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1 *Article*

2 Differential effects of DHA- and EPA-rich oils on 3 sleep in healthy young adults: A randomised 4 controlled trial

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17 **Abstract:** Emerging evidence suggests that adequate intake of omega-3 polyunsaturated fatty acids 18 (n-3 PUFAs), which include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), may be 19 associated with better quality sleep. N-3 PUFAs, which must be acquired from dietary sources, are 20 typically consumed at suboptimal levels in Western diets. Therefore, the current placebo controlled 21 double blind randomized trial investigated the effects of an oil rich in either DHA or EPA on sleep 22 quality in healthy adults habitually consuming low amounts of oily fish. Eighty-four participants 23 aged 25–49 years completed the 26–week intervention trial. Compared to placebo, improvements in 24 actigraphy sleep efficiency ($p = 0.030$) and latency ($p = 0.026$) were observed following the DHA-rich 25 oil. However, these participants also reported feeling less energetic compared to placebo ($p = 0.041$), 26 and less rested ($p = 0.017$) and there was a trend towards feeling less ready to perform ($p = 0.075$) 27 than those given EPA-rich oil. A trend towards improved sleep efficiency was identified in the EPA- 28 rich group compared to placebo ($p = 0.087$), along with a significant decrease in both total time in 29 bed ($p = 0.032$) and total sleep time ($p = 0.019$) compared to DHA-rich oil. No significant effects of 30 either treatment were identified for urinary excretion of the major melatonin metabolite 6- 31 sulfatoxymelatonin. This study is the first to demonstrate some positive effects of dietary 32 supplementation with n-3 PUFAs in healthy adult normal sleepers, and provides novel evidence 33 showing the differential effects of n-3 PUFA supplements rich in either DHA or EPA. Further 34 investigation into the mechanisms underpinning these observations including the effects of n-3 35 PUFAs on sleep architecture are required.

36 **Keywords:** docosahexaenoic acid; eicosapentaenoic acid; omega-3; sleep; actigraphy; SMEDS
37

38 1. Introduction

39 The relationship between diet, which includes both specific dietary components and eating 40 behaviours, and sleep quality and duration is complex and bi-directional in nature [1]. Whilst 41 emerging evidence suggests that obesity and following a high fat, high carbohydrate diet may be 42 detrimental to sleep, conversely, improving micronutrient status (e.g. iron, zinc, magnesium, vitamin 43 D, vitamin B12) and consumption of particular whole foods (e.g. milk, kiwi, tart cherries, oily fish)

44 may have beneficial effects [2,3]. Oily fish is rich in the omega-3 polyunsaturated fatty acids (n-3
45 PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are not easily
46 produced endogenously in humans and so must be acquired from the diet. EPA and DHA are
47 incorporated into the membranes of cells throughout the body, and DHA is particularly enriched in
48 the brain. As such, adequate intake of these important fatty acids ensures proper functioning across
49 multiple systems. Indeed, low levels of circulating n-3 PUFAs have been observed in a wide range of
50 psychopathologies including attention deficit hyperactivity disorder, major depression and
51 Alzheimer's disease [4].
52 Converging evidence suggests n-3 PUFAs are also important for sleep. N-3 PUFA dietary
53 deficiency studies in animals have revealed a number of mechanisms by which DHA specifically may
54 affect sleep regulation including impaired functioning of the superchiasmatic nuclei [5], altered
55 melatonin release [6] and disruption to endocannabinoid signaling [7]. With regard to behavioural
56 effects, n-3 PUFA deficiency in rodents results in disorganized sleep patterns [6], an observation that
57 was paralleled in children during a period of total parenteral nutrition devoid of lipids [8]. In humans,
58 higher maternal levels of DHA appear to be linked with more mature infant sleep patterns [9,10].
59 Further, lower levels of DHA and a lower ratio of DHA to arachidonic acid (an n-6 PUFA) were
60 negatively associated with parent ratings of children's total sleep disturbance [11]. Likewise, the
61 concentration of n-3 PUFAs in adipose tissue of patients with obesity suffering from sleep apnea was
62 positively associated with sleep efficiency and minutes spent in slow wave sleep and rapid eye
63 movement (REM) sleep [12].
64 Results from an exploratory pilot trial in children (n = 43, age 7–9 years) indicated that dietary
65 supplementation with DHA may improve objectively measured sleep [11]. However, more data are
66 needed. In addition, to our knowledge no studies have evaluated the effects of EPA, which may also
67 be relevant given the previously observed effects of this n-3 PUFA on serotonin release [13] and the
68 production of prostaglandins [14]; prostaglandin D2 in particular is a potent somnogen known to
69 mediate the sleep/wake cycle [15]. Therefore, the present study investigated the effects of 26 weeks'
70 supplementation with oils rich in either DHA or EPA on subjective and objective sleep quality in
71 healthy, adult, low consumers of oily fish.

72 **2. Materials and Methods**

73 *2.1. Study Design*

74 This study employed a randomised, placebo controlled, double blind, parallel groups design.
75 Participants were randomly assigned to receive one of three treatments for 26 weeks (placebo, DHA-
76 rich oil, EPA-rich oil).

77 *2.2. Participants*

78 Prior to screening, all participants received information about the study and its procedures and
79 signed an informed consent form. Participants were aged between 25–49 years and had to pass a
80 physical/lifestyle screening to demonstrate they were in good health. Participants self-reported
81 consumption of oily fish of less than once per week, measured via a DHA food frequency
82 questionnaire [16]. Having good health was identified as being a non-smoker, free from prescription,
83 herbal, illicit or recreational drugs (females taking the contraceptive pill were included), free from
84 major illnesses, having a blood pressure lower than 159/99 mmHg and a BMI between 18.5 and 35
85 kg/m^2. All participants were recruited via posters, adverts placed on social media websites or emails
86 sent out to university staff and students and were either students or staff attending/working at
87 Northumbria University or individuals living in the Newcastle-upon-Tyne surrounding area.
88 Ninety-five males and females were screened for eligibility, 90 were enrolled into the study and
89 84 completed all study requirements. Of the six participants that did not complete the study, four
90 were lost to follow up after completion of the baseline testing visit, one withdrew consent and one
91 was advised to stop adhering to the consumption of the supplements due to reporting minor adverse

events. Participant disposition through the trial is displayed in Figure 1, demographic data are shown in Table 1 and outcomes from the DHA food frequency questionnaire are shown in Table 2.

