, memory consolidation, mood and their efficacy for cognitive function in healthy, young adults. Prior to conducting this programme of studies, fifteen RCTs investigating one or more of these parameters in healthy, young adults had been conducted (Fontani et al., 2005a; 2005b; Antypa et al., 2009; Cornu et al., 2010; Hamazaki-Fujita et al. 2011; Bauer et al., 2011; Kiecolt-Glaser et al., 2011; Jackson et al. 2012a; 2012b; 2012c; Stonehouse et al. 2013; Bauer et al., 2014a; Hansen et al., 2014; Giles et al., 2015; Watanabe et al., 2018). Moreover, only four of these previous studies used both a DHA-rich and EPA-rich treatment, resulting in a limited amount of knowledge concerning what the similar and separate effects of DHA and EPA are within healthy, young adults. Additionally, healthy, young adult samples were specifically of interest throughout this thesis as they represent a sample, the majority of whom do not meet the recommended intake of n-3 PUFAs (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018) but are otherwise healthy. In addition, it is suggested that this population are also undergoing natural age-related patterns concerning overall cerebral blood flow and their ability to mobilise additional local blood flow during neural activity and cognition (Salthouse, 2009; Salthouse, 2016; Salthouse, 2019), that may be ameliorated or reduced when n-3 PUFA intake is increased.

The previous literature was also limited by several methodological issues concerning the n-3 PUFA treatments, with regards to the formulations used. Attempts to increase the bioavailability and absorption of the treatments throughout the supplementation periods were lacking, with none of the previous studies employing galenic formulations (i.e. emulsifications) in an attempt to maximise uptake of the fatty acids throughout the trials. Furthermore, other factors that may impact absorption, including the time of supplementation and the stability of the oils in terms of oxidation had also been overlooked. To this end SMEDS formulated oils, containing traces of the stabilising antioxidants tocopherols (vitamin E) and ascorbyl palmitate (vitamin C) to stabilise the oils, dosed at bedtime, were employed throughout the entire thesis in an attempt to increase the bioavailability of the supplements. Bed time dosing was specifically chosen in an attempt to ensure that the n-3 PUFAs were at the site of action in the intestines paralleled with the peak time for lipid absorption and digestion during the very beginning of the active/awake phase (Bray & Young, 2011; Jackson et al., unpublished data; Appendix IX; Appendix X).

As examination of the literature revealed that there was a previous lack of focus placed on the effects of n-3 PUFAs in healthy, young adults, further investigation was warranted to expand upon the limited previous findings. In order to address this, Chapter 3 aimed to investigate the effects of both DHA-rich and EPA-rich treatments on cerebral blood flow whilst attempting to correct previous issues regarding neuroimaging methodologies. Chapter 4 investigated the effects of both DHA-rich and EPA-rich treatments on objective, subjective and biological parameters of sleep, and Chapter 5 investigated the effects of both DHA- and EPA-rich treatments on learning-memory tasks, employing a novel methodological paradigm that assessed these parameters before going to sleep and upon waking, in an attempt to allow for overnight memory consolidation to occur. Finally, Chapter 6 aimed to investigate the effects of both DHA-rich and EPA-rich supplements on measures of cognitive function and mood.

7.2 Associations between Dietary n-3 PUFAs and Brain Function

7.2.1 Cognitive Function

Concerning the effects on cognitive function, no effects of either the EPA-rich or DHA-rich supplement were observed in Chapter 3. In Chapter 5, the DHA-rich supplement improved word recognition reaction times whilst also increasing displacement errors on an object location learning task, compared to placebo. In Chapter 6, the EPA-rich supplement resulted in improvements in global accuracy and speed, as well as improving reaction times on the Stroop and word recognition tasks, compared to placebo. Additionally, the EPA-rich supplement also lead to improvements in the delayed word recall task and accuracy of memory compared to the DHA-rich groups.

Previously, Bauer et al., (2011; 2014a) has also identified positive effects of EPA supplementation on reaction times during the Stroop task, consistent with the findings from Chapter 6. As well, Stonehouse et al., (2013) has identified positive effects of a DHA-rich supplement on reaction times during the word recognition task, consistent with the findings from Chapter 5. However, a significant effect was also identified following supplementation with the EPA-rich treatment on word recognition reaction times in Chapter 6. As Stonehouse et al., (2013) did not employ an EPA-rich treatment this finding is difficult to interpret. Furthermore, supplementation with the EPA-rich oil in Chapter 6 was seen to improve performance on the delayed word recall task and accuracy of memory, compared to the DHA-rich oil. This may imply that the ratio of EPA:DHA is important with regards to performance on episodic memory tasks and memory more generally. This finding concerning improved reaction times following the EPA-rich supplement may also be consistent with the previous conclusions of Bauer et al., (2011; 2014a; 2014b), which suggests that EPA has a greater

influence on neural efficiency than that of DHA. Potentially suggesting that intakes higher in EPA are more beneficial with regards to performance on higher order tasks. Indeed, this idea is further supported by the findings observed in Chapter 6 concerning the significantly higher global accuracy and speed scores within the EPA-rich group compared to both the DHA-rich group and placebo. This increase in relation to EPA has been observed previously in older samples and samples suffering with MCI and AD (Chiu et al., 2008; Nishihira et al., 2016), yet no study thus far has reported an effect on global cognition in a sample of healthy, young adults following supplementation with an EPA-rich treatment. Together, the positive findings regarding the effects of EPA on cognitive function are interesting given that brain EPA levels are typically 250-300 times lower than DHA (Chen et al., 2013). This therefore suggests that EPA may exert its effects on cognition via different mechanism than incorporation into brain, potentially via eicosanoid lipid mediators (Chen & Bazinet, 2015) and/or by having direct actions on mitochondrial enzymes that produce ATP within the brain (Bauer et al., 2014b). Overall, the findings observed throughout this thesis in relation to cognitive function appear to support increased intake of EPA over DHA in healthy, young adults. The specific focus on DHA in previous RCTs may represent one of the biggest oversights within the research area thus far and therefore future RCTs should ensure to also include EPA-rich supplements rather than solely focussing on the effects of DHA-rich supplements.

7.2.2 Cerebral Haemodynamics and Neural Efficiency

Chapter 3 of this thesis aimed to investigate the chronic effects of 26 weeks' supplementation of both DHA- and EPA-rich treatments on cerebral haemodynamics via the employment of a "quantitative" fd-NIRS device that allowed for the assessment of gross changes across the supplementation period, a measurement that was missing from previous studies. A trend towards a significant decrease in quantities (μM) of $Hb0_2$ was identified in the right hemisphere during completion of serial subtraction tasks, following supplementation with both the DHA- and EPA-rich treatments. Furthermore, negative correlations supporting these findings were also identified at baseline and week 26 showing n-3 index to be inversely associated with quantities of THb and $Hb0_2$. The observed decreases in $Hb0_2$ were also not seen to be associated with any decreases in cognitive performance, suggesting there may have been an increase in neural efficiency following supplementation with n-3 PUFAs. Indeed, positive correlations were identified both at baseline and at week 26 in both hemispheres, between THb and $Hb0_2$ neural efficiency scores and n-3 index during completion of the serial 17 subtraction task. However, it should be noted that no significant main effects of treatment were identified to show increases in neural efficiency scores. Though, this may be due to the study not being statistically powered to detect any cognitive effects and the neural efficiency scores

representing both standardised haemoglobin quantities and standardised performance scores for the serial subtraction tasks.

EPA specifically has been suggested to influence neural efficiency, with Bauer et al., (2014b) previously concluding that EPA over DHA is more advantageous in reducing "brain effort". Additionally, Fontani et al., (2005) argues that because EPA-rich supplementation reduced the alpha to theta wave ratio and improved cognitive performance in their study, EPA may have induced a state of neural efficiency whereby the brain activates less areas to perform to the same or better standard than before supplementation. These findings for EPA contributing towards increased neural efficiency may be supported by the results of Chapter 6 which favour the EPA-rich supplement for improving cognitive performance compared to placebo and the DHA-rich supplement. This was seen in reductions of the reaction times during a delayed word recall task and accuracy of memory, compared to the DHA-rich supplement and trends towards improvements in reaction times during the Stroop task and significant improvements in reaction times on the word recognition task and global accuracy and speed scores, compared to placebo. These findings support the idea that EPA may increase neural efficiency and potentially results in greater activation in brain regions specifically associated with memory retrieval, such as the medial temporal lobe and hippocampus (Rugg & Vilberg, 2013). However, as the observed correlations in Chapter 3 were with n-3 index, not EPA or DHA specifically, and as DHA is known to influence n-3 index in RBCs to a greater extent than EPA, the limited positive findings for DHA in Chapter 6 do not support any potential further increases in neural efficiency bar those relating to memory. As a result, large scale trials utilising fMRI scans, capable of accurately measuring specific locations of neural efficiency and levels of activation in the medial temporal lobe and hippocampus during completion of episodic memory tasks, following supplementation of EPA- and DHA-rich treatments may be needed to further investigate these findings. The use of fMRI may also help to provide useful information regarding activation, as increased neural efficiency may be a result of increased activation of relevant brain regions and decreased activation in irrelevant regions.

Concerning the methodological use of the fd-NIRS, as a way to measure gross chronic changes rather than the cw-NIRS employed in previous studies (Hamazaki-Fuijita et al., 2011; Jackson et al., 2012), it appears that the findings are in support of the employment of the fd-NIRS. Throughout Chapter 3 significant differences were detected by the fd-NIRS between the baseline and week 26 assessments, whilst also being able to detect significant differences between the levels of haemoglobin in both the right and left hemispheres and during completion of the three separate tasks (Appendix V), suggesting that the fd-NIRS was appropriate for measuring both chronic and acute changes in cerebral blood flow. However,

regarding the type and length of tasks chosen during Chapter 3, there may be a few methodological concerns. The choice of one minute long tasks may questionably not have been long enough to induce the desired cerebral haemodynamic responses and additionally providing only one minute of rest between each repetition of the task may not have been long enough to allow the cerebral haemodynamic response to return to resting levels (Allen et al., 2007). Though, participants did have longer two minute rest periods whilst changing between the serial 3, 7 and 17 tasks, which may have potentially helped to contribute to the significant effects often observed between tasks but not during repetition of the same task (Appendix V). Additionally, the type of task employed may not have been the most appropriate, as although the subtraction task allowed for clear manipulation of the task difficulty, Chapter 6 revealed no significant effects of either the EPA- or DHA-rich treatment during completion of the serial subtraction tasks perhaps suggesting that working memory tasks were not the most appropriate choice of task to elicit the greatest cerebral haemodynamic response. In light of the findings from Chapter 6 it may have been more appropriate to employ a range of executive functioning tasks to activate the cerebral haemodynamic response. For example, the peg and ball task could have been used as it has been employed in a previous RCT using the fd-NIRS following supplementation with a *Sideritis scardica* (Greek mountain tea) extract (Wightman et al., 2018).

7.2.3 Sleep

Sleep is recognised as a vital part of public health with longitudinal studies showing people reporting sleep disorders are at a greater risk for developing cardiovascular disease (Cappuccio et al., 2011), hypertension (Wang et al., 2012), type 2 diabetes (Cappuccio et al., 2010), obesity (Bos et al., 2018) anxiety and depression (Sullivan & Ordiah, 2018) and indirectly contributing to higher risks of mortality (Åkerstedt et al., 2019). Populations in western societies as a whole often report insufficient amounts of sleep or a high prevalence of sleep problems (Cirelli et al., 2016; Kerkhof, 2017). Specifically, Rössler et al., (2017) report that sleep problems were highly prevalent among young and middle aged adults in a sample of 1,274 from Switzerland, further identifying that all subtypes of sleep problems were associated with poorer mental health and particularly depression. Consequently, RCTs focused on alleviating sleep problems and insufficient amounts of sleep are of the upmost importance in relation to public health.

The findings of Chapter 4 identified beneficial effects of supplementation with the DHA-rich treatment on measures of sleep latency and sleep efficiency compared to placebo and, interestingly, showed those supplemented with the DHA-rich treatment as having the longest

sleep times whilst those supplemented with the EPA-rich treatment had the shortest sleep times. It should be noted however, that all three treatment groups slept for between 7-8 hours' per night which does not indicate any abnormal sleep times and match with sleep durations reported in epidemiological studies (Adams et al., 2017). With regards to increased sleep length, previous studies have associated this with increased athletic performance (Mah et al., 2010) and decreased vulnerability to accidents and injuries (Swanson et al., 2011). As shorter sleep times have previously been associated with poor cognitive performance (Gruber et al., 2010; Lo et al., 2014), one peculiar finding in Chapter 4 was the EPA-rich group recording the shortest amount of sleep time, as this group also reported the greatest cognitive performance in Chapter 6. As the EPA-rich treatment was seen to have a trend towards significantly improved sleep efficiency compared to placebo and findings from Chapter 3 potentially suggesting that EPA may be driving increases in neural efficiency, it could be that those supplemented with EPA required less sleep to feel replenished or that the underlying processes that occur during sleep occurred slightly faster following supplementation with EPA. It could also be that improvements in other aspects of sleep, such as, sleep architecture i.e. amounts of SWS and REM sleep or processes related to neurogenesis, brain plasticity or clearing of adenosine from the cortex and forebrain occurred at a faster rate, resulting in shorter sleep times. Indeed, the AA synthesised prostaglandin D_2 is known to increase extracellular levels of adenosine in the basal forebrain promoting sleep (Satoh et al., 1996; Huang, Urade & Hayaishi, 2011). It could be that, as EPA competes with AA in the production of eicosanoids, increased dietary intake of EPA leads to reductions in prostaglandin D_2 within the basal forebrain resulting in a decreased promotion of sleep. If this is the case, then this reduction in sleep promoting prostaglandins did not appear to affect the quality of sleep, as the EPA-rich group also experienced trends towards increased sleep efficiency.

