Original research article

Diurnal rhythm of plasma EPA and DHA in healthy adults

Philippa A. Jacksona,b,*, Cathrine Husbergb, Svein-Olaf Hustvedtb, Philip C. Calderc,d, Julie Kham, Hannah Averyb, Joanne Forsterb, David O. Kennedya

a Brain Performance and Nutrition Research Centre, Northumbria University, Newcastle Upon Tyne NE1 8ST, United Kingdom
b Human Development and Health, Faculty of Medicine, University of Southampton., Southampton SO16 6YD, United Kingdom
c National Institute of Health Research Southampton Biomedical Research Centre, University Hospital Southampton National Health Service Foundation Trust and University of Southampton, Southampton SO16 6YD, United Kingdom

d Corresponding author.
E-mail address: philippa.jackson@northumbria.ac.uk (P.A. Jackson).

Knowledge of the diurnal variation in circulating omega-3 polyunsaturated fatty acids (n-3 PUFAs) may be an important consideration for the development of dosing protocols designed to optimise tissue delivery of these fatty acids. The objective of the current study was to examine the variation in plasma concentrations of eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) over a 24-h period in healthy adults under eating and sleeping conditions generally approximate to a free-living environment. Twenty-one healthy participants aged 25–44 years took part in a single laboratory visit encompassing an overnight stay. EPA and DHA were measured in plasma samples collected every two hours from 22:00 until 22:00 the following day, with all meals being provided at conventional times. Cosinor analysis was used to estimate the diurnal variation in each fatty acid from pooled data across all participants. A significant diurnal variation in the pooled plasma concentrations of both fatty acids was detected. However, evidence of distinct rhythmicity was strongest for DHA. The timing of the peak concentration of DHA was 17:43 with a corresponding nadir at 05:43. In comparison, the observed acrophase for EPA was delayed by three hours, occurring at 20:41, with a nadir at 08:41. This is the first time that the diurnal variation in these important bioactive fatty acids has been described in a sample of healthy adults following a normal pattern of eating and sleeping. In the absence of any dietary intake of EPA and DHA, circulating levels of these fatty acids fall during the overnight period and reach their lowest point in the morning. Consumption of n-3 PUFAs at night time, which counteracts this pattern, may have functional significance.

1. Introduction

Following several decades of investigation into the effects of the bioactive omega-3 polyunsaturated fatty acids (n-3 PUFAs) eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) on cognition, firm conclusions regarding their specific effects are still to be drawn. Evidence from cross-sectional studies is generally consistent: across the lifespan, increased consumption of n-3 PUFAs is associated with better cognitive function [1–5]. On the other hand, critical and systematic reviews of clinical trials are more tentative, often citing methodological variations underpinning the inconsistency in the evidence [6–8]. Recommendations regarding dose, duration of intervention, outcome measures and populations from which study samples are drawn are now being proposed, which may lead to stronger evidence in time [6,7,9,10].

Due consideration is also being given to the formulation of the n-3 PUFAs intervention itself, so that maximal delivery of DHA and/or EPA to tissues is ensured [11–13]. With regards optimal digestion and absorption, a further factor that may influence the efficacy of n-3 PUFAs is the time at which the supplement is consumed. Evidence from rodent studies suggests circadian variation in lipid metabolism that may be an important consideration. For example, under normal light-dark conditions where animals have ad libitum access to food, the peak time for lipid digestion and absorption and for fatty acid oxidation occurs at the very beginning of the active/awake phase [14], suggesting consumption of fats is more desirable in the morning.

Specific investigation of the diurnal variation of blood levels of n-3 PUFAs in humans is limited to two studies, the results from which do
not agree. Cornelissen et al. [15] estimated peak whole blood (finger- 
prick) concentrations of total n-3 PUFAs (EPA, DHA, alpha linolenic 
acid, docosapentaenoic acid) collected from one male and one female 
to occur shortly after 08:00, with an estimated nadir occurring at 20:00. 
On the other hand, the highest concentrations of plasma EPA and DHA 
were reported by Dallmann et al. [16] at around noon in their sample of 
ten males, after which they rapidly declined. Whilst both studies re-
cruited a small number of participants, key methodological differences 
between these studies may have influenced the reported findings. For 
example, both participants in the study by Cornelissen et al. had a 
number of health issues, and the authors reported that one of the par-
ticipants followed an odd schedule on the day of data collection that 
the authors suspected may have affected the results. On the other hand, 
despite the fact that all of the participants recruited to the study re-
ported by Dallmann et al. were healthy, the blood samples were col-
lected under a strict regimen of hourly feeding around the clock and 
participants were not able to sleep under a constant routine protocol. 
The current study therefore aimed to investigate the diurnal rhythm of 
plasma concentrations of EPA and DHA in humans under normal eating 
and sleeping conditions. The results described below comprise a sec-
ondary outcome from a larger study that also evaluated the bioavail-
ability of acute doses of n-3 oils, which together informed the design of 
a randomised controlled trial investigating the effects of n-3 oils on 
cognitive function.