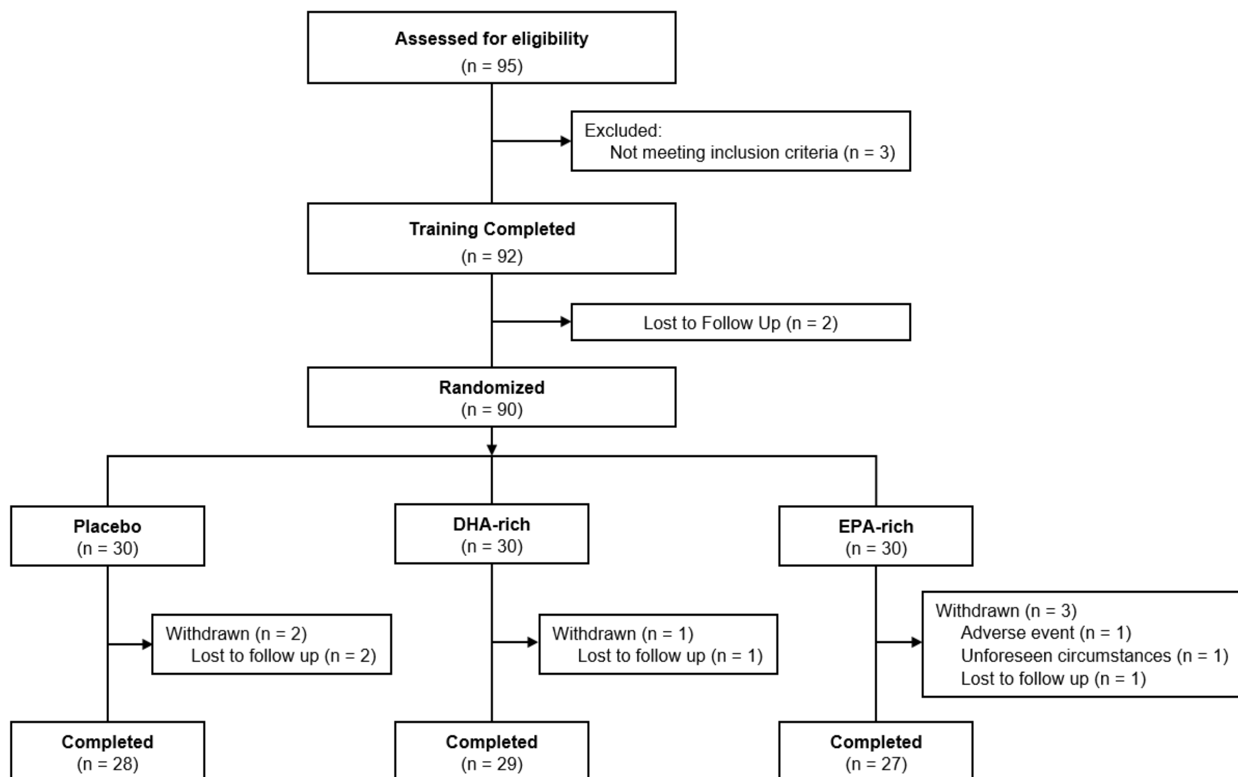

Figure 1. Participant disposition through the trial. Figure depicts the disposition of participants throughout the study, culminating in n = 84 of the 90 who were randomized.

Table 1. Participant demographic information and baseline characteristics for the 84 participants who completed all aspects of the study. Means ± SD are given where appropriate. Baseline differences were assessed using separate one-way ANOVAs or Chi-Square tests; resulting p values from these analyses are also presented.

Variable	Treatment	Mean	SD	*p*
n (Males/Females)	Placebo	8/20	-	
	DHA-rich	7/22	-	0.886
	EPA-rich	8/19	-	
% of EPA in RBC	Placebo	0.82	0.21	
	DHA-rich	0.89	0.28	0.169
	EPA-rich	1.05	0.67	
% of DHA in RBC	Placebo	4.87	0.94	
	DHA-rich	4.74	0.92	0.637
	EPA-rich	5.04	1.50	
n-3 index (EPA + DHA)	Placebo	5.69	1.01	
	DHA-rich	5.63	1.06	0.455
	EPA-rich	6.10	2.00	
Age (years)	Placebo	36.89	7.78	
	DHA-rich	37.41	7.28	0.768
	EPA-rich	35.89	8.73	
Systolic BP (mmHg)	Placebo	122.80	11.19	
	DHA-rich	120.21	13.04	0.699

	EPA-rich	120.50	13.43	
Diastolic BP (mmHg)	Placebo	81.00	8.12	0.805
	DHA-rich	79.45	9.67	
	EPA-rich	79.81	9.82	
Heart Rate (BPM)	Placebo	71.70	12.36	0.439
	DHA-rich	69.50	11.38	
	EPA-rich	73.43	10.42	
Weight (kg)	Placebo	73.92	17.45	0.656
	DHA-rich	70.37	12.20	
	EPA-rich	72.11	14.40	
Height (cm)	Placebo	168.83	9.85	0.614
	DHA-rich	166.59	6.01	
	EPA-rich	167.65	8.48	
BMI (kg/m^2)	Placebo	25.76	4.59	0.936
	DHA-rich	25.36	4.24	
	EPA-rich	25.62	4.48	
Years in Education	Placebo	16.28	1.10	0.239
	DHA-rich	15.56	1.74	
	EPA-rich	15.96	1.61	
Fruit & Vegetable (portions per day)	Placebo	4.13	1.78	0.359
	DHA-rich	4.48	2.21	
	EPA-rich	4.11	1.93	
Alcohol (units per day)	Placebo	1.00	0.71	0.157
	DHA-rich	1.27	1.05	
	EPA-rich	1.23	0.85	

102 **Table 2.** Outcomes from the DHA food frequency questionnaire for the 84 participants who
103 completed all aspects of the study. Group differences were assessed using separate one-way
104 ANOVAs; resulting p values from these analyses are also presented.