Conversely, the positive effects of supplementation with the DHA-rich treatment on certain sleep parameters were not consistent with positive effects on memory consolidation in Chapter 5 or on cognitive function in Chapter 6. This could potentially be a result of the effects of DHA on memory consolidation being too trivial in healthy, young adults. However, the positive effects of DHA on the objective measures of sleep not matching with any positive effects in Chapter 5 or 6 are further complicated with the negative effects on subjective ratings of sleep that were identified in Chapter 4. These negative VAS ratings of subjective sleep could also be seen as consistent with the negative VAS ratings of task difficulty identified in Chapter 3 following supplementation with the DHA-rich treatment, with both of these findings occurring simultaneously with what appears to be a positive effect on the respective objective measures. These incongruous findings in Chapter 4 may potentially be explained by 'sleep state misconception' (Edinger & Krystal, 2003) observed in patients with a relatively normal sleep

continuity and architecture in spite of large subjective complaints of disturbed sleep. Additionally, the increased ratings of task difficulty observed in Chapter 3 may be explained by research that shows hedonic state during cognitive testing can impact on subjective mood ratings, with previous research showing that subjective ratings of alertness were increased following a drink of water in a group rated as “high in thirst” (Rogers & Kainth, 2001). This potentially could offer insights into the sensitive nature of subjective rating scales and may explain why there is a discrepancy between subjective and objective scores in this group. However, it is definitely of interest that it is consistently the DHA-rich group that produces these incongruent findings in both Chapter 3 and Chapter 4 whilst these effects were not seen within the EPA-rich group. Together, these findings appear to suggest that the EPA:DHA ratio of the supplement is important in relation to subjective ratings as the EPA-rich treatment also contained DHA yet reported no negative effects concerning subjective measurements.

With regards to the measures used in Chapter 4 to assess sleep parameters, it can be concluded that the use of actigraphy watches were the most practical objective measure available for the study presented in Chapter 4. However, these watches are related to certain methodological limitations regarding the lack of measurement of sleep architecture and issues with the algorithms used to score the data, mostly due to the complex nature of sleep. However, as PSG or overnight laboratory methodologies were not available and also present their own limitations, actiwatches appeared to be the most suitable choice for Chapter 4. Additionally, the measurement of aMT6s as a physiological measure of sleep allowed for a more holistic measurement of sleep as a whole. Though, previous literature does seem to advocate for longer collection periods than just one night and/or for the direct measurement of melatonin via blood samples that should definitely be considered in future trials (Benloucif et al., 2008). Finally, the use of the LSEQ over other subjective measures of sleep, such as the Pittsburgh Sleep Quality Index, can be defended as the LSEQ was specifically designed to monitor subjectively perceived changes in sleep and next morning behaviour during investigations and behavioural interventions.

7.2.4 Memory Consolidation

The study presented in Chapter 5 of this thesis was the first to employ a methodological paradigm which involved overnight learning and recall tasks with the aim of allowing overnight memory consolidation to occur. A significant effect of treatment was identified for the reaction times during the immediate word recognition tasks with the DHA-rich group having significantly faster reaction times compared to placebo. Although the task employed within Chapter 5 was an immediate word recognition task, these findings can still be seen to be consistent with

previous studies from Yurko-Mauro et al., (2010) and Stonehouse et al., (2013) who identified improvements in delayed word recognition following supplementation with a DHA-rich treatment. Conversely, this finding is contradictory to the findings identified in Chapter 6 as it was the EPA-rich group which showed significantly faster reaction times during the word recognition task whilst the DHA-rich group showed no significant difference compared to placebo. Furthermore, a negative effect was also identified for total location learning displacement following supplementation with the DHA-rich treatment compared to placebo. Although a greater amount of displacement in the computerised location learning task was observed in the DHA-group, this was not consistent with any negative effects on the rate of learning the locations of the items or on the recall of the locations of the items rendering it difficult to interpret and could simply be a chance result. Moreover, as the DHA-rich group was seen to have faster reaction times during completion of the word recognition tasks paired with an increased number of errors in the location learning tasks it may be that this group experienced a speed-accuracy trade off whilst completing the learning trials.

Considering that DHA has previously been the main focus of RCTs that measure cognition due to it being highly enriched in the brain, the lack of effects in the DHA-rich groups on cognitive performance throughout the thesis was not anticipated. Null effects of DHA were identified in Chapter 6 and on the cognitive tasks completed in Chapter 3, although this study was not powered to detect cognitive differences between the groups. Nonetheless, these null effects are consistent with a large body of the literature that also identify null or conflicting results in relationship between n-3 PUFAs and cognitive function in healthy samples (reviewed in Cooper et al., 2015; Rangel-Huerta & Gil, 2017; Solfrizz et al., 2018). The lack of findings concerning the learning-recall tasks in Chapter 5 following supplementation with the DHA-rich treatment seems to contradict the positive effects on objective sleep that were observed in Chapter 4. However, as SWS has previously been associated with memory consolidation (Rothschild, Eban & Frank, 2017), it could be that the positive effects of DHA observed in Chapter 4 were not directly related to SWS specifically but other aspects of sleep, i.e. reduced sleep latency or increased amounts of REM sleep. Indeed, Feige et al., (2008) has previously shown that patients with higher amounts of REM sleep tended to report more minutes of subjective wakefulness. If the DHA-rich group did experience increases in REM sleep rather than SWS then the evidence from Feige et al., (2008) would also match with the negative subjective ratings of sleep identified within the DHA-rich group in Chapter 4. Moreover, Lutchman and Song (2013) describe how the effects of n-3 PUFAs on cognitive function are more often observed, in developing (potentially n-3 deficient) and aged or cognitively impaired cohorts. This may suggest that the healthy, young adult samples used throughout the studies that comprise this thesis may have potentially not been adequately deficient in n-3 PUFA

status. Indeed, some previous recommendations suggest that it may be reasonable to exclude participants with a baseline n-3 index greater than 4% total fatty acid content in RBC (Block et al., 2008; Harris, 2010; Harris, Del Gobbo & Tintle, 2017), yet only 7% of all participants randomised into the studies throughout this thesis were found to meet this criteria.

One interesting outcome from Chapter 5, with regards to the negative subjective ratings of feeling energetic and rested following supplementation with the DHA-treatment in Chapter 4, is that no negative effect of DHA on ratings of morning alertness or performance on the attention and executive functioning tasks were identified. This appears to suggest that, although participants rated that they felt less rested and energetic following the DHA-rich treatment, this did not appear to impact upon how alert participants felt, as well as, did not have an impact upon performance on the attention and executive functioning tasks upon waking. These nuances in subjective state following sleep should be investigated further in future research in an attempt to provide further insight into the underlying mechanisms of these findings.

Concerning the overnight methodological paradigm employed in Chapter 5, although it allowed for the measurement of a learning and recall task to occur prior to and after sleeping, it could be argued that the measurement of this over a single night was not adequate to capture memory consolidation. As the study presented in Chapter 4 measured sleep parameters over a seven day period due to nightly variations in sleep occurring, perhaps it was also practical to measure the learning-recall tasks over a similar seven day period. If nightly variations in quality and length of sleep can occur, then it also seems likely that nightly variations in memory consolidation also occur consistently with a better or worse night's sleep. Furthermore, this extended paradigm would also benefit from actiwatch measures as objective improvements or decreases in sleep parameters could then be matched with improvements or decreases in performance on the morning recall tasks. Additionally, just as aMT6s was measured in Chapter 4 to provide a more holistic measure of sleep, perhaps measures of BDNF could also have been included to Chapter 5 as a biological measure of synaptic plasticity. BDNF levels are seen to be readily detectable in human serum and can be measured in serum, plasma or whole blood (Polacchini et al., 2015), although this was unfortunately not possible within the scope of this thesis and should therefore be considered in future trials.

Furthermore, it was also hypothesised that night-time supplementation may support night time processes, such as neurogenesis and synaptogenesis, though the null findings identified in Chapter 5 appear to refute this hypothesis. The null effects of night time dosing in Chapter 5 are unexpected given that Jackson et al., (unpublished data; Appendix X) has previously

observed that night time supplementation with the same DHA- and EPA-rich oils resulted in significant increases in plasma DHA for 6 hours post dose, with concentrations of plasma EPA having similar peaks but also being significantly greater for the entire 24 hour period following. Given the findings from Jackson et al., (unpublished data; Appendix X), as well as previous evidence to support the role of DHA and EPA in processes such as LTP and brain plasticity, it appears reasonable to have anticipated significant effects of the active treatment on overnight memory consolidation. However, as the study presented in Chapter 5 was the first of its kind the methodological paradigm that was employed has not yet been proven to be capable of measuring learning and memory consolidation overnight. This may potentially explain the lack of findings concerning the effects of n-3 PUFA supplementation of memory consolidation. Indeed, the performance scores identified within Chapter 5 were found to be worse than those reported in previous studies (Holz et al., 2012a; Haskell-Ramsay et al., 2018). Therefore, future research should aim to identify whether COMPASS tasks performed on tablet computers outside of the laboratory setting are consistent with outcomes of the same tasks on the COMPASS software conducted within the laboratory under the supervision of the researcher. It could also be argued that additional learning memory tasks, such as the paired associate learning task or name to face association task could also be added to the paradigm in future trials as additional measures of learning and memory. Furthermore, the use of the recall phase of the learning-memory tasks as a proxy measure of memory consolidation may not have been the most appropriate measure. For example, tDCS could potentially have been used instead as a more direct measurement of LTP and the mechanisms underpinning learning and memory. A growing body of evidence suggests that tDCS can have beneficial effects on long-term memory, synaptic plasticity and adult neurogenesis (Di Lazzaro et al., 2013; Coffman et al., 2014; Leone et al., 2014, 2015; Podda et al., 2014), as well as a consistent body of evidence that indicates that tDCS exerts modulatory effects of LTP (Ranieri et al., 2012; Rohan et al., 2015; Podda et al., 2016; Kronberg et al., 2017). The measurement of tDCS-induced LTP and synaptic plasticity pre and post supplementation with DHA and EPA may provide a more sound and objective methodological paradigm to further assess the relationship between n-3 PUFAs and memory consolidation and even memory processes more generally.

7.2.5 Mood

One of the main aims throughout this thesis was to measure aspects of cognition and mood, with the study presented in Chapter 6 being the largest RCT to date that measures the effects of both EPA-rich and DHA-rich treatments on cognition and mood in healthy, young adults. No significant effects of treatment were identified on any components of the POMS

questionnaire, which was mostly inconsistent with the small number of RCTs employing healthy, adult samples that have found significant improvements previously following supplementation with EPA-rich treatments (Fontani et al., 2005a, 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011). One potential explanation for the null findings in Chapter 6 regarding ratings of mood may be due to the use of a healthy, young adult sample that were free from mood disorders. For instance, Hallahan et al., (2016) explains how changes in mood in non-clinical populations are not the same as within clinically depressed populations and so positive or negative impacts on mood may not necessarily translate into positive or negative outcomes in clinical depression, which are populations that produce a more consistent body of evidence for the beneficial effects n-3 PUFAs on mood (Mocking et al., 2016; Hallahan et al., 2016; Deacon et al., 2017). Combined with the null results in Chapter 6, this may potentially suggest that there could be a ceiling effect of n-3 PUFAs in relation to mood in healthy non-clinically depressed populations that already score well on measures of mood and depression. Indeed, some literature reviews agree that evidence of the mood enhancing effects of n-3 PUFAs in non-clinical populations is limited and still requires further investigations (Giles et al., 2013; Hallahan et al., 2016). Additionally, it could be that if effects of n-3 PUFAs on mood in healthy adults exist then these effects may be transient and occur quickly when deficiencies in the n-3 PUFA status of low consumers are addressed. Previous studies that report beneficial effects of n-3 PUFAs within this sample supplemented between only 1 and 4 months (Fontani et al., 2005a, 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011) and other nutritional intervention studies have also identified acute changes in mood within healthy, adult samples (Macpherson et al., 2015; Haskell et al., 2017; Khalid et al., 2017; Haskell-Ramsay et al., 2018). If the effects of n-3 PUFAs on mood are transient in healthy adults then future RCTs supplementing for longer durations should ensure to include measures of mood at regular intervals from the beginning of supplementation. However, it should be noted that the findings from Chapter 6 do appear to suggest that there may be little or no effects of n-3 PUFA supplementation on mood in healthy, young adults.