2. Materials and methods

2.1. Participants

All study procedures were reviewed and approved by Yorkshire and 
The Humber - Bradford Leeds National Health Service Research Ethics 
Committee (REC 16/YH/0224) and were conducted according to the 
principles of the Declaration of Helsinki. All participants gave their 
written informed consent before inclusion in the study. The trial is re-
istered at ClinicalTrials.gov (NCT02661698).

A convenience sample of 21 adults aged 25–44 years, recruited from 
within the staff and student population of Northumbria University, 
completed the study (n = 10 males, 11 females; mean age 31.86 years, 
mean BMI 25.30 kg/m² and self-reporting an average intake of 0.5 
portions of oily fish per week). Participants declared themselves to be 
good health. Participant demographics are displayed in Table 1. Ex-
clusion criteria were: consumption of more than one oily fish meal per 
week; BMI < 18 or > 35 kg/m²; high blood pressure (defined as sys-
tolic > 159 mmHg or diastolic > 99 mmHg); smoking; food allergies or 
insensitivities; pregnancy; breast feeding; currently taking any dietary 
supplements; sleep disturbances and/or taking sleep aid medication; 
chronic health conditions; gastrointestinal problems; active infections 
and any health condition that would prevent the fulfillment of the study 
requirements. The study was conducted from March to October 2016 at 
the Brain, Performance and Nutrition Research Centre (BPNRC), 
Northumbria University, UK.

Table 1

<table>
<thead>
<tr>
<th>Participant demographics. N = 21 including 10 males and 11 females.</th>
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<tbody>
<tr>
<td>Age (years)</td>
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<td>Systolic BP (mmHg)</td>
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<td>Diastolic BP (mmHg)</td>
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<td>Hispanic</td>
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2.2. Study design

Participants attended four identical study visits. During the first of 
these visits, all participants received a placebo treatment (olive oil 
capsules) in a single blind design. This approach fulfilled a dual pur-
pose; the first was to meet the objective of the current paper, namely to 
investigate the diurnal variation in plasma EPA and DHA. The second 
was to serve as a ‘practice’ visit for a randomised controlled trial as-
sessing the enrichment of plasma fatty acids following bedtime con-
sumption of n-3 oils—which comprised the remaining three visits—as it 
was anticipated some participants might not feel comfortable with the 
extended, overnight laboratory protocol. Only data collected at the first 
visit are presented here. Sample size was calculated for the intervention 
study; the effect size from a previous in-house bioavailability study 
(unpublished data) showed that the effect size for 24-h Area Under the 
Curve (AUC) increase in plasma DHA and EPA concentrations following 
DHA-enriched oil was f = 0.49. Assuming similar, large effect size, an 
a priori calculation suggested that in order to achieve 80% power and 
a 95% statistical confidence, the sample size required was 24, inclusive of 
a 20% drop out rate.

The placebo treatment comprised three soft gelatine capsules pro-
viding a total of 3 g refined olive oil. The blinding and labelling of the 
treatment was carried out on site by a third party who had no further 
involve in the study.

2.3. Procedure

Participants arrived at the Brain, Performance and Nutrition 
Research Centre (BPNRC) at Northumbria University at 19:30 following 
a minimum two-hour fast. They were immediately fitted with a cannula 
and a sample was drawn to check the participant was comfortable with 
the procedure and to check for patency; this sample was discarded. At 
20:00, participants were given an evening meal consisting of pasta, 
tomato sauce, grated cheese and olive oil (624 Kcal, 26 g fat). They then 
rested until 22:00 when the treatment (i.e. olive oil) was administered 
with 200 mL of water. Participants were then settled into their room in 
the Northumbria Centre for Sleep Research (adjacent to BPNRC) and 
lights out was at 22:30. Lights on was at 06:00 the following morning 
and a breakfast of cereal and toast was served at 07:00 (504 Kcal, 13 g 
fat). Participants spent the remainder of the visit in a comfortable 
waiting room in BPNRC where they read, watched TV, listened to music 
etc. Lunch, comprising a cheese sandwich, an apple and a sweet biscuit 
(479 Kcal, 17 g fat), was provided at 12:00, and an afternoon snack was 
provided at 16:00 comprising a yoghurt, a banana and a packet of 
savoury cracker snacks (402 Kcal, 15 g fat). The identical evening meal 
to the previous day was given at 20:00 and participants went home by 
public transport or by taxi at 22:15. Hot drinks (e.g. tea, coffee) were 
offered along with the study meals and participants drank water ad 
libitum throughout the visit.

In total, from each participant, thirteen blood samples were drawn 
for analysis via cannula, with the first being drawn immediately prior to 
treatment administration at 22:00. Samples were then collected every 
two hours on the hour for the remainder of the study visit. The last 
sample was drawn at 22:00, twenty-four hours post treatment. Samples 
were always collected prior to the study meals. To keep the vein patent 
throughout the entire study visit, after each draw the vein was flushed 
with 0.5 mL saline solution (PosiFlush, BD, UK).

2.4. Fatty acid analysis

Blood was collected in EDTA vacutainers that were refrigerated at 
4 °C for a maximum of four hours before being processed. Samples were 
then centrifuged for 20 min at 3000 x g at 4 °C. Resulting plasma was 
pipetted off and frozen at −80 °C prior to analysis, which took place at 
the University of Southampton.