N-3 food source	Treatment	Mean	SD	*p*
Oily fish, servings per month[a]	Placebo	1.43	1.45	0.522
	DHA-rich	1.79	1.55	
	EPA-rich	1.85	1.48	
Fish, servings per month[b]	Placebo	2.07	1.54	0.834
	DHA-rich	2.05	1.76	
	EPA-rich	2.33	2.41	
Fish/shellfish, servings per month[c]	Placebo	1.36	1.70	0.402
	DHA-rich	1.90	1.81	
	EPA-rich	1.39	1.50	
Liver, servings per month[d]	Placebo	2.04	4.78	0.381
	DHA-rich	0.78	1.84	
	EPA-rich	1.93	4.12	
Egg yolks, servings per week	Placebo	4.11	3.62	0.890
	DHA-rich	4.52	4.12	
	EPA-rich	4.69	5.77	
Poultry, servings per week	Placebo	2.93	2.36	

	DHA-rich	3.53	2.59	0.534
	EPA-rich	2.87	2.44	

[a] includes: bluefish, blue fin tuna, cisco (smoked), herring, mackerel, pollock, sardines, salmon, whitefish; [b] includes: bass, calamari, catfish, drumfish, flounder, grouper, hailbut, mussles, perch, redfish, rockfish, shark, snapper, sole, squid, swordfish, trout, tuna (canned 6 oz), whiting; [c] includes: carp, clams, cod, crab, crayfish, fish patties/squares, fish sticks, haddock, lobster, mullet, oysters, pike, pompano, scallops, shrimp (14 medium), surgeon; [d] includes: chicken liver, turkey liver or beef liver.

2.3. Sample size

Sample size was calculated based on a medium effect size reported by Montgomery et al. [11] 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, was 30 participants per treatment arm, inclusive of a 10% anticipated dropout rate. Power calculations were made using GPower 3.1.3.

2.4. Randomization

Treatment group was assigned randomly according to a randomisation schedule produced using the website www.randomization.com. To ensure blinding was maintained throughout the study, a 3rd party within the same university created the randomisation schedule and coded treatments before the treatments and randomisation schedule were delivered to the research team. Capsules were provided in opaque containers. Therefore, both the research team and participants were blind as to which participants received which treatment until after data analysis was complete.

2.5. Treatment

All treatment capsules were supplied by BASF AS. Treatment was provided as three 1 g capsules. The DHA-rich capsules provided 900 mg DHA/d and 270 mg EPA/d (Accelon™ DHA EE EU capsules), the EPA-rich capsules provided 360 mg DHA/d and 900 mg EPA/d (Accelon™ EPA EE EU capsules) and the placebo capsules contained 1 g refined olive oil. Each capsule of Accelon High DHA contained 600 mg oil with at least 420 mg omega-3 fatty acid EEs including EPA, DHA, C18:3 n-3, C18:4 n-3, C20:4 n-3, C21:5 n-3, C22:5 n-3. The amount of DHA was at least 300 mg and EPA at least 90 mg per capsule. Similarly, each capsule of Accelon High EPA contained 600 mg oil with at least 450 mg omega-3 fatty acid EEs including the same fatty acids as above. The amount of DHA was at least 120 mg and EPA at least 300 mg per capsule. The additional capsule fill, 400 mg/capsule for both formulations, was food additives (permitted for use in food supplements). The active treatments using the Accelon™ technology also contained a proprietary mixture of surfactants and co-solvents. When exposed to the contents of the stomach, these ingredients are designed to spontaneously emulsify the oils, forming microdroplets. Known as a self-microemulsifying delivery system (SMEDS), this approach improves the absorption of the n-3 PUFAs contained within the treatments [17]. Participants were instructed to take their capsules with a glass of water at their usual bedtime. Placebo and treatment capsules were identical in size and shape, and similar in appearance.

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. Formal written consent was provided.

Before the baseline and week 26 assessments, participants were required to visit the centre 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 urine samples the night before and morning of 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 of 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 at least 1 hour prior to arrival at the laboratory (but to avoid any caffeinated products) or to not have breakfast if they usually skipped breakfast. 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 Supplementary Figure 1 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 Leeds Sleep Evaluation Questionnaire (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 the treatment and sleep diaries, all remaining treatments, completion of a treatment guess questionnaire 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 red blood cell fatty acid profile.

2.7. Outcomes

2.7.1. Subjective Measures

The LSEQ is a 10-item VAS scale designed specifically to measure changes in subjective sleep following a pharmacological intervention [18]. The questionnaire measures 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 each presented on a 100 mm line with one end representing a negative and the other representing a positive response to the question. Higher scores on these scales represent more positive feelings of the respective items.

Visual analogue scales (VAS) measured items related to participant's subjective 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 the following questions: "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 stronger feelings of the respective items.

2.7.2. Biological Measures

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 as 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 participant's study details. The samples were taken to the laboratory at the baseline and week 26 testing visits for further labelling and immediate storage at -80ºC for later analysis of the major melatonin metabolite 6-sulfatoxymelatonin (aMT6s) by radioimmunoassay [19].