Bedtime dosing may potentially have also influenced the null findings concerning mood. The findings from Jackson et al., (unpublished data; Appendix X) suggest that night time supplementation with EPA and DHA results in increased levels in circulating plasma throughout the night, which may potentially then impact serotonin levels. Indeed, EPA is known to impede the formation of E_2 series prostaglandins, which inhibit the release of serotonin (Schlicker, Fink & Göthert, 1987; Günther et al., 2010) and DHA's effects on membrane fluidity have been seen to influence serotonin receptor function (Patrick & Ames, 2015) which may result in an increase of serotonin throughout the night. This increase in the circulating plasma levels of EPA and DHA within the body could therefore allow for the effects

of EPA and DHA on serotonin to be enhanced throughout the night, due to the increased bioavailability of the n-3 PUFAs in circulation. Although the precise timings of the effects of EPA and DHA on serotonin are still not currently well documented within the literature, previous literature has indeed identified associations between higher plasma concentrations of n-3 PUFAs, particularly DHA, and higher concentrations of serotonin metabolites (Hibbeln et al., 1998). It could be that night time supplementation with n-3 PUFAs impacts upon the natural circadian rhythm of serotonin. Serotonin is seen to be highest during the active wake phase (Challet, 2007) and lowest during low levels of light (Ushijima et al., 2005; 2012) and therefore, increased circulating levels of DHA and EPA through the night may also result in increased levels of serotonin throughout the night, altering the natural circadian rhythm of serotonin. As serotonin is most often described for its role in the aetiology of mood and neuropsychiatric disorders, it could be that matching the timing of n-3 intake with the natural circadian rhythm of serotonin would be the most beneficial for positively influencing serotonin levels. This would therefore appear to suggest that morning intake of n-3 PUFAs would be most beneficial in regards to mood due to the potential increase in day time serotonin levels that may follow morning intake.

Concerning the measures of mood, the use of the POMS questionnaire in Chapter 6 allowed for the measurement of a wider range of aspects of mood compared to other more specific scales such as the Beck's Depression Inventory (BDI), HADS or the generalised anxiety disorder assessment. However, the POMS has previously been criticised for its inability to appropriately measure positive and pleasant feelings (Andrade et al., 2010), which may be relevant in this healthy sample. As a large portion of the literature focuses specifically on depression and not overall mood, it could be argued that a more specific measure of depression levels, such as the BDI, should have also been employed in Chapter 6 to more closely measure aspects of depression specifically.

7.2.6 Separate effects of DHA and EPA

The studies conducted throughout this thesis allowed for the direct comparison of both the DHA- and EPA-rich treatments as a secondary analysis, an aspect that is often missing within the research area. The effects of both the DHA- and EPA-rich treatments that were observed in Chapter 3 in regards to CBF were comparable, with both treatments showing a trend towards reducing the amounts of $Hb0^2$ in the right prefrontal cortex during the subtraction tasks. However, the DHA-rich treatment was seen to increase ratings of task difficulty whereas the EPA-rich treatment did not. Additionally, in Chapter 4 opposite effects were observed between the active treatments for total minutes in bed, total sleep time and subjective ratings

of feeling rested, with the DHA-rich group having the longest sleep times and lowest feeling rested ratings whilst the EPA-rich group had the shortest sleep times and highest feeling rested ratings. Although, both n-3 treatments were seen to improve sleep efficiency compared to placebo. In Chapter 5 the DHA-rich treatment improved reaction times during the word recognition task, whilst also increasing the number of errors during the learning phase of the location learning task, whereas no such findings were observed within the EPA-rich group. Finally, in Chapter 6 differences were identified for delayed word recall, accuracy of memory global accuracy and global speed with the EPA-rich group producing the highest scores and the DHA-group producing the lowest scores in all instances.

As the full extent of the separate and similar effects of DHA and EPA are still poorly understood (Drouin et al., 2019), it should be noted that across the breadth of this thesis only a limited number of common effects of the active treatments were identified when compared to placebo. These included both n-3 treatments resulting in a trend towards decreased quantities of $Hb0^2$ during completion of serial subtraction tasks identified in Chapter 3 and improvements in sleep efficiency in Chapter 4. All other findings identified throughout the thesis were seen to be related specifically to either the EPA- or DHA-rich treatment or were opposite effects. The separate effects of both the DHA- and EPA-rich supplements that were identified are most likely due to the separate actions DHA and EPA have within the body. In a recent systematic review, Innes & Calder (2018) concluded that DHA and EPA actually appear to have different effects on cardiometabolic risk factors such as LDL cholesterol size, platelet activity, vascular function, heart rate and blood pressure even though the majority of clinical trials that have been conducted thus far have been focused on administering both DHA and EPA together, with only a small number of comparator trials existing within the literature. Furthermore, Gorjão et al., (2009) have previously identified that EPA and DHA have been seen to have different effects on leukocyte functions such as cytokine production, chemotactic response and phagocytosis as well as modulating different gene expressions in lymphocytes and activation of intracellular signalling pathways involved with lymphocyte proliferation. This may provide insights into why supplementation with the DHA- and EPA-rich treatments resulted in a number of separate effects across the studies. Another important factor to consider concerning the two active treatments, is the ratio of the n-3 PUFAs that were administered. For example, the DHA-rich treatment was seen to increase overall n-3 index more than the EPA-rich treatment, yet this increased n-3 index did not result in positive effects in relation to cognitive function or subjective ratings in any of the experimental chapters. This may then suggest that achieving higher DHA levels parallel to diminished increases in EPA levels was less efficacious than achieving lower DHA levels but higher EPA levels, even if this resulted in a lower overall n-3 index. Additionally, the findings from this thesis provide evidence to support

the idea that EPA may actually be more important than previously thought, at least with regards to cognitive function and subjective ratings of task difficulty, feeling energetic, rested and ready to perform.

The different outcomes identified in this thesis following supplementation with either the DHA- or EPA-rich treatment, further supports the notion that there is a definite need for RCTs in the research area to employ methodologies that use both a DHA- and EPA-rich treatment and not just one n-3 treatment, to further establish what the respective effects of DHA and EPA actually are. Indeed, it is clear in the literature that both the compound specific and shared effects of DHA and EPA are actually still poorly understood (Cottin, Sanders & Hall, 2011; Alexander et al., 2017; Innes & Calder, 2018; AbuMweis et al., 2018; Drouin et al., 2019) and this will be, at least in part, due to a plethora of research employing designs with only one active n-3 treatment or multiple n-3 treatments that are all higher in just one of the n-3 PUFAs. Furthermore, the data collected throughout this thesis appears to support the importance of achieving an optimal EPA:DHA ratio, with higher quantities of EPA compared to higher DHA being associated with an increased number of positive findings across the experimental chapters of this thesis. Taken together, the findings from this thesis provides evidence for the impact of varying ratios of EPA:DHA on aspects of cognitive function, sleep and cerebral haemodynamics, highlighting the importance of the EPA:DHA ratio of n-3 supplements as well as their administration.

7.3 Night-time Dosing with SMEDS Formulated Treatments

Whilst reviewing the current literature in Chapter 1, it became apparent for the need for RCTs to pay greater attention to employing strategies and methodologies that will be beneficial with regards to increasing bioavailability of the fats throughout the duration of the trials. This may be one of the reasons why previous research has identified null effects of n-3 PUFAs, as the bioavailability of a substance is one of the key factors required to show an effect within nutritional trials (Khan & Singh, 2016). As n-3 PUFAs have extremely low water solubility and show slow nutrient dissolution rates and poor absorption in the gastrointestinal tract when administered orally, it has been recommended that n-3 PUFA supplements be consumed with a meal (Schuchardt & Hahn, 2013). However, the nutritional components of this meal, in particular the fat content can significantly influence the bioavailability of n-3 PUFAs (Kling et al., 2011; Davidson et al., 2012), which would be an issue in most RCTs as each participant will consume their treatments with a meal that may not be matched in terms of its nutritional content compared to the other participants. As it is not possible to provide daily meals during long clinical trials, other methodologies can be employed such as SMEDS formulations

(Garaiova et al., 2007; Müllertz et al., 2010; Wakil et al., 2010; West et al., 2018; Maki & Dicklin, 2019) to not only increase the absorption of n-3 PUFAs without the additional consumption of a high fat meal (Maki & Dicklin, 2019) but to also help to standardise study protocols in terms of treatment consumption throughout the trial. To this end, each study comprised in this thesis used SMEDS formulated treatments in an attempt to increase absorption and uptake of the n-3 PUFAs.

Furthermore, the rationale for the delivery of the treatments at night-time was two-fold. Firstly, previous research has begun to show consistent circadian rhythms for DHA and EPA that peak around noontime (Dallamann et al., 2012; Gooley & Chua, 2014) with peak absorption occurring during the morning (Bray & Young, 2011). However, due to the resistance of n-3 PUFAs to intestinal lipases it seemed most appropriate for the n-3 PUFAs to be consumed the previous night so they have time to reach the intestines in time for the peak morning absorption period. Secondly, increases in EPA and DHA overnight following night time dosing may acutely affect processes that support cognitive function including memory consolidation and/or neurogenesis known to occur overnight, and even sleep more generally. Although these theories were based on previous findings presented in the literature, the latter did not appear to be supported by the findings obtained in Chapter 5 as it was found that night time dosing with either EPA- or DHA-rich treatments did not result in improvements in measures of memory consolidation via the learning-recall tasks. One potential explanation for these may come from Jackson et al's., (unpublished data; Appendix IX; Appendix X) findings, which show that EPA and DHA levels appear to peak four hours post-supplementation within blood plasma. Therefore, this data suggests that bedtime supplementation actually results in peak blood n-3 levels a few hours earlier than the nadirs seen to occur at 05:43am for DHA and 08:41am for EPA. This may then suggest that the peak absorption that occurs during the morning (Bray & Young, 2011) was actually missed by a few hours following the bedtime dosing utilised throughout this thesis. However, as the combined data from Jackson et al., (unpublished data; Appendix IX; Appendix X) and Bray and Young (2011) would therefore suggest that the optimum time of dosage in relation to absorption is between 02:00-04:00am, bedtime dosing may still provide the most practical time to supplement with n-3 PUFAs with regards to absorption during future RCTs. With regards to the bioavailability of the n-3 PUFAs, the employed methodology concerning both night-time dosing and SMEDS formulations does seem to have been effective, with the observed incorporation of the n-3 PUFAs into the erythrocyte membranes in Chapter 6, matching closely with and even resulting in greater incorporation, when compared to similar previous studies such as Stonehouse (2013; Appendix VII). Taken together, the findings obtained throughout this thesis, particularly following supplementation with the EPA-rich treatment, suggest that membrane incorporation

of n-3 PUFAs may not be the main mechanism underpinning effects relating to cognitive functioning. If this is true, then this highlights the underestimation of previous studies that focussed on DHA-rich treatment based off its greater membrane accretion when compared to EPA.

7.4 Use of Olive Oil as a Placebo

Selection of an appropriate control or placebo treatment is crucial in all RCTs but is an issue that is particularly challenging within n-3 PUFA supplementation studies, mainly due to the distinct fish taste associated with consumption of n-3 supplements. The majority of n-3 placebo controlled trials opt to use similar looking oils such as olive oil (Cornu et al., 2010; Dangour et al., 2010; Stonehouse, 2013; Giles et al., 2015) or oils containing n-6 PUFAs (Peet et al., 2001; Sinn et al., 2012). Although the use of both of these constitutes an "n-3 deficient" treatment, their use as a "placebo" causes issue for debate, as both olive oil and n-6 PUFAs are known to have bioactive properties. The use of n-6 PUFA supplements as a control treatment is questionable due to the potent effects n-6 AA is known to have on inflammation and eicosanoid production (Innes & Calder, 2018) as well as n-6 PUFAs competing for the same enzymes used to convert n-3 PUFAs in each of their respective conversion pathways. Additionally, extra virgin olive oil is known to contain high levels of polyphenols (Gorzynik-Debicka et al., 2018) and has previously been associated with a reduction in anti-inflammatory biomarkers and cardiovascular disease incidence (Wongwarawipat et al., 2018). However, pomace, refined and common olive oil are actually low in vitamins, polyphenols, phytosterols, and other low molecular natural ingredients (Kamm et al., 2001; Bianco et al., 2002; Naczk & Shahidi, 2004) and therefore have less widely reported effects in clinical trials. Though, the high oleic acid (n-9 monounsaturated fatty acid) content in olive oil has previously been linked to some reports of reductions in blood pressure (Terés et al., 2008) and reduced prevalence's of neurodegenerative diseases (Vejux et al., 2009; Casas, Sacanella & Estruch, 2014). Still, pomace, refined or common olive oils can be argued to be the most appropriate control treatment, especially in terms of compounds that are capable of matching the appearance of n-3 supplements, an important feature for maintaining the blinding of separate treatment arms throughout clinical trials.

The control treatment chosen to be applied in the trials that comprise this thesis was an olive oil supplement high in oleic acid, with 75.37% of the olive oil specified as oleic acid. This olive oil was consistently shown to be an effective n-3 absent treatment throughout chapters 3-6 with no observed effects on increasing n-3 PUFA status in RBC in the control groups. Additionally, this choice could be further defended over the use of n-6 PUFA oils as AA is

known to compete with n-3 PUFAs for incorporation into tissue membranes and so supplementation with n-6 PUFAs in the control groups would actually result in a detrimental effect on the percentage of n-3 PUFA in RBC, as well as altering the n-3/n-6 ratio in this group, which is regarded as of high importance within the literature (Simopoulos, 2008; 2016). Nonetheless, as increased oleic acid has been associated with positive health outcomes, including reductions in blood pressure (Terés et al., 2008), reduced prevalence of neurodegenerative diseases (Vejux et al., 2009; Casas, Sacanella & Estruch, 2014), regulation of hepatic lipogenesis (Ducheix et al., 2017) and improvements in insulin sensitivity (Palomer et al., 2018), it does not constitute as an ideal placebo within RCTs. Potentially, these previously identified benefits of increased intake of oleic acid may offer insights into some of the negative findings observed, following supplementation with the DHA-rich treatment, or may have masked some of the potential effects of the n-3 treatments. It could be that the oleic acid within the placebo treatment actually resulted in the positive health outcomes mentioned above, influencing the between group comparisons. Concerning subjective ratings throughout the thesis the placebo groups rated the subtraction tasks as the least difficult in Chapter 3 and were the most rested group in Chapter 4, potentially contributing to the significant difference identified between the DHA-rich and placebo groups for these outcomes.