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 [20] and urinary levels of aMT6s are seen to parallel those of melatonin in the blood, saliva, and urine [21].

199 2.7.3. Objective Measures

200 Participants were instructed to complete sleep diaries to record time in and out of bed and to
201 wear actigraphy WGT3X-BT watches (ActiGraph LLC, FL, USA) on the non-dominant wrist for seven
202 consecutive days and nights both prior to commencing and before completing the 26-week
203 supplementation period. The devices are small and lightweight and can detect body accelerations in
204 the vertical, horizontal (right to left) and frontal (front and back) planes at varying sample rates. The
205 data from the watches were collected in 1-minute epochs. Utilizing Actilife software (version 6.1,
206 ActiGraph) and the Cole-Kripke algorithm **[22]**, the following parameters could then be calculated:

207 • Sleep latency *(The difference in minutes between in bedtime and sleep onset)*

208 • Sleep efficiency *(Number of sleep minutes divided by the total number of minutes the participant*
209 *was in bed; i.e., the difference between the In-Bed and Out Bedtime)*

210 • Total sleep time *(The total number of minutes scored as "asleep")*

211 • Total minutes in bed *(The total number of minutes in bed both awake and asleep)*

212 • Wake after sleep onset *(The total number of minutes awake after sleep onset occurred)*

213 • Number of awakenings *(Total number of awakenings from the time spent in bed)*

214 • Average awakening length *(The average length, in minutes, of all awakening episodes)*

215 • Sleep Fragmentation Index *(The sum of the Movement Index – Total of scored awake minutes*
216 *divided by Total time in bed in hours x 100 and Fragmentation Index - Total of 1-minute scored*
217 *sleep bouts divided by the total number of sleep bouts of any length x 100)*

218 *2.8. Red blood cell fatty acid measurements*

219 Blood samples were collected by trained phlebotomists via venepuncture into
220 ethylenediaminetetraacetic acid vacutainers (6 ml). The samples were stored in an ice box, or at 5°C,
221 until they could be processed which was within 8 hours of collection. Blood was centrifuged at 2000
222 rpm (913 x g) for 10 minutes at room temperature. The top layer of plasma was then removed and
223 discarded. One ml of the red blood cell (RBC) pellet was collected, transferred to a 15 ml centrifuge
224 tube and made up to 15 ml with phosphate-buffered saline (PBS). The mixture was inverted and the
225 centrifuged at 1200 rpm (350 x g) for 10 minutes at room temperature with a low brake. The PBS was
226 then removed and the washing process repeated for a second time. After the second wash the RBC
227 pellet was transferred into 1.5 ml microtubes and immediately frozen at -80°C prior to analysis.
228 RBC fatty acid composition was analysed by gas chromatography [23]. The RBC pellet was
229 washed twice with 5 ml of 0.9% sodium chloride (NaCl) and then total lipid was extracted using
230 chloroform-methanol (2:1) containing 50 mg/l butylated hydroxytoluene as antioxidant. The lipid
231 phase was dried down under nitrogen, redissolved in a small volume of toluene, and then heated for
232 2 hours at 50°C with dry methanol containing 2% sulphuric acid. This procedure cleaves fatty acids
233 from more complex lipids (e.g. membrane phospholipids) and simultaneously methylates them to
234 produce fatty acid methyl esters (FAMEs). At the ed of the reaction tie the sample was neutralized
235 and FAMEs extracted into hexane. FAMEs were concentrated and then separated on an Agilent 6890
236 gas chromatograph fitted with a 30 m long SGE BPX-70 fused silica capillary column. The split ratio
237 was 25:1. The injector port temperature was 300°C and helium was used as the carrier gas. The oven
238 was held at 115°C for 2 minutes, then increasing at a rate of 10°C per minute up to 200°C where it was
239 held for 18.5 minutes. Oven temperature was then increased sat a rate of 60°C per minute to 245°C
240 where it was held for 4 minutes. The flame ionization detector was held at 300°C. FAMEs were
241 identified by comparison with tun times of authentic standards. Peak areas were calculated using
242 ChemStation software and each FAME was expressed as a weight% of the total. The n-3 index was
243 calculated as % EPA + % DHA.

244

245 *2.9. Compliance and treatment guess*

246 As each participant was provided with 600 treatment capsules throughout the supplementation
247 period, treatment compliance (%) could be calculated in order to measure adherence to the study
248 protocol concerning appropriate consumption of the study treatments. Treatment compliance was
249 calculated by comparing the number of capsules that were returned by each participant at the end of
250 the study with the number of capsules that should have been returned.
251 Additionally, at the end of the study, all participants were provided with a treatment guess
252 questionnaire and asked to choose between whether they had received an active or placebo treatment
253 throughout the supplementation period, to verify the blinding procedure. Responses from the
254 treatment guess questionnaire were analyzed via Chi-square test comparing the number of correct
255 and incorrect responses given by each treatment group.

256 *2.10. Statistical methods*

257 Statistical analyses were performed with IBM SPSS statistics software (version 25; IBM Corp).
258 Full data handling and cleaning procedures are described in full in Supplementary Materials Section
259 2. Descriptive and comparison statistics (independent t test, 2 tailed or Chi-square test) of all baseline
260 characteristics were based on all participants who were randomised and consumed at least one dose
261 of treatment. All other analyses conducted were from the intention-to-treat (ITT) population. The
262 general statistical approach selected to analyse the repeated measures data by treatment group was
263 via linear mixed models (LMM) with treatment (DHA-rich, EPA-rich, Placebo) and night (1-7) as
264 factors in the objective sleep models and treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13
265 and week 26) in the subjective models. For each model that was run, the covariance matrix structure
266 was chosen based on the structure that produced the lowest Schwarz's Bayesian Criterion (BIC), an
267 indication of the best fitting model for the data [24]. Changes within outcome variables during the
268 treatment period were assessed via LMMs that adjusted for respective baseline scores. Significant
269 main or interaction effects of treatment ($p < 0.050$) were investigated further with Sidak corrected
270 comparisons to account for multiple group comparisons.

271 *2.11. Ethics*

272 This study was pre-registered via www.clinicaltrials.gov (NCT03559361) and conducted at the
273 University of Northumbria according to the guidelines of the Declaration of Helsinki (2013). Ethical
274 approval for the trial was obtained from the University of Northumbria Department of Psychology
275 Ethics Committee (SUB023), and written informed consent was obtained from all participants. All
276 paper study data were stored in a locked filing cabinet and electronic data on a secure network drive
277 with access granted only to those working within the research centre. The trial described in this
278 manuscript was a sub-study of a larger study investigating the effects of the EPA- and DHA-rich oils
279 on cognitive function (NCT02763514).

280 **3. Results**

281 The flow of participants through the study is summarized in Figure 1. The final analysis was
282 conducted in 84 participants (n = 28 in the placebo group; n = 29 in the DHA-rich oil group; n = 27 in
283 the EPA-rich oil group) for whom baseline and end of study data were available. Baseline
284 characteristics of subjects are summarised in Table 1. No significant differences between the
285 treatment groups were identified for any of the baseline demographics.

286 *3.1. Compliance*

287 For participants who completed the study, compliance was observed to be very good in all three
288 groups (95.21% Placebo, 96.42% DHA-rich, 95.64% EPA-rich) with one way ANOVA identifying no
289 significant differences for compliance percentage by treatment group [F (2, 81) = 0.274, p = 0.761]. A
290 Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was
291 completed at the end of the final visit and revealed no significant differences in participants' ability

292 to correctly identify whether they had been administered an active or placebo treatment between the
293 three groups [χ^2 (2) = 3.84, p = 0.147]. Analysis of RBC fatty acid profiles further supports the
294 compliance data (Table 3).

295 **Table 3.** Red blood cell EPA, DHA and n-3 index for placebo, DHA-rich and EPA-rich treatment
296 groups. Data are mean ± SD at Baseline, Week 26 and Change (from baseline).

Variable	Treatment	Baseline (n = 80)	Week 26 (n = 70)	Change* (n = 69)
% of EPA in RBC	Placebo	0.82 ± 0.21	0.80 ± 0.27	-0.03 ± 0.19
	DHA-rich	0.88 ± 0.28	2.16 ± 0.57	1.24 ± 0.57
	EPA-rich	1.03 ± 0.43	2.73 ± 1.02	1.68 ± 1.03
% of DHA in RBC	Placebo	4.82 ± 0.96	4.77 ± 0.82	0.03 ± 0.78
	DHA-rich	4.71 ± 0.91	7.69 ± 1.31	2.94 ± 1.42
	EPA-rich	5.04 ± 1.48	6.12 ± 0.95	1.08 ± 1.43
n-3 index (EPA + DHA)	Placebo	5.63 ± 1.03	5.57 ± 0.95	-0.00 ± 0.80
	DHA-rich	5.59 ± 1.06	9.85 ± 1.64	4.18 ± 1.69
	EPA-rich	6.07 ± 1.94	8.85 ± 1.60	2.75 ± 2.19

*Change values are only calculated for those participants who had data at both Baseline and Week 26.

297 *3.2. Mixed Models Analysis*

298 Due to the number of possible interactions between the factors, only those which revealed
299 significant main or interaction effects including treatment are reported.

300 3.2.1. Objective Measures

301 See Table 4 for a summary of all objective sleep results. A significant main effect of treatment for
302 sleep efficiency was identified [F (2, 79.79) = 3.68, p = 0.030] with post hoc comparisons identifying
303 the DHA-rich group (92.02%; p = 0.037) as having significantly higher sleep efficiency with a trend
304 towards significantly higher sleep efficiency in the EPA-rich group (91.85%; p = .087) compared to
305 placebo (90.30%) (Figure 2A).
306 Analysis identified a significant main effect of treatment for sleep latency [F (2, 322) = 3.68, p =
307 0.026] with post hoc comparisons identifying the DHA-rich (3.76; p = 0.021) but not the EPA-rich (3.98;
308 p = 0.276) group as showing significantly shorter sleep latency compared to placebo (4.31) (Figure
309 2B).
310 Analysis also identified a significant interaction between treatment and night for sleep latency
311 [F (12, 322) = 2.28, p = 0.009] with post hoc comparisons identifying the DHA-rich group (3.31) as
312 having a significantly shorter latency period compared to both the placebo (6.43; p = 0.003) and EPA-
313 rich (5.80; p = 0.023) groups on night 1 and both the DHA-rich (3.36, p = 0.017) and EPA-rich (3.34, p
314 = 0.021) groups as having a significantly shorter latency period compared to placebo (4.53) on night
315 6.
316 Analysis identified a significant main effect of treatment for total minutes in bed [F (2, 328) =
317 3.29, p = 0.039] with post hoc comparisons identifying no significant differences between the active
318 and placebo groups but the DHA-rich group (484.51 minutes) spent significantly more time in bed
319 than the EPA-rich group (467.10; p = 0.032) (Figure 2C).
320 A significant main effect of treatment was also identified for total sleep time [F (2, 323) = 4.06, p
321 = 0.018] with post hoc comparisons identifying no significant differences between the active and
322 placebo groups but the DHA-rich group (455.17 minutes) spent significantly more time asleep than
323 the EPA-rich group (427.28; p = 0.019) (Figure 2D).
324 Analysis also identified a significant interaction between treatment and night for sleep
325 fragmentation index [F (12, 227.64) = 1.90, p = 0.025] with post hoc comparisons identifying the DHA-

rich group (15.88; $p = 0.003$) as having significantly less sleep fragmentation compared to placebo (26.85) on night 2 only.