Although the use of an olive oil placebo, high in oleic acid, throughout the studies that comprise this thesis is not faultless, it can still be argued as a more appropriate n-3 deficient alternative to supplements high in n-6 PUFAs. Nevertheless, the previously identified benefits of oleic acid may potentially have influenced the results throughout this thesis concerning the placebo comparisons between the groups. To avoid this issue, future trials may consider crossover designs similar to those employed by Bauer et al., (2011; 2014a), though it should be noted that these designs come with their own limitations and therefore researchers must thoroughly consider the most appropriate study designs for future trials.

7.5 Healthy Adult Samples

The healthy, young adult samples employed throughout this thesis offered several benefits. Firstly, intake of n-3 PUFAs is known to fall short of recommended doses within the general population (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018) and so young adults comprise a group likely be deficient with regards to their n-3 PUFA intake but are otherwise healthy. Secondly, previous findings suggest that this sample may be beginning to experience natural age related decreases in some aspects of cognitive function (Salthouse, 2009), as normal cognitive ageing is characterised by almost linear declines, from early adulthood, in

measures of speed, and accelerated declines in memory and reasoning (Salthouse, 2016; Salthouse, 2019). Thirdly, as there is currently limited findings from n-3 PUFA intervention studies in older populations and those already suffering with AD (Cederholm, Salem & Palmbald, 2013), it could be that n-3 PUFA supplementation is more beneficial earlier in the lifespan to help to maintain both long and short-term brain health that lasts into the final stages of the lifespan. Finally, very little emphasis has been placed previously on this demographic group (Derbyshire, 2018) and therefore the effects of n-3 PUFA supplementation within this population are not actually well understood or described within the literature. As a result, healthy young adults may offer insights into the functions of n-3 PUFAs on brain function in the absence of any health issues or periods of neurodevelopment and neurodegeneration, occurring in children and older adults. Previously, RCTs including healthy, young adults that aimed to address specific nutrient deficiencies have identified both acute and chronic effects on cognitive function (Lamport et al., 2016; Kennedy et al., 2016; Pettersen, 2017), suggesting that this sample is still susceptible to improvements in cognitive function following dietary manipulation.

The findings from Chapter 6 with regards to the observed improvements in reaction times following supplementation with the EPA-rich treatment suggest that there is a positive impact of EPA on cognitive function in healthy, young adults. Additionally, the correlations between n-3 index and neural efficiency scores observed in Chapter 3 may also provide evidence for a protective role of n-3 PUFAs in maintaining optimal brain function, even in healthy samples. On the other hand, the null findings on measures of subjective mood observed in Chapter 6, whilst previous studies have reported beneficial effects on mood in depressed populations (Mocking et al., 2016; Hallahan et al., 2016), may suggest there could be a ceiling effect of n-3 PUFAs in relation to mood in healthy non-clinically depressed populations. As healthy adults should not specifically require an increase in their mood, it may be that supplementation with n-3 PUFAs alone is insufficient to induce increases in moods in healthy, young populations or that any effects are trivial. Yet, changes in mood have been observed within healthy, young adults in other nutritional interventions (Scholey et al., 2010; Pipingas et al., 2013; Haskell-Ramsay et al., 2017) as well as in previous n-3 PUFA RCTs (Fontani et al., 2005a; 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011). However, the observed improvements in mood in previous RCTs have been acute effects which may suggest that if there are effects of n-3 PUFAs on mood within healthy, young adults then the effects may be transient. Although, it should be stated that the findings from this thesis suggest that supplementation with n-3 PUFAs does not have an effect on mood in healthy, young adults.

Concerning the null effects of treatment on most of the cognitive outcomes across the thesis, these findings are actually consistent with a number of previous RCTs with healthy, young adult samples. A systematic review from Rangel-Huerta and Gil (2017) identified only six RCTs that assessed the effects of n-3 PUFAs on cognition in healthy, young adults aged 18-34 years with interventions lasting between 1-3 months. Five studies reported no significant effects on cognitive function after supplementation (Karr, Grindstaff & Alexander, 2012; Jackson et al., 2012a; Jackson et al., 2012b; Jackson et al., 2012c; Giles et al., 2015) with only Bauer et al., (2014a) identifying significant effects on reaction times following supplementation with EPA. Additionally, Rangel-Huerta and Gil (2017) also identified a further seven RCTs that included healthy adults aged 18-75 years with interventions lasting between two months and six months with primary objectives to assess cognitive function. Five studies again reported no significant effects on cognitive function (Nilsson et al., 2012; Stough et al., 2012; Antypa et al., 2012; Dretsch et al., 2014; Pase et al., 2015; Mazereeuw et al., 2016) with only Stonehouse et al., (2013) and Witte et al., (2013) identifying significant effects of n-3 PUFAs over high-oleic sunflower placebo oils. This might suggest that the effects of n-3 PUFA supplementation over relatively short periods of 6 months' or less may not be large enough to be fully observed within these RCTs. However, evidence exists that supports the positive relationship between intake of n-3 PUFAs and cognitive function in older populations (Konagi et al., 2013; Duffy et al., 2015; Boespflug et al., 2016; Bo et al., 2017; Hooper et al., 2017). Therefore, it could be that supplementation with n-3 PUFA is indeed required at the beginning of natural age-related decrements in cognitive functioning, known to be occurring from young adulthood, with longitudinal studies required to more accurately observe lifelong effects of increased n-3 PUFA intake. Finally, as the samples for the studies presented throughout this thesis were mainly recruited from a university it could be that the samples were of an above average cognitive status which may have then influenced the findings. Indeed, previous research has identified that more educated individuals tend to have healthier diets in general (McCabe-Sellers et al., 2007; Choi et al., 2011; Wang & Chen, 2011; Hiza et al., 2013) which may then make them less susceptible to the impact of dietary manipulation. Therefore, the recruitment methods used in future RCTs should consider capturing an equal spread of participants from a range of educational backgrounds.

7.6 Limitations

The studies that comprise this thesis are respectively the largest RCTs thus far that employ both DHA- and EPA-rich supplements, in samples of healthy, young adults. Additionally, the employment of novel methodologies, such as fd-NIRS, allowed for the correction of the shortcomings of previous studies and the employment of a 26 week supplementation period

across all trials (recommended by Stonehouse et al., 2013) helps to address the limitations of previous studies in terms of supplementation periods that were too short. However, and as with all research, it is essential to consider any potential methodological limitations that have arisen throughout the studies presented in this thesis, some of which have only been briefly mentioned in previous chapters.

One potential issue that arises throughout the studies was solely relying on the fish consumption questionnaire (Benisek et al., 2002) as a screening tool to ensure participants were low consumers of fish. There is an extensive debate over the exclusion of participants in RCTs with a baseline n-3 index that is too high (McLennan & Pepe, 2015; Nestle et al., 2015; Rice et al., 2016; Alexander et al., 2017; McLennan and Pepe, 2017) with some suggesting that low-fish intake individuals should have a baseline n-3 index no greater than 4% (Harris, Del Gobbo and Tintle, 2017). The studies presented throughout this thesis did not exclude participants based on this recommendation and additionally may not have been possible within both the time and financial restraints of the thesis. As an alternative to this, a DHA and EPA consumption questionnaire was used as a screening tool throughout the studies (Benisek et al., 2002; Appendix I) with participants who reported eating oily fish more than once per week being excluded from participating due to failing to meet the “low-fish consumers” inclusion criteria. However, total DHA and EPA consumption, calculated from the questionnaire, was not used as an exclusion criteria and therefore it was possible for high consumers of DHA and EPA to meet the inclusion criteria as long as this was not obtained from more than one oily fish meal per week. Future users of the DHA questionnaire should therefore not only exclude based on the consumption of oily fish, but also from a total DHA and EPA consumption that is equal to or greater than the recommended daily intakes, currently between 250-500mg/d (Thompson et al., 2019).

Another potential issue that arises throughout the studies is the unbalanced samples, with regards to sex, in favour of females. Although it was not stated as a requirement for any of the studies to recruit samples stratified by gender, research by Stonehouse et al., (2013) has previously identified treatment by sex interactions with regards to episodic memory, a recent systematic review by Groot et al., (2019) has identified sex as an influencing factor on human n-3 PUFA levels and Howe et al., (2018) has also suggested that it is important for future studies to take account of the sex differences in responses to n-3 supplementation. Across all four of the studies the samples were in favour of female participants, with 51%, 68%, 77% and 69% of participants being female respectively. As the samples in Chapter 4, 5 and 6 are greater than 2/3 in favour towards females, splitting the analysis by sex would leave the male group underpowered in any exploratory analyses. Nonetheless, as the literature is beginning

to report the importance of sex on the effects of n-3 PUFA supplementation it seems of the upmost importance for future studies to attempt to stratify samples evenly by sex.

Another methodological consideration concerns the lack of any measurements of exercise and dietary habits and changes therein across the supplementation period. Although the DHA consumption questionnaire (Benisek et al., 2002) was used as a tool during the screening of participants, continued adherence to the inclusion criteria in relation to low-fish consumption was not measured throughout the duration of the study. As the supplementation period spanned 26 weeks it could be that participant's diets changed, especially between different seasons, i.e. enrolment in summer and completing in winter, as previous research has discussed how the types of foods that are available to us changes across seasons and influences seasonal variations in human gut microbiome composition (Davenport et al., 2014). Potentially, a more comprehensive measure of participant's diet could have been employed, such as the FFQ, pre and post supplementation in all of the studies, to allow for any changes in diet that may have occurred to be measured. Additionally, no measures of physical activity levels or how these may have also changed across the supplementation period were employed throughout any of the studies. Measurement of physical activity levels or any changes would be directly relevant in all of the studies conducted throughout the thesis as exercise has previously been associated with mood (Powers, Asmundson & Smits, 2015; Hearing et al., 2016; Crush, Frith & Loprinzi, 2018; Yang, Ko & Roh, 2018), cognition (Reiner et al., 2013; Prakash et al., 2015; Donnelly et al., 2016) and sleep (Chennaoui et al., 2015; Dolezal et al., 2017; Wilckens, Erickson & Wheeler, 2018). A measure of physical activity, such as the IPAQ, could have also been employed throughout the supplementation period to allow for activity levels and any changes therein to be observed. Again, the employment of additional questionnaires, such as the FFQ and IPAQ, would have put additional financial constraints on the studies, as well as, increased time restraints resulting from the increased data handling during the studies conducted throughout the thesis. However, future studies that measure changes in both diet and physical activity across the supplementation period would benefit from more detailed datasets and analyses when being able to account for these factors.

7.7 Future Research

In addition to addressing the limitations of this thesis, there are also a number of exciting findings that could be investigated further in future trials. However, it does also seem apparent that the aforementioned limitations identified within the thesis, as well as in the literature as a whole, must be addressed for the research area to be able to continue to develop. Therefore

future research should ensure to employ methodological paradigms with appropriate sample sizes that account for potential differences that occur by sex and genotype, that account for the baseline n-3 index of participants to ensure they are not only “low-fish” consumers but also “low n-3” consumers and that supplement for an appropriate length of time with both DHA- and EPA-rich oils, delivered with consideration towards the absorption and bioavailability of the PUFAs. Despite the limitations of this thesis several significant findings were able to be detected within a population that often shows null or inconclusive findings, most likely a direct result of the attempts that have been made throughout this thesis to correct previous methodological limitations.

The results presented in Chapter 3 appear to provide additional support that n-3 PUFA supplementation and n-3 index is related to increased neural efficiency, previously identified and discussed by Bauer et al., (2011; 2014a). The results appear to show that, although supplementation with the DHA- and EPA-rich supplements did not significantly improve cognitive performance, they resulted in a trend towards less $Hb0_2$ during completion of the tasks. This suggests that the brain required less $Hb0_2$ to complete the subtraction tasks to the same standard. This same association between increased n-3 index and reduced $Hb0_2$ was also identified both at baseline and post-supplementation, suggesting that this relationship already existed prior to the supplementation period as well as existing post-supplementation. To investigate these findings further, it would be appropriate to employ IC in conjunction with NIRS to also measure overall cellular respiration and energy expenditure, from oxygen consumption and carbon dioxide production, during completion of cognitive tasks (Oshima et al., 2017). Additionally, neural efficiency has previously been associated with an increased activation in specific brain regions and decreased activation in less relevant regions, resulting in a more efficient communication between only the relevant regions of the brain and therefore increasing efficiency (Langer et al., 2012; Bauer et al., 2014; Fairclough et al., 2019). It could be that future research should employ the use of fMRI and or multi-channel NIRS in order to measure the levels of activation within the brain. Furthermore, the employment of other methodologies such as TMS may be useful tools when measuring the efficiency of neural pathways as research has previously shown increases in cortico-cortical pathways associated with specific functions during TMS (Chiappini et al., 2018), or tDCS as a growing body of evidence suggests that tDCS can have beneficial effects on long-term memory, synaptic plasticity and adult neurogenesis (Di Lazzaro et al., 2013; Coffman et al., 2014; Leone et al., 2014, 2015; Podda et al., 2014) which may all be relevant to neural efficiency.