Table 4. Objective 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.

Variable	Treatment	Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Latency (minutes)	Placebo	74	4.31[a]	0.21	Treatment	3.68	0.026
	DHA-rich		3.76[a]	0.26			
	EPA-rich		3.98	0.27	Treatment*Night	2.28	0.009
Efficiency (%)	Placebo	72	90.30[a,T]	0.50	Treatment	3.68	0.030
	DHA-rich		92.02[a]	0.49			
	EPA-rich		91.85[T]	0.57	Treatment*Night	1.47	0.138
Total Minutes in bed (minutes)	Placebo	74	484.51	8.13	Treatment	3.29	0.039
	DHA-rich		494.85[b]	6.63			
	EPA-rich		467.10[b]	8.55	Treatment*Night	.851	0.598
Total Sleep Time (Minutes)	Placebo	73	437.91	7.56	Treatment	4.06	0.018
	DHA-rich		455.17[b]	6.18			
	EPA-rich		427.28[b]	8.08	Treatment*Night	1.20	0.281
Wake after Sleep Onset (minutes)	Placebo	72	42.02	2.42	Treatment	2.55	0.084
	DHA-rich		35.84	2.14			
	EPA-rich		34.77	2.74	Treatment*Night	1.29	0.225
Number of Awakenings	Placebo	74	17.50	0.99	Treatment	.813	0.446
	DHA-rich		15.87	0.88			
	EPA-rich		16.20	1.08	Treatment*Night	1.19	0.289
Average Awakening Length (minutes)	Placebo	74	2.44	0.11	Treatment	.576	0.564
	DHA-rich		2.29	0.09			
	EPA-rich		2.38	0.12	Treatment*Night	1.50	0.126
Sleep Fragmentation Index	Placebo	74	22.89	1.28	Treatment	.802	0.451
	DHA-rich		20.80	1.11			
	EPA-rich		22.22	1.38	Treatment*Night	1.90	0.036

[a] = significant difference between active and placebo groups below $p < 0.050$; [b] = significant difference between the active treatment groups below $p < 0.050$; [T] = trend towards a significant difference between active and placebo groups below $p < 0.100$

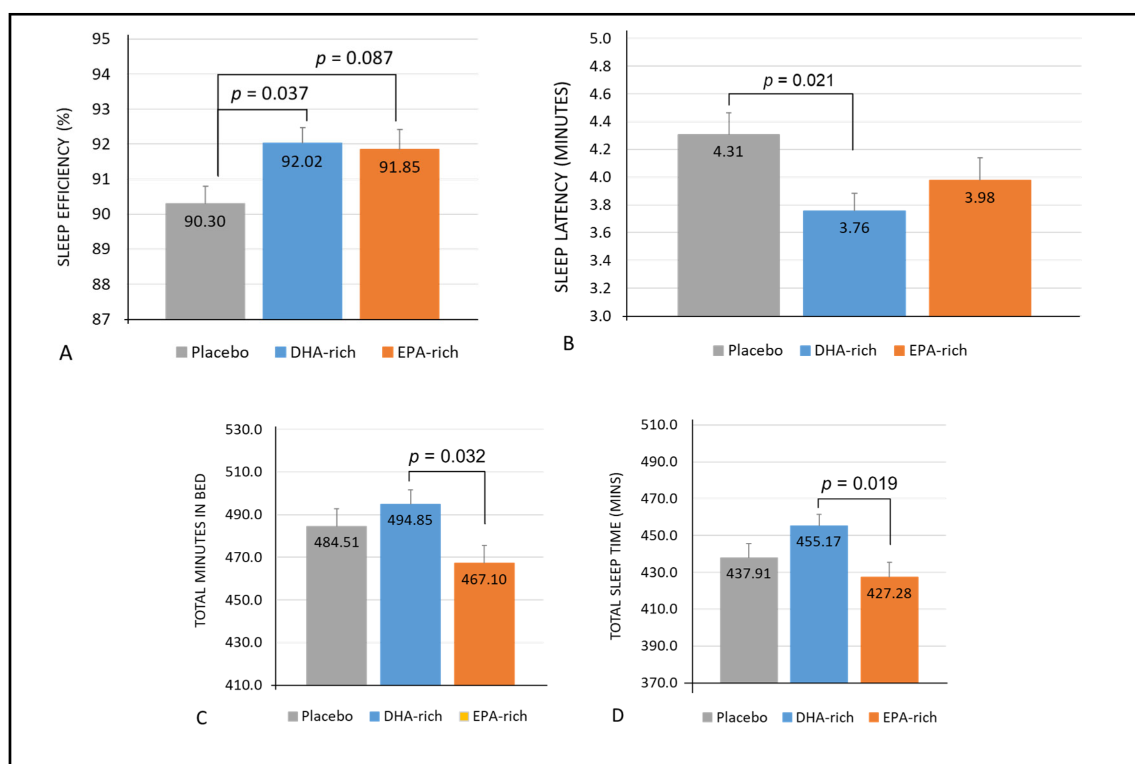

Figure 2. Estimated marginal means and standard error (SE) for post-dose values of sleep efficiency (A), sleep latency (B), total minutes in bed (C), total sleep time in minutes (D).

3.2.2. Subjective Measures

See Table 5 for a full summary of subjective sleep results. A significant effect of treatment for feeling energetic was also identified [F (2, 79.35) = 3.545, p = 0.034], with post hoc comparisons identifying the DHA-rich (53.79; p = 0.041) but not the EPA-rich (64.94; p = 0.970) group as feeling significantly less energetic compared to placebo (62.47) (Figure 3).

A significant effect of treatment for feeling rested was identified [F (2, 76.42) = 4.71, p = 0.017], with post hoc comparisons identifying no significant difference between the active and placebo groups, but the DHA-rich group (53.55) were significantly less rested than the EPA-rich group (64.94; p = 0.017) (Figure 3).

A significant effect of treatment for feeling ready to perform was identified [F (2, 84.12) = 3.211, p = 0.045], with post hoc comparisons identifying no significant difference between the active and placebo groups but the DHA-rich group (59.12) showed a trend towards being significantly less ready to perform than the EPA-rich group (66.65; p = 0.075) (Figure 3).

A significant treatment by visit interaction for behaviour following waking was observed [F (2, 77.01) = 5.03, p = 0.009]. However, post hoc comparisons identified no significant differences between any of the groups at either week 13 or 26. No other effects of treatment were observed for any other subjective measures.

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Table 5. 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.

Variable	Treatment		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	0.243	0.785
	DHA-rich		177.13	7.28	177.05	6.56			
	EPA-rich		176.04	7.09	167.88	6.98	Treatment*Visit	0.557	0.575
Quality of Sleep (0 - 200)	Placebo	86	118.12	6.65	112.38	6.74	Treatment	0.438	0.647
	DHA-rich		118.47	7.19	118.64	6.53			
	EPA-rich		109.22	7.02	112.26	6.92	Treatment*Visit	0.392	0.677
Awake Following Sleep (0 – 200)	Placebo	86	107.23	6.01	113.52	6.12	Treatment	0.518	0.598
	DHA-rich		118.80	6.62	115.34	5.91			
	EPA-rich		112.80	6.37	113.05	6.27	Treatment*Visit	0.379	0.686
Behaviour Following Wakening (0 – 300)	Placebo	86	191.09	7.68	180.02	7.79	Treatment	0.814	0.447
	DHA-rich		181.98	8.38	165.39	7.57			
	EPA-rich		169.66	8.20	188.93	8.09	Treatment*Visit	5.03	0.009
Rested (%)	Placebo	86	66.21[a]	3.67	59.80	3.74	Treatment	4.71	0.012
	DHA-rich		56.44[a]	4.06	50.65	3.60			
	EPA-rich		68.79	3.91	61.09	3.82	Treatment*Visit	0.034	0.966
Energetic (%)	Placebo	86	65.69[a]	3.21	60.20	3.26	Treatment	3.55	0.034
	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	0.354
Relaxed (%)	Placebo	86	64.87	3.21	65.82	3.26	Treatment	1.37	0.260
	DHA-rich		61.12	3.49	58.81	3.14			
	EPA-rich		65.60	3.40	65.47	3.35	Treatment*Visit	0.191	0.827
Irritable (%)	Placebo	86	26.74	3.70	27.70	3.77	Treatment	1.46	0.238
	DHA-rich		31.95	4.09	35.10	3.64			
	EPA-rich		28.70	3.92	27.25	3.85	Treatment*Visit	.196	0.822
Ready to Perform (%)	Placebo	86	65.70	2.81	66.23	2.86	Treatment	3.21	0.045
	DHA-rich		61.56[b]	3.08	56.68	2.76			
	EPA-rich		66.88[b]	2.98	66.43	2.92	Treatment*Visit	0.668	0.515
Good Night's Sleep (%)	Placebo	86	65.88	4.30	59.53	4.38	Treatment	1.61	0.205
	DHA-rich		63.06	4.72	50.85	4.23			
	EPA-rich		68.37	4.54	62.69	4.45	Treatment*Visit	0.392	0.677

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

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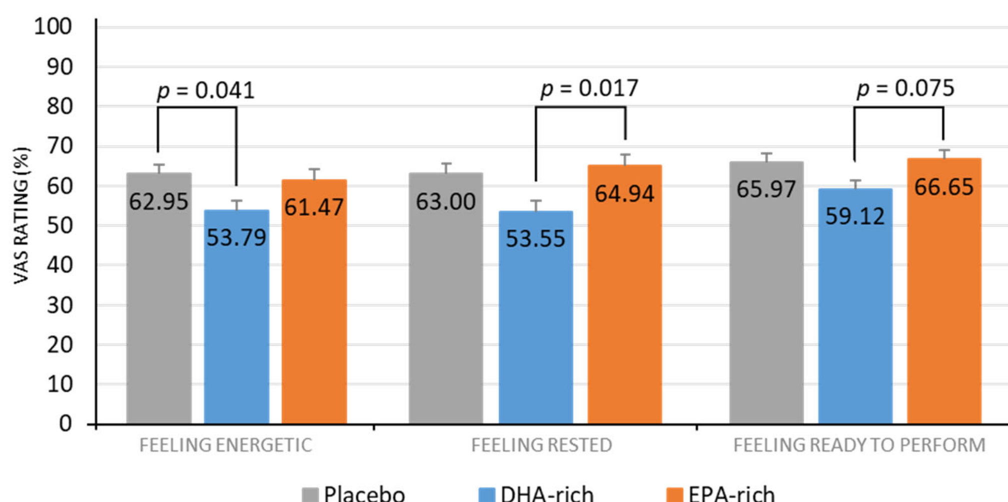

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392 **Figure 3.** Estimated marginal means and standard error (SE) for post-dose subjective ratings for
393 feeling energetic, feeling rested and feeling ready to perform, by treatment group.

394 3.2.3. Biological Measures

395 No significant main effects of treatment were observed for urinary aMT6s (Table 6).

396 **Table 6.** aMT6s outcomes for placebo, DHA-rich and EPA-rich treatment groups. Post-dose estimated
397 marginal means and standard error (SE) are presented with F and p values of the main effects from
398 the linear mixed models.