Equally, future research is also required to further develop the findings presented in Chapter 4 concerning effects on sleep efficiency, sleep time and sleep latency to identify which

mechanisms may be underpinning these findings. Future research in this area should consider either the use of 24/48 hour collection periods of urinary aMT6s or the collection of blood samples to allow for greater resolution and sensitivity than sampling via urine or saliva (Benloucif et al., 2008). Moreover, the employment of PSG or EEG in future studies would allow for a more detailed account concerning the 'objective' measures of sleep and would allow for more specific insights into the potential effects of n-3 PUFAs on the architecture of sleep in relation to the amounts of REM and NREM sleep. Recently, significant progress has been made with mobile EEGs and the development of concealed, unobtrusive ear-centered EEG acquisitions (cEEGrid). The cEEGrid provides the advantage of not requiring individualised electrodes, a larger number of channels and increased inter-electrode distances compared to previous ear-EEG devices (Mikkelsen et al., 2019). Comparisons between the cEEGrid and standard PSG confirm the suitability of cEEGrid for capturing sleep staging data (Sterr et al., 2018) with additional advantages of being mobile and less invasive than PSG or EEG devices. Therefore, use of the cEEGrid in future n-3 PUFA interventions may be a useful method for collecting detailed sleep data. Additionally, the more detailed measurement of sleep architecture would also be of use if combined with measures of memory consolidation which have previously been associated with SWS (Rothschild, Eban & Frank, 2017), along with longer periods of urinary melatonin or aMT6s collection to investigate this relationship further in regards to supplementation with n-3 PUFAs.

Despite the limited findings, the study presented in Chapter 5 was the first n-3 PUFA RCT to employ a methodological paradigm that attempts to investigate memory consolidation overnight and therefore future research that directly addresses the limitations of the study would be of use to the research area. Methodological improvements or potential adaptations could relate to the employment of tDCS as a more direct measurement of LTP and the mechanisms underpinning learning and memory. A growing body of evidence suggests that tDCS can have beneficial effects on long-term memory, synaptic plasticity and adult neurogenesis (Di Lazzaro et al., 2013; Coffman et al., 2014; Leone et al., 2014, 2015; Podda et al., 2014), as well as a consistent body of evidence that indicates that tDCS exerts modulatory effects of LTP (Ranieri et al., 2012; Rohan et al., 2015; Podda et al., 2016; Kronberg et al., 2017). In addition to this, convergent evidence from both animal and human studies points to BDNF as a critical determinant of the effects of tDCS and provides a rationale for further investigation of the involvement of BDNF in tDCS-induced modulation of cognitive functions (Cocco, Podda & Grassi, 2018). The measurement of tDCS-induced LTP and synaptic plasticity combined with measurements of BDNF in blood samples pre- and post-supplementation with DHA and EPA supplements may provide a more sound and objective

methodological paradigm for future investigations in this area than the one employed in Chapter 5.

In addition, the findings presented in Chapter 6 concerning the effects of the EPA-rich treatment in reducing the reaction times during the Stroop and word recognition tasks, may provide insights into the types of tasks that should be focussed on in future studies, as similar studies have also identified significant effects of n-3 PUFA supplementation on these types of tasks (Bauer et al., 2011; Stonehouse et al., 2013; Bauer et al., 2014a). The findings from these previous studies, together with those identified in Chapter 6, would therefore support the future employment of executive functioning and episodic memory tasks in RCTs measuring cognition. Additionally, consideration towards baseline n-3 index and physical activity levels should be made in future trials that aim to measure cognition and mood, as these appear to be factors that are often overlooked in the research area.

In relation to the collection of blood samples, future studies should include measurements of ApoE4 carrier status, which has been previously identified as an influencing factor on n-3 PUFA status. A number of human studies have retrospectively reported that the cognitive benefits associated with fish intake were actually lower or absent in ApoE4 carriers (Huang et al., 2005; Whalley et al., 2008; Quinn et al., 2010) and Stonehouse et al., (2013) have previously identified some indications of APOE × sex × treatment interactions. This interaction may be due to previously observed sex differences between males and female ApoE4 carriers in terms of gene expression patterns (Hsu et al., 2019). This sex difference may be related to the overall heightened immune response and increased risk for AD observed in females (Taneja, 2018) and suggests that female ApoE4 carriers are at greater risk of developing AD at younger ages compared to male ApoE4 carriers (Neu et al., 2017). Additional blood analyses to identify ApoE4 carriers were not possible in the studies conducted throughout this thesis. However, it does appear to be an important variable that should be collected in future research.

Finally, future research could focus on directly evaluating the impact of time of dosing on increasing the absorption and bioavailability of both EPA and DHA. Unfortunately, it was not within the scope of this thesis to be able to assess this and so future studies should ideally employ a 26 week parallel groups design with two DHA-rich and two EPA-rich treatment arms that consist of: SMEDS formulated DHA- or EPA-rich supplements dosed in the morning or SMEDS formulated DHA- or EPA-rich supplements dosed at night-time. A study following this design would allow for the direct comparisons between: morning and night time dosing with SMEDS formulated EPA- and DHA-rich oils. This would provide knowledge to the research

area in terms of which methods should be applied to best increase absorption of n-3 PUFAs across supplementation periods.

7.8 General Conclusions

The aim of this thesis was to establish the efficacy of both DHA-rich and EPA-rich SMEDS formulated supplements, dosed at bedtime, on cognitive function in healthy, young adults and to also explore related mechanisms that may impact this overall effect, such as cerebral haemodynamics, sleep and memory consolidation. Concerning the results identified in Chapter 3, it appears that 26 weeks' supplementation with both the DHA- and EPA-rich treatments resulted in a trend towards reduced quantities of $Hb0^2$ in the PFC during serial subtraction tasks, whilst having no effect on cognition, compared to placebo. Suggesting that increases in n-3 index may be relevant to increased neural efficiency. However, as neural efficiency was not a primary outcome measure for this study these findings are difficult to interpret and require further investigation with methodologies that are more capable of directly measuring neural efficiency, such as fMRI or a combination of ICD and NIRS or EEG and NIRS.

With regards to the relationship between n-3 PUFAs and sleep parameters investigated in Chapter 4, it appears that there was evidence to support the role of n-3 PUFAs, particularly DHA, at improving objective measurements of sleep. These included a reduction in sleep latency following the DHA-rich treatment and an overall increase in sleep efficiency following both of the active treatments. However, these improvements were not consistent with subjective ratings of sleep in the DHA-rich group. These results support the effects of DHA on parameters of sleep in healthy, young adults, although, further investigation into the effects of DHA on the architecture of sleep and how it impacts the serotonin/melatonin synthesis pathway or production of relevant eicosanoids is still required to further knowledge around the effects of n-3 PUFAs on sleep in healthy, young adults.

Concerning the study presented in Chapter 5, it is the first to investigate the potentially separate effects of DHA-rich and EPA-rich supplements, dosed at bed time, on learning, memory consolidation and morning alertness. The study identified improvements in reaction times during completion of immediate word recognition tasks in the DHA-rich group. On the other hand, a negative finding in relation to the ability to identify the correct locations of objects during learning trials was also identified within the DHA-rich group, which may potentially suggest there was a speed-accuracy trade off during the learning trials within this group. However, considering that the methodological paradigm employed throughout Chapter 5 has not yet proven to be capable of accurately measuring overnight memory consolidation, it could

be that other methodologies that are capable of measuring the mechanisms underpinning memory consolidation more directly should be employed in future investigations, such as tCDS and biological measures of BDNF. Nonetheless, the study presented in Chapter 5 attempted to provide knowledge to a substantial gap within the current literature and should therefore inspire future research to develop methodologies best suited to measuring the effects of both DHA and EPA on overnight memory consolidation.

The study presented in Chapter 6 is one of the largest RCTs thus far to supplement with both a DHA- and EPA-rich treatment in healthy, young adults. The findings identified within Chapter 6 appear to provide support for the effects of supplementation with n-3 PUFAs, specifically EPA, at improving reaction times during both executive functioning and episodic memory tasks, as well as improving global cognition scores in healthy, young adults. No effects of either the DHA- or EPA-rich treatment on measures of mood were identified, which have been identified previously (Fontani et al., 2005a; 2005b; Antypa et al., 2009; Kiecolt-Glaser et al., 2011; Watanabe et al., 2018), but findings overall are still inconsistent across healthy, young samples (Kiecolt-Glaser et al., 2012; Giles et al., 2013; Hallahan et al., 2016). It could be that ceiling effects of n-3 PUFAs in relation to mood in healthy non-clinically depressed populations exist, that the effects in this demographic group are trivial, or that effects of n-3 PUFAs in these samples are transient rather than chronic. Nonetheless, the findings from Chapter 6 concerning cognitive function are consistent with previous studies conducted by (Bauer et al., 2011; 2014a) and Stonehouse (2013) in healthy, young adult samples and provide evidence that supports the employment of EPA-rich supplements in future n-3 PUFA RCTs that aim to measure cognitive function.

Overall the relationships between n-3 PUFAs and brain function, sleep, memory consolidation and mood are highly complex. Both human and animal studies consistently suggest that there are both biological and behavioural consequences of low intakes of n-3 PUFAs, whilst populations in general appear to not meet the recommended amounts of n-3 PUFAs in their diets (Gopinath et al., 2017; Sioen et al., 2017; Yau et al., 2018). Despite this population deficiency, findings across RCTs remain inconclusive, especially in healthy, young samples and this can be attributed, at least in part, to methodological limitations. The significant effects of n-3 PUFAs observed on cerebral haemodynamics, sleep and cognitive function identified within this thesis are most likely a direct result of the novel research questions addressed throughout the thesis, paired alongside rigorous methodologies that attempted to address the shortcomings of previous research to produce more effective interventions. However, the mechanisms that underpin the observed effects of n-3 PUFAs on brain function, sleep,

memory consolidation and mood in healthy, young adults, still require further investigation and offer several exciting opportunities for future research.

APPENDICES

APPENDIX I: Fish Consumption Questionnaire (Benisek et al., 2002)

DHA Food Frequency Questionnaire®

Subject ID #: _____ **Initials:** ____ ____ ____ (F M L) **Date:** ____ ____ ____ (MM DD YY)

Estimate your consumption over the past 2 months of the following foods.
Use the food model forms to help estimate portion sizes.

	Servings	DHA	EPA
1. How many 3oz servings of the following fish do you eat **monthly**? bluefish, blue fin tuna, cisco, smoked, herring, mackerel, pollock, sardines, salmon, whitefish	_____	x 22 _____	x 14 _____
2. How many 3oz servings of the following fish do you eat **monthly**? bass, calamari, catfish, drumfish, flounder, grouper, halibut, mussels, perch, redfish, rockfish, shark, snapper, sole, squid, swordfish, trout, tuna, canned (6oz can), whiting	_____	x 10 _____	x 5 _____
3. How many 3oz servings of the following fish/shellfish do you eat **monthly**? carp, clams, cod, crab, crayfish, fish patties/squares, fish sticks, haddock, lobster, mullet, oysters, pike, pompano, scallops, shrimp (14 med.), sturgeon	_____	x 5 _____	x 6 _____
4. How many 3oz servings of liver (chicken, turkey or beef) do you eat **monthly**?	_____	x 7 _____	x 2 _____
5. How many egg yolks do you eat **weekly** (including eggs yolks used in cooking)?	_____	x 3 _____	x 0.25 _____
6. How many 3oz servings of chicken, turkey or other poultry (not including livers) do you eat **weekly**?	_____	x 5 _____	x 3 _____
7. Any omega-3 dietary supplements or functional foods (i.e., flax, fish oil, Neuromins, DHA Gold, high DHA eggs)? amount or strength _____ frequency _____		mg/d	mg/d
Sub-Total:		_____ DHA (mg/d)	_____ EPA (mg/d)
TOTAL= Rounded to Nearest Whole #:		_____ DHA (mg/d)	_____ EPA (mg/d)

*All calculations are based on USDA Nutrient Database (USDA, Nutrient Database for Standard Reference release 14, 2002).

APPENDIX II: Treatment Guess Questionnaire

Study Code: 44N3 Date: ____/____/____

Subject ID:

Randomisation No.:

Visit: 2

Which treatment do you think you were administered? (please circle)

1. Placebo (dummy pill)
2. Omega-3 (active treatment)

What is your reason(s) for thinking this?

_____

_____

_____

URINE SAMPLE AND ACTIWATCH DIARY

Study Title: Efficacy Evaluation of 26 Weeks' Dietary Supplementation with DHA- and EPA-enriched Oils on sleep efficiency in Healthy Adults

Principal Investigator: Philippa Jackson

Lead Researcher: Michael Patan

Clinical Trial Number: CTN 007 16205

Subject ID: |__ |__ |__ |

Date Subject Diary dispensed: |__ |__ |- |__ |__ |- |__ |__ | *(dd-mm-yy)*

Visit Urine Diary was dispensed: |___ |

If you have any questions or any problems occur please contact:

Name: Michael Patan
E-mail: michael.j.patan@northumbria.ac.uk

Or any member of the research team on 0191 2437252 (office hours).

Sample Instructions

You will take samples on the night prior to and morning of your Testing Visits. These will include the final void at bedtime, and the first and second voids of the following day. It is anticipated that the second void of the day may take place at Northumbria University on the morning of the respective Testing Visit. Should you also need to urinate during/throughout the night, then these voids should also be collected in the same manner as described below.

You should collect each sample into the measuring container provided. You should then place the measuring container on a level surface and record the total volume of the sample (ml) along with the time that the sample was collected in the table below. Once the details have been recorded you should transfer 10ml of the sample from the measuring container into the 10ml sample tubes provided, which will be pre-labelled with your Subject ID and the sample number, starting from Sample 1. Once sealed in the sample tube you can pour away the excess waste. All sample tubes should then be returned to the BPNRC 4th floor of Northumberland Building on the morning of your testing visit.

Sample Number	***Time of Sample (am/pm)***	***Total Sample Volume (ml)***
1 (bedtime void)		
2 (first void in morning)		
3 (second void in morning)		
Any Additional Voids	***Time of Sample (am/pm)***	***Total Sample Volume (ml)***
4 (first additional void)		
5 (second additional void)		

Bedtime/Waking

Please complete the following table with the approximate time you went to bed/awoke the following morning for each of the seven days. The date for each day should also be completed.

Day of the week	***Date***	***Approximate Bedtime***	***Approximate waking time the following morning***

Procedural Deviations

Please fill in any issues/problems you have experienced throughout the week relating to your urine samples or sleep watches in the space below.

Date	Deviation from procedure

TREATMENT DIARY

Study Code: 44N3
Study Title: Efficacy Evaluation of 26 Weeks' Dietary Supplementation with DHA- and EPA-enriched Oils on Cognitive Function and Mood in Healthy Adults
Principal Investigator: Philippa Jackson
Lead Researcher: Michael Patan

Subject ID: |__ |__ |__|

Random Number: |__ |__ |__|

Date Subject Diary dispensed: |__ |__ |-|__ |__ |-|__ |__| *(dd-mm-yy)*

Week 13 Exchange Visit: _____

Dear Participant,

For the success of the study it is very important that you follow the instructions of the study team very carefully. Therefore please find a short summary of the most important points.

Treatment consumption instructions

Take 3 tablets per day, with your evening meal. Take tablets with water or a cold drink. Do not take tablets on an empty stomach and do not exceed the recommended dose.

Diet

During the course of the whole study you should not change your dietary habits.

Medication / Therapy or Health Problems or Symptoms

If you take any new medication or therapy or if you have any health problems or unusual symptoms you should document in as much detail as possible in this diary on the appropriate pages and talk about it to the researcher during the next Study Day. You do not need to write down routine medications which have already been discussed at Study Day 1.

In general you should avoid, if possible, the use of non-prescription drugs during the study. However, if you intend to use non-prescription drugs within the week preceding the next Study Day, **please contact the research team**. There is a chance that the Study Day might be postponed.

Before Treatment Exchange Instructions

To remember prior to your week 13 exchange:

Please bring this diary to the treatment exchange.

If you have any questions or any problems occur please contact:

Name: Michael Patan
E-mail: michael.j.patan@northumbria.ac.uk

Or any member of the research team on 0191 2437252 (office hours).

Please remember to take your treatment with your evening meal every day. In the table below, please record the date and time each day that you take the treatment. If you forget to eat it, please leave this box blank. Any ill health or intake of non-routine medications/therapy should be recorded on pages 7-10.

Week 1		**Week 2**		**Week 3**	
Date	***Time***	***Date***	***Time***	***Date***	***Time***

Please remember to take your treatment with your evening meal every day. In the table below, please record the date and time each day that you take the treatment. If you forget to eat it, please leave this box blank. Any ill health or intake of non-routine medications/therapy should be recorded on pages 7-10.

Week 4		**Week 5**		**Week 6**	
Date	***Time***	***Date***	***Time***	***Date***	***Time***

Please remember to take your treatment with your evening meal every day. In the table below, please record the date and time each day that you take the treatment. If you forget to eat it, please leave this box blank. Any ill health or intake of non-routine medications/therapy should be recorded on pages 7-10.

Week 7		**Week 8**		**Week 9**	
Date	***Time***	***Date***	***Time***	***Date***	***Time***

Please remember to take your treatment with your evening meal every day. In the table below, please record the date and time each day that you take the treatment. If you forget to eat it, please leave this box blank. Any ill health or intake of non-routine

Week 10		**Week 11**		**Week 12**		**Week 13**	
Date	***Time***	***Date***	***Time***	***Date***	***Time***	***Date***	***Time***

Have you taken any medication or dietary supplements?

☐ No

☐ Yes: *please give details in the table below. Please include the product/drug/therapy name in full, the amount (e.g. 1x200mg tablet), the date you took it and what you took it for.*

Product/drug/therapy name	**Quantity**	**Dose**	**Date**	**What you took it for?**
Example: paracetamol	*2 / day*	*500 mg/tablet*	*25-Apr-12*	*headache*

Product/drug/therapy name	*Quantity*	*Dose*	*Date*	*What you took it for?*

Have you experienced any new health problems or unusual symptoms?

☐ No

☐ Yes*, please give details in the table below. Please include your symptom(s), the date(s) it/they started and stopped if applicable, its/their severity and any action you took to relieve it/them.*

Symptom	**Date symptom started**	**Please rate the severity of the symptom in terms of how it affects your daily functioning 1: Mild 2: Moderate 3: Severe**	**If resolved please give the date it stopped**	**Did you do anything to relieve your health problems/symptoms (like drug, therapy)?**
Example: headache	*25-Apr-12*	*Moderate*	*25-Apr-12*	*Paracetamol*

Symptom	Date symptom started	Please rate the severity of the symptom in terms of how it affects your daily functioning 1: Mild 2: Moderate 3: Severe	If resolved please give the date it stopped	Did you do anything to relieve your health problems/symptoms (like drug, therapy)?

APPENDIX V: Chapter 3 Main Effects

Table V.1 F and *p* values of the main effects from the linear mixed models during the 5 minute resting period. Main effects of Ox%, THb, $Hb0_2$ and Hb are presented.

		Main Effects	
		F	*p*
Ox (%)	Treatment	.297	.744
	Hemisphere	.069	.794
	Treatment*Hemisphere	.871	.423
THb (µM)	Treatment	.834	.439
	Hemisphere	**7.06**	**.010**
	Treatment*Hemisphere	.068	.935
$Hb0_2$ (µM)	Treatment	.711	.495
	Hemisphere	**4.41**	**.039**
	Treatment*Hemisphere	.005	.995
Hb (µM)	Treatment	.363	.697
	Hemisphere	**7.26**	**.009**
	Treatment*Hemisphere	.356	.702

Table V.2 F and *p* values of the main effects from the linear mixed models for efficiency index scores. Main effects of Ox%, THb, $Hb0_2$ and Hb are presented.

	Main Effects		
		F	*p*
Ox (%)	Treatment	1.30	.280
	Task	.360	.698
	Hemisphere	.006	.939
	Treatment*Task	.946	.439
	Treatment*Hemisphere	1.57	.213
	Treatment*Hemisphere*Task	.296	.938
THb (µM)	Treatment	1.90	.162
	Task	.054	.948
	Hemisphere	**22.55**	**<.001**
	Treatment*Task	.023	.999
	Treatment*Hemisphere	**3.65**	**.027**
	Treatment*Hemisphere*Task	.030	1.000
$Hb0_2$ (µM)	Treatment	2.13	.131
	Task	.106	.899
	Hemisphere	**19.31**	**<.001**
	Treatment*Task	.034	.998
	Treatment*Hemisphere	**4.95**	**.008**
	Treatment*Hemisphere*Task	.047	1.000
Hb (µM)	Treatment	.891	.418
	Task	.015	.986
	Hemisphere	**18.07**	**<.001**
	Treatment*Task	.266	.900
	Treatment*Hemisphere	1.08	.340
	Treatment*Hemisphere*Task	.031	1.000

Table V.3 F and *p* values of the main effects from the linear mixed models for the serial subtraction task and VAS. Main effects for total number of responses, number of errors and task difficulty are presented.

		Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Total Responses	Placebo	75	15.42	0.39	Treatment	.234	.792
	DHA-rich		15.82	0.46	**Task**	**7.08**	**.001**
	EPA-rich		15.49	0.39	Treatment*Task	1.42	.235
Number of Errors	Placebo	75	1.21	0.11	Treatment	.336	.716
	DHA-rich		1.07	0.13	Task	.594	.554
	EPA-rich		1.19	0.11	Treatment*Task	.192	.942
Task Difficulty (0-100%)	Placebo	75	**42.01[a]**	1.97	**Treatment**	**3.91**	**.026**
	DHA-rich		**50.18[a]**	2.30	**Task**	**16.04**	**<.001**
	EPA-rich		43.65	1.94	Treatment*Task	.997	.412

Table V.4 F and *p* values of the main effects from the linear mixed models during the NIRS: active period. Main effects of Ox%, THb, $Hb0_2$ and Hb are presented.

			Post-dose			Main Effects		
			n	Mean	SE		*F*	*p*
Ox	Left	Placebo	71	0.07	0.04	Treatment	.312	.734
		DHA-rich		0.10	0.05	**Task**	**22.56**	**<.001**
		EPA-rich		0.09	0.04	**Hemisphere**	**19.61**	**<.001**
	Right	Placebo		0.15	0.04	Treatment*Task	.096	.984
		DHA-rich		0.22	0.05	Treatment*Hemisphere	.390	.678
		EPA-rich		0.18	0.04	Treatment*Hemisphere*Task	.978	.441
THb	Left	Placebo	71	0.27	0.05	Treatment	.167	.846
		DHA-rich		0.28	0.05	**Task**	**32.41**	**<.001**
		EPA-rich		0.31	0.05	**Hemisphere**	**11.12**	**.001**
	Right	Placebo		0.33	0.05	Treatment*Task	.080	.989
		DHA-rich		0.37	0.05	Treatment*Hemisphere	.272	.762
		EPA-rich		0.36	0.05	Treatment*Hemisphere*Task	.079	.998
$Hb0_2$	Left	Placebo	71	0.29	0.04	Treatment	.168	.846
		DHA-rich		0.30	0.05	**Task**	**32.07**	**<.001**
		EPA-rich		0.32	0.04	**Hemisphere**	**11.23**	**.001**
	Right	Placebo		0.34	0.04	Treatment*Task	.076	.990
		DHA-rich		0.39	0.05	Treatment*Hemisphere	.362	.697
		EPA-rich		0.37	0.04	Treatment*Hemisphere*Task	.113	.995
Hb	Left	Placebo	71	0.36	0.05	Treatment	.185	.832
		DHA-rich		0.35	0.06	**Task**	**22.00**	**<.001**
		EPA-rich		0.39	0.05	Hemisphere	1.52	.218
	Right	Placebo		0.34	0.05	Treatment*Task	.131	.971
		DHA-rich		0.33	0.06	Treatment*Hemisphere	.010	.990
		EPA-rich		0.36	0.05	Treatment*Hemisphere*Task	.054	.999

APPENDIX VI: Chapter 4 Average Times in and out of Bed

Table VI.1 Average time getting in bed, getting out of bed and time spent in bed, split by treatment group at week 26.

Treatment	Time in bed	Time out of bed	Time in bed (mins)
Placebo	23:13	07:24	491
DHA-rich	23:25	07:29	484
EPA-rich	23:21	07:18	477

APPENDIX VII: Chapter 6 Blood Values Compared to Stonehouse et al., (2013)

Table VII.1 Comparison of the bloods analysis in Chapter 6 compared to Stonehouse et al., (2013), split by treatment group. Mean ± SD are provided for baseline, week 26 and change values are presented for DHA, EPA and n-3 index

		Placebo			DHA-rich		
		Baseline	26 weeks	Change	Baseline	26 weeks	Change
DHA	Chapter 6	4.95 ± 1.07	4.73 ± 1.05	-0.19 ± 0.95	4.79 ± 1.19	7.43 ±1.57	2.61 ± 1.56
	Stonehouse et al (2013)	5.06 ± 1.76	4.98 ± 1.60	-0.08 ± 0.88	5.28 ± 1.35	7.91 ± 1.65	2.62 ± 1.27
EPA	Chapter 6	0.86 ± 0.32	0.85 ± 0.36	-0.03 ± 0.36	0.87 ± 0.35	2.07 ± 0.82	1.23 ± 0.73
	Stonehouse et al (2013)	0.54 ± 0.31	0.52 ± 0.31	-0.01 ± 0.25	0.61 ± 0.34	0.81 ± 0.41	0.20 ± 0.22
n-3 index	Chapter 6	5.81 ± 1.25	5.58 ± 1.24	-0.21 ± 1.12	5.66 ± 1.40	9.50 ± 2.25	3.81 ± 2.04
	Stonehouse et al (2013)	5.59 ± 1.90	5.50 ± 1.72	-0.09 ± 0.98	5.89 ± 1.42	8.72 ± 1.72	2.82 ± 1.34

APPENDIX VIII: Fish Consumption Data

Table VIII.1 Average number of servings per week of various n-3 food sources and the equivalent DHA, EPA and n-3 (± SD). Full lists of food sources are outlined in the corresponding questions on the DHA food frequency questionnaire (Appendix I).