Variable	Treatment	Post-dose			Main Effects		
		n	Mean	SE		*F*	*p*
Total aMT6s (ng)	Placebo	67	15,289.27	1,267.50	Treatment	0.558	0.575
	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	0.130
	DHA-rich		468.62	123.42			
	EPA-rich		805.34	117.08			

399 **4. Discussion**

400 The results from the current study show that supplementation with DHA-rich oil in healthy
401 adults who do not habitually consume oily fish resulted in a significant increase in sleep efficiency
402 and a significant decrease in sleep latency compared to placebo. Interestingly, despite these
403 improvements in the objective actigraphy sleep measures in the DHA-rich group, it was also found
404 that this group reported feeling less rested compared to placebo, and less energetic and ready to
405 perform than those given EPA-rich oil. A significant decrease in sleep fragmentation index was also
406 observed in the DHA-rich group compared to placebo. However, the latter effect was found to only
407 be evident during the second night of the seven nights recorded, and must be interpreted with
408 caution. With regards to the EPA-rich oil, a trend towards a significant increase in sleep efficiency
409 was identified in this group, compared to placebo. The EPA-rich oil also resulted in a significant
410 decrease in both total time in bed and total sleep time compared to the DHA-rich group, although no
411 significant differences were identified between either treatment group and placebo for these
412 measures. Finally, no significant effects of treatment were identified for urinary aMT6s excretion.
413 The beneficial effects of DHA in increasing sleep efficiency and reducing sleep latency are
414 consistent with previous animal models [25] and exploratory data from an intervention study in
415 children [11], providing further evidence to support the beneficial role of DHA in sleep. Indeed,
416 enzymatic transformation of serotonin to melatonin by aralkylamine N-acetyltransferase [26] is

supported by DHA via its positive effects on membrane fluidity [27] and serotonin levels in the prefrontal cortex [28], which may help to modulate the transition between sleep and wakefulness [29]. Given the above, the null findings of treatment on urinary aMT6s may suggest that DHA affects sleep via mechanisms other than the melatonin synthesis pathway. However, it may also be the case that the period of urinary collection over a single night was simply not sensitive enough to identify an effect on aMT6s. Therefore, in order to better evaluate the relationship between n-3 PUFAs, melatonin and sleep, future research should consider either the use of 24/48 hour urinary collection periods or the analysis of melatonin in blood which allows for greater resolution and sensitivity [30].

The negative subjective ratings identified in the DHA-rich oil group are inconsistent with the actigraphy data. One potential explanation for this may be informed by investigations of patients suffering from insomnia. For instance, Feige et al. [31] explain how a major enigma of insomnia research constitutes the frequently noted discrepancy between the subjective experience of sleep (measured by sleep questionnaires) and the polysomnographic (PSG) findings. PSG studies often demonstrate that patients suffering from insomnia tend to underestimate their nocturnal sleep time [32,33] leading to terms such as 'sleep state misperception' for patients with a relatively normal sleep continuity and architecture, despite subjective complaints of disturbed sleep [34]. Due to the issues with objectively defining sleep parameters (e.g. sleep efficiency/latency [35]), focusing on the architecture of sleep may offer additional explanations for these conflicting data. For example, Feige et al. [32] showed that differences between subjectively 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. Further investigation using PSG would therefore provide valuable insights into the effects of n-3 PUFAs on the sleep architecture in relation to the amounts of REM and non-REM sleep, which could then be evaluated alongside subjective effects.

Regarding the observed effects of EPA-rich oil on sleep, the differential pattern of results compared to placebo and the direct differences between the effects of each treatment do suggest specific roles of DHA and EPA in sleep. The shortened sleep times identified within the current study following EPA-rich oil compared to DHA-rich oil may potentially be explained by the role of EPA inhibiting the formation of E_2 series prostaglandins which in turn inhibit the release of serotonin [36]. As serotonin promotes wakefulness and inhibits REM sleep [37], it may be that increased levels of circulating EPA indirectly upregulate promotion of wakefulness, resulting in decreased sleep time. It should be noted that although participants in the EPA-rich oil group reported the shortest sleep times, this did not appear to lead to any 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 increases in the time spent awake, number of awakenings or decreased ratings of subjective sleep quality. This may potentially suggest that EPA is beneficial for regulating a healthy sleep cycle and could help protect against suboptimal sleep (i.e., too little or too much sleep), which is known to be detrimental for health [38,39].

The current study is the first to investigate the separate effects of DHA and EPA on sleep, in a sample of healthy, young adults, with a rigorous study protocol that collected both objective and subjective measurements of sleep. Additionally, the measurement of aMT6s offered the potential to gain insight into possible mechanisms underpinning the relationship between n-3 PUFAs and sleep. The study had good compliance as confirmed by measuring RBC EPA, DHA and n-3 index. However, the study is not without its limitations and several challenges were faced with regards 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—resulted in a reduced sample size in the actigraphy datasets, although this reduction is in line with missing data observed in previous actigraphy studies [11,40]. Furthermore, future research may wish to take body composition into account when recruiting participants. As overweight and obese individuals are seen to have increased inflammatory profiles [41,42] and are more likely to experience sleep disorders [43], this may be a factor which future trials control for more strictly or consider

during data analysis. As the current study included participants with a BMI ≤35 kg/m^2, it could be that a more conservative range of BMI should be used in future.

Overall, this study provides additional support for the beneficial role of n-3 PUFAs, particularly DHA, for sleep. These include an overall increase in sleep efficiency and a reduction in sleep latency, although these positive measures of increased sleep quality measured using actigraphy were not consistent with subjective ratings following DHA-rich oil. Further investigations into the relationship between n-3 PUFAs and the serotonin/melatonin synthesis pathway and effects on sleep architecture are required. 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 sleep.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Schematic showing the study progression from enrolment to completion 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.

Author Contributions: The study was conceived and designed by PAJ, MJP, DOK, CH, SOH. MJP, JK, JF collected the data. MJP, BM and PCC analyzed the data. All authors contributed to preparing the draft and gave final approval of the manuscript for publication.

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