Food Source	Number of Servings (per week)	DHA (mg/d)	EPA (mg/d)	n-3 (mg/d)
Oily Fish (Q1)	1.64 ± 1.43	34.20 ± 31.74	21.76 ± 20.20	55.97 ± 27.31
Fish (Q2)	2.01 ± 1.99	19.02 ± 19.89	9.51 ± 9.95	28.53 ± 16.41
Fish/Shellfish (Q3)	1.45 ± 1.61	6.84 ± 8.01	8.21 ± 9.61	15.05 ± 8.87
Liver (Q4)	1.22 ± 3.46	8.07 ± 23.65	2.30 ± 6.76	10.37 ± 17.60
Egg Yolks (Q5)	3.83 ± 3.51	10.87 ± 10.56	0.91 ± 0.88	11.78 ± 8.99
Poultry (Q6)	3.23 ± 2.74	15.09 ± 13.43	9.16 ± 8.28	24.25 ± 11.54
Total (Q1-Q6)	13.38 ± 8.30	94.09 ± 62.01	51.85 ± 34.76	145.94 ± 95.87

APPENDIX IX: Diurnal Rhythms of EPA and DHA in Blood Plasma (Jackson et al., unpublished data)

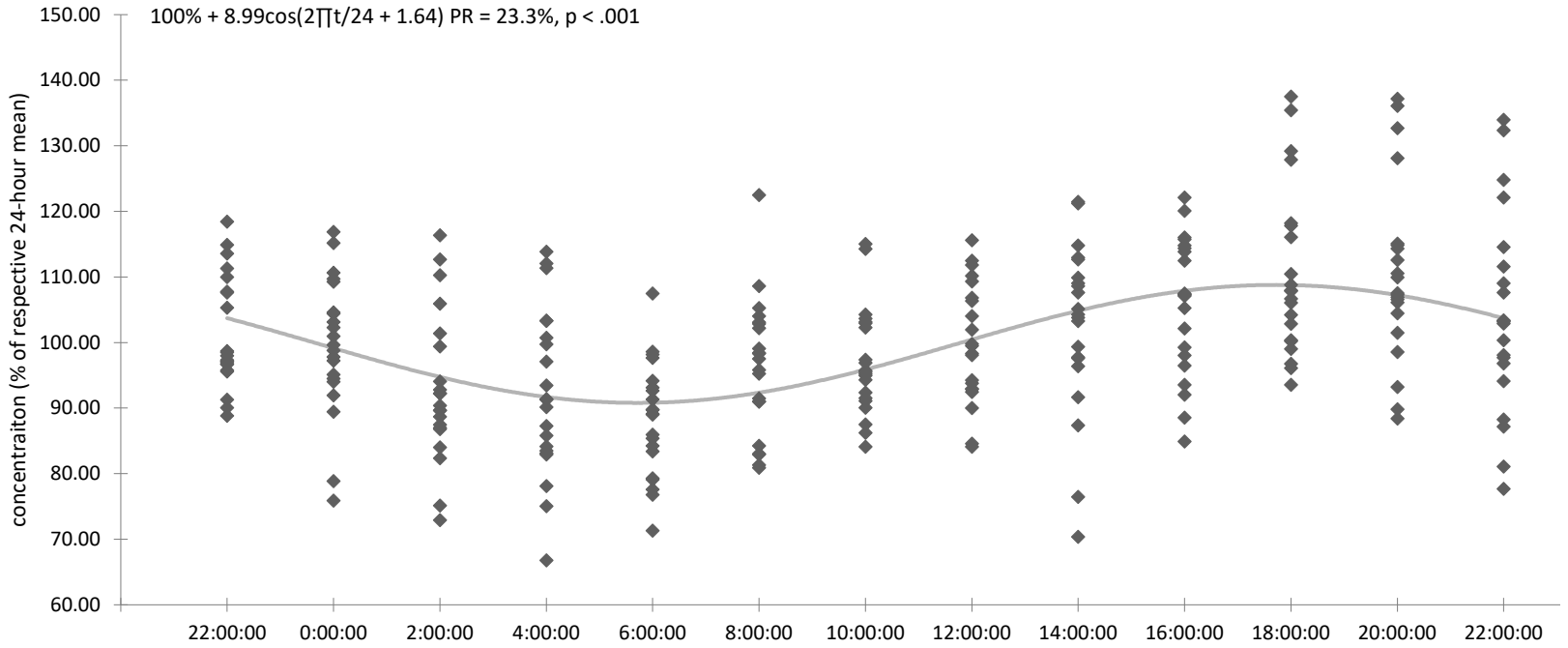

Figure IX.1. Diurnal variation of pooled data (N = 21) in plasma concentrations of DHA expressed as a percentage of their respective mean 24-hour value. A significant rhythm is detected for DHA. The equation of the cosine curve fitted by least squares to the data is presented on the graph. PR, percentage rhythm or proportion of variance in the data accounted for by the cosine wave (R^2 x 100).

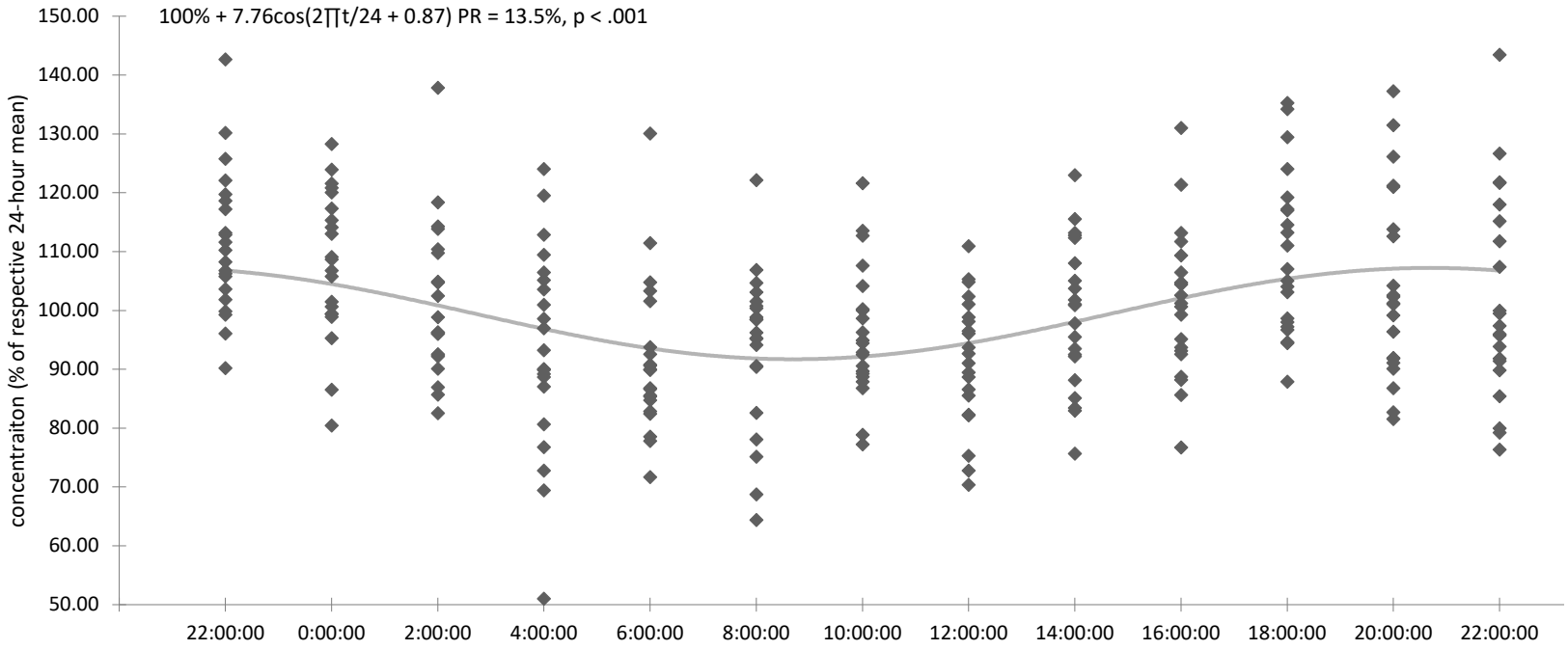

Figure IX.2. Diurnal variation of pooled data (N = 21) in plasma concentrations of EPA expressed as a percentage of their respective mean 24-hour value. A significant rhythm is detected for EPA. The equation of the cosine curve fitted by least squares to the data is presented on the graph. PR, percentage rhythm or proportion of variance in the data accounted for by the cosine wave (R^2 x 100).

APPENDIX X: Bioavailability of EPA and DHA in Plasma (Jackson et al., unpublished data)

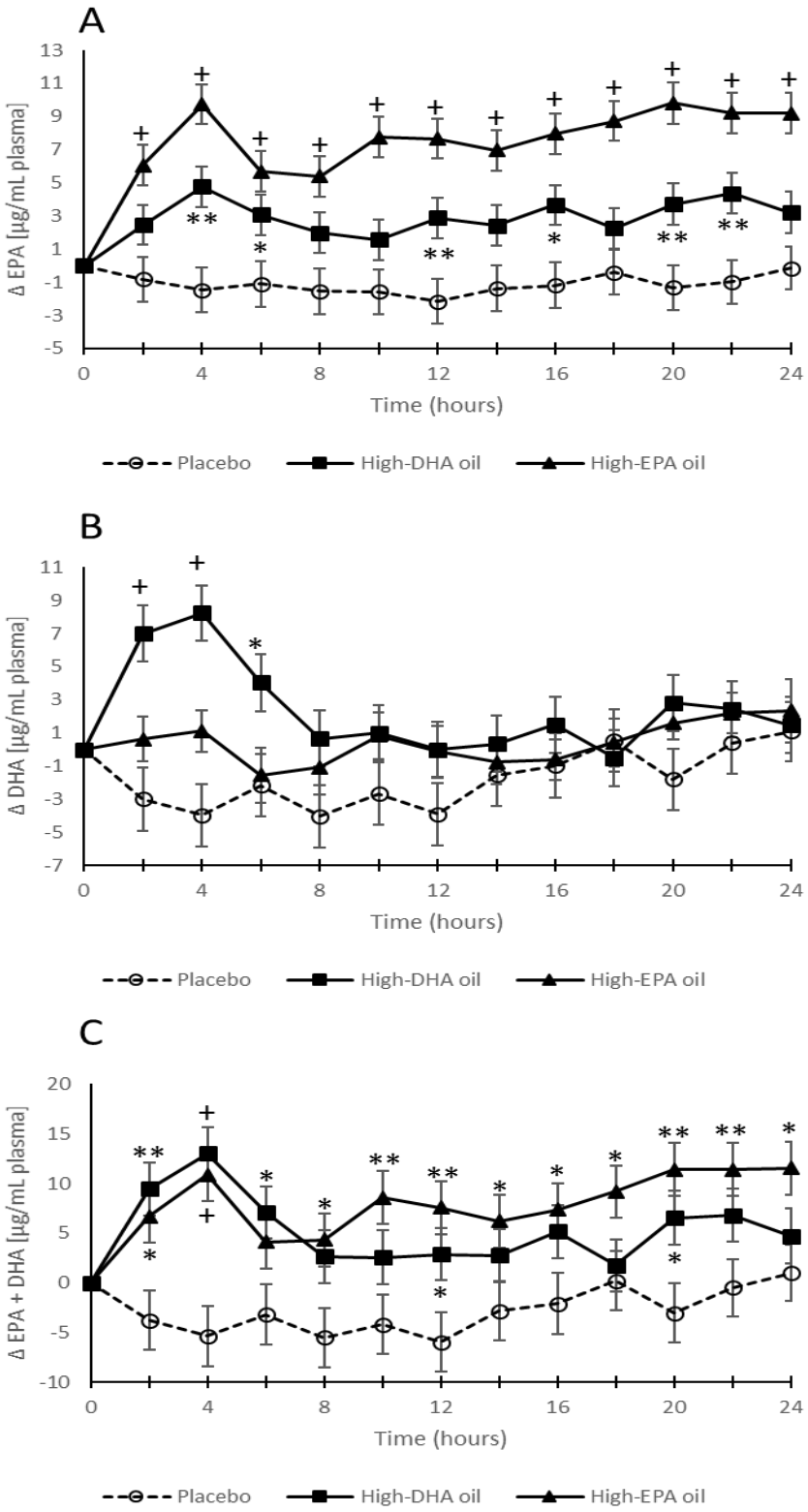

Figure X.1. Change in plasma EPA (A), DHA (B) and EPA+DHA (C) concentrations following single dosing of EPA-rich SMEDS formulation, DHA-rich SMEDS formulation and placebo at 22:00. Data are displayed as change from pre-dose baseline and represent estimated means and standard errors derived from linear mixed models including treatment, time and sequence as fixed effects and subject as a random effect. Pairwise comparisons between placebo and each of the SMEDS formulated oils are displayed. *p < 0.05; **p < 0.01; +p < 0.001. N = 17.

APPENDIX XI: Blood Fatty Acid Profiles from Chapter 3, 4 & 5

The analysis of the blood fatty acid profiles from the study presented in Chapter 3 revealed a significant effect of treatment for RBC EPA [F (2, 59) = 27.58, p <.001] with both the EPA-rich (2.67%; p <.001) and DHA-rich (2.32%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (0.92%). A significant effect of treatment was also identified for RBC DHA, [F (2, 59) = 51.50, p <.001] with both the EPA-rich (6.09%; p = .003) and DHA-rich (8.13%; p <.001) groups having significantly greater levels of blood DHA than the placebo group (4.95%). A significant effect of treatment was also identified for n-3 index, [F (2, 59) = 41.21, p <.001], with both the EPA-rich (8.71%; p <.001) and DHA-rich (10.48%; p <.001) groups having a significantly greater n-3 blood index than the placebo group (5.88%) (Figure XI.1).

Analysis of the blood fatty acid profiles from the study presented in Chapter 4 revealed a significant effect of treatment for RBC EPA, [F (2, 65) = 42.36, p <.001] with both the EPA-rich (2.67%; p <.001) and DHA-rich (2.17%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (0.86%). A significant effect of treatment for RBC DHA, [F (2, 65) = 47.73, p <.001] with both the EPA-rich (6.06%; p <.001) and DHA-rich (7.69%; p <.001) groups having significantly greater levels of blood DHA than the placebo group (4.80%). A significant effect of treatment for n-3 index, [F (2, 65) = 47.73, p <.001] with both the EPA-rich (8.75%; p <.001) and DHA-rich (9.86%; p <.001) groups having significantly greater levels of blood DHA than the placebo group (5.64%). (Figure XI.2).

Analysis of the blood fatty acid profiles from the study presented in Chapter 5 revealed a significant effect of treatment for RBC EPA, [F (2, 125) = 79.80, p <.001] with both the EPA-rich (2.87%; p <.001) and DHA-rich (1.92%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (0.85%). Analysis also revealed a significant effect of treatment for RBC DHA, [F (2, 125) = 49.78, p <.001] with both the EPA-rich (5.91%; p <.001) and DHA-rich (7.05%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (4.65%). The analysis revealed a significant effect of treatment for RBC n-3 index, [F (2, 125) = 63.20, p <.001] with both the EPA-rich (8.76%; p <.001) and DHA-rich (8.99%; p <.001) groups having significantly greater levels of blood EPA than the placebo group (5.50%) (Figure XI.3).

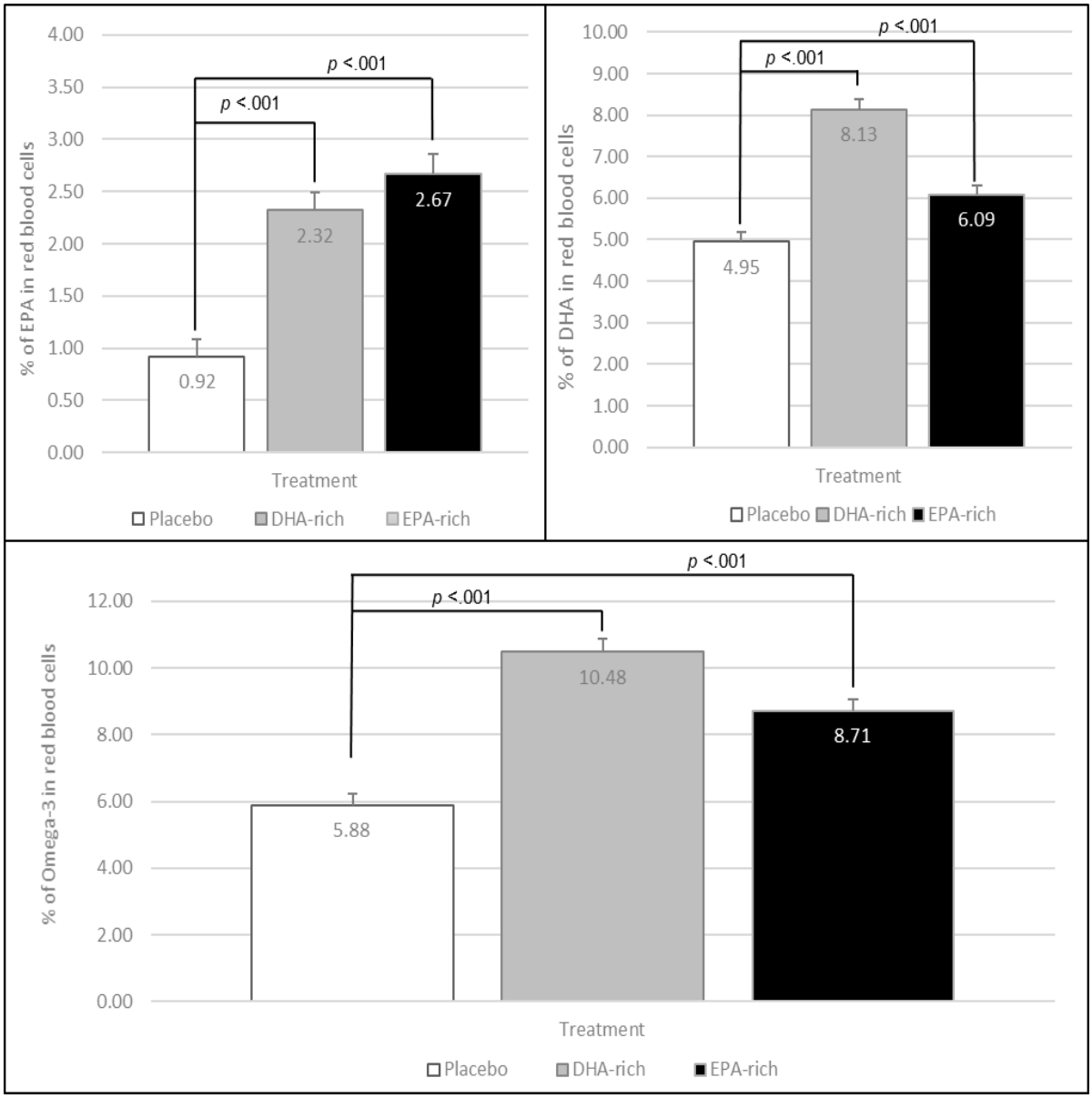

Figure XI.1. Estimated marginal means and standard errors (SE) for the week 26 percentage of EPA **(top left)** percentage of DHA **(top right)** and total percentage of EPA+DHA **(bottom)**, out of total fatty acids, in red blood cells by treatment group for the study presented in Chapter 3.

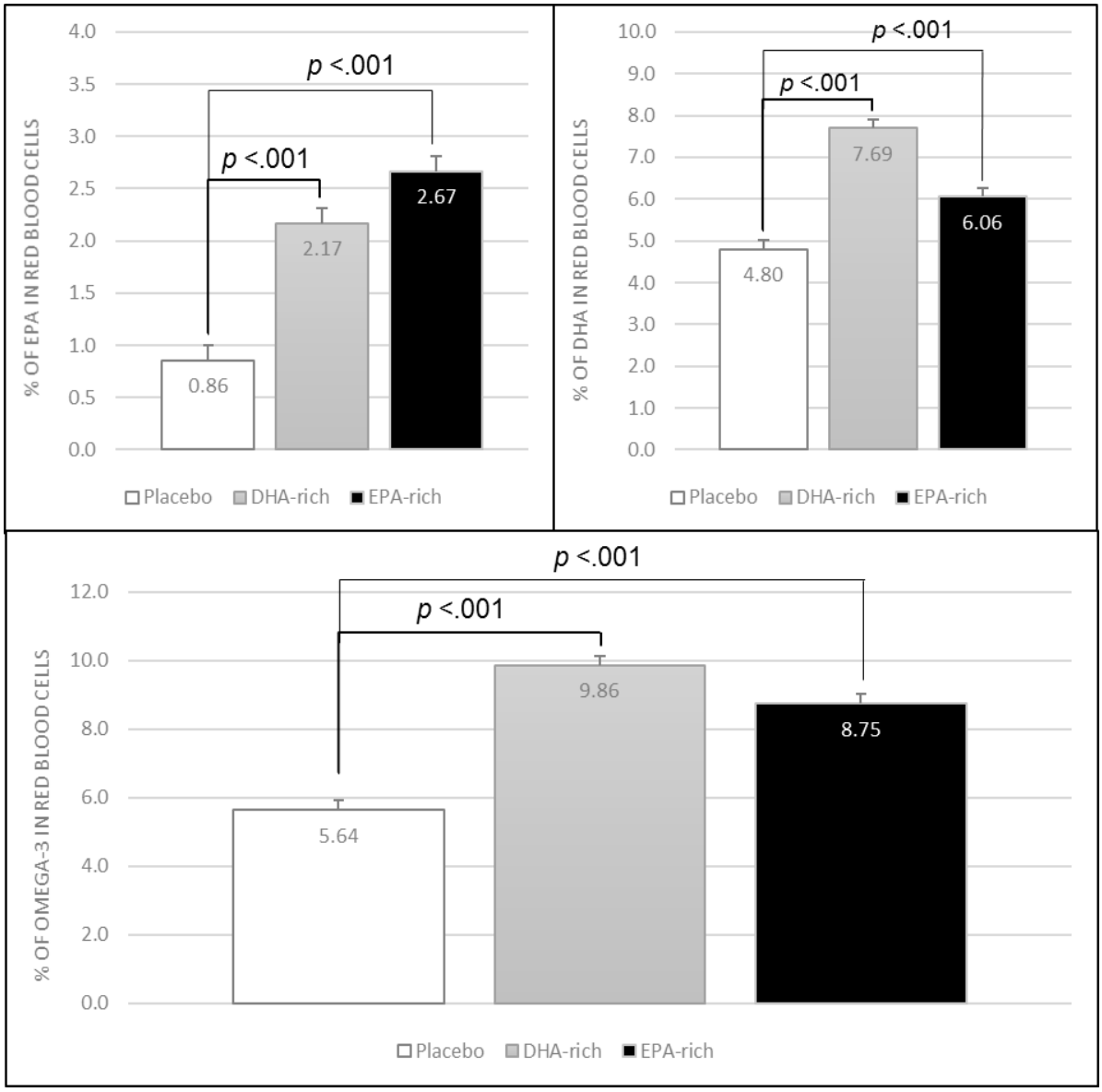

Figure XI.2. Estimated marginal means and standard errors (SE) for the post-dose percentage of EPA **(top left)** percentage of DHA **(top right)** and total percentage of EPA+DHA **(bottom)**, out of total fatty acids, in red blood cells by treatment group for the study presented in Chapter 4.

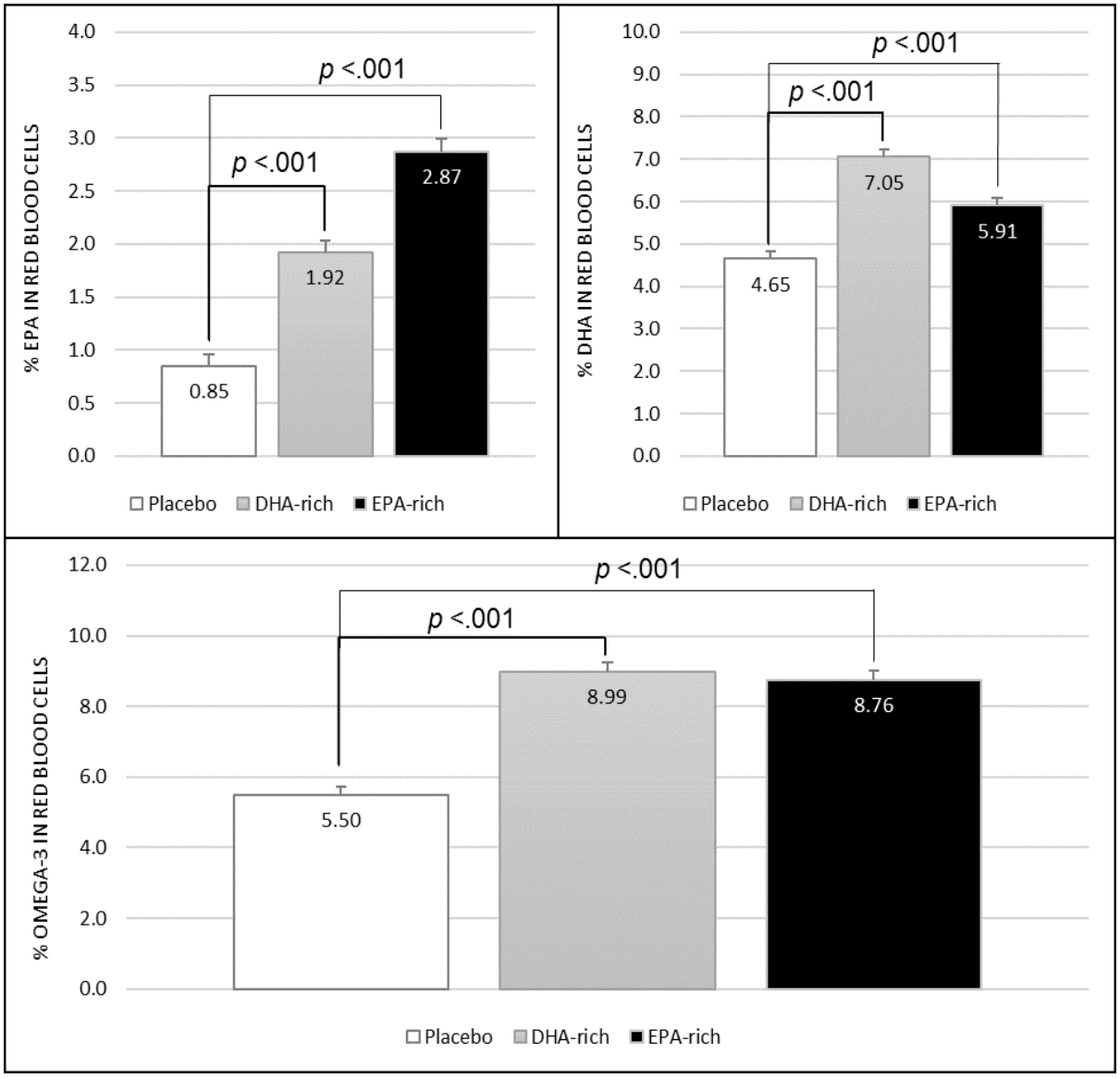

Figure XI.3. Estimated marginal means and standard errors (SE) for the post-dose percentage of EPA **(top left)** percentage of DHA **(top right)** and total percentage of EPA+DHA **(bottom)**, out of total fatty acids, in red blood cells by treatment group for the study presented in Chapter 5.

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