Lipid was extracted from plasma using 5 mL of chloroform/
methanol (2:1; v/v) containing 0.2 M butyraldehyde as antioxidant. 1 M sodium chloride (1 mL) was added and the sample vortexed and then centrifuged. The lower solvent phase was aspirated and evaporated to dryness under nitrogen at 40 °C. The lipid extract was redissolved in 0.5 mL toluene and fatty acids were released from esterified lipids and simultaneously derivatized to methyl esters by incubation with 1 mL 2% H2SO4 in methanol for a minimum of 2 h at 50°C to form fatty acid methyl esters. The samples were then neutralized and fatty acid methyl esters transferred into hexane for analysis by gas chromatography. Fatty acid methyl esters were separated on a BPX-70 fused silica capillary column (30 m x 0.2 mm x 0.25 µm, manufactured by SE) in a HP6890 gas chromatograph fitted with a flame ionisation detector. Gas chromatography run conditions were as described elsewhere [17]. Dipentadecanoyl phosphatidylcholine added into the initial plasma sample was used as an internal standard for quantification purposes and a Supelco® 37 Component FAME Mix was used as a calibration reference standard (Sigma-Aldrich). Plasma concentrations of EPA and DHA are expressed as µg/mL.

2.5. Statistical analysis

In order to investigate the diurnal variation in plasma EPA and DHA, data collected over the 24-h period were evaluated using cosinor analysis. In this procedure, a cosine curve is fitted to the data based on the least squares method from which the MESOR (Midline Estimating Statistic of Rhythm; a mean value), amplitude (the difference between the MESOR and peak plasma concentrations) and acrophase (time of the peak plasma fatty acid concentration) parameters are estimated [18,19]. In order to establish the presence of a common rhythm between participants, data points (µg/mL) from each participant were firstly expressed as a percentage of their respective mean value and then pooled for the cosinor analysis [15]. Four data points were missing due to technical issues during blood sampling. These missing data were imputed by averaging the value of the samples drawn immediately prior to and after the missing sample. Linear regression was used to determine the goodness of fit of the estimated cosine curve where the $R^2$ value, referred to here as Percentage Rhythm (PR), relates to the percentage of the variability accounted by the curve and the $p$ value of the F test is the likelihood of the data fitting a straight line as opposed to a cosine curve [20].

A post-hoc exploratory analysis was carried out to assess the difference between males and females at each of the sampling time points. This was conducted using the MIXED procedure in SPSS and included the terms sample and sex. Subject was included as a random effect.

Significance was set at $p < 0.05$. Calculations of the cosine curve parameters were made in Microsoft Excel 2016 and the regression analyses were carried out in SPSS version 24 (IBM, USA).

In an exploratory analysis, the DHA and EPA data were analysed separately for males and females. For both fatty acids, the rhythm was phase advanced in females although this was more evident for DHA. Specifically, the acrophase was estimated to occur at 16:16 in females and 18:50 in males for DHA. For EPA, the estimated acrophase occurred at 19:36 and 20:18 for females and males, respectively. Raw individual data points, plotted as a function of time, are presented together with the 24-h cosine curve for DHA and EPA in males and females separately in Supplementary Material Fig. 2.

3. Results

Of the 25 participants who received treatment, four participants withdrew from the study; in all cases this occurred during the study visit due to feeling uncomfortable with the overnight blood sampling. Mean (± SD) plasma fatty acid concentrations for EPA and DHA collected at the first pre-treatment sample (22:00) were 14.27 ± 9.08 µg/mL and 32.41 ± 14.07 µg/mL, respectively. When expressed as relative percentages of total fatty acids (EPA, 0.87 ± 0.79; DHA, 1.94 ± 0.95), these values are largely in line with what has been observed in other samples from the UK population [21].

Results from the cosinor analysis of the pooled data are presented in Table 2. This table includes the cosinor analysis of all twenty-one fatty acids that were quantified in the plasma samples, although only EPA and DHA were of interest in the current study. A cosine curve, indicating diurnal variation, was fitted to the data with statistical significance for both DHA and EPA. In clock time, the acrophase for DHA and EPA was estimated to occur at 17:43 and 20:41, respectively, with the nadir of each occurring exactly 12 h previously (i.e. at 05:43 and 08:41, respectively). A similar pattern was observed for all analysed fatty acids. Individual data points, plotted as a function of time, are presented together with the 24-h cosine curve for DHA and EPA in Fig 1. Plasma concentrations of DHA and EPA across the 24-h period for each individual are presented in Supplementary Material Table 1.

The results of the linear mixed model analysis showed no main effect of sex or interaction with time with regards the raw plasma concentrations (both $p < 0.05$). Data are displayed in Supplementary Material Table 1.

4. Discussion

The current study aimed to investigate the diurnal variation of plasma concentrations of the n-3 PUFAs EPA and DHA under eating and sleeping conditions generally approximate to what might be considered a ‘normal’ pattern for adults living in the UK. The cosinor analysis revealed a significant diurnal rhythm for both DHA and EPA. Whilst there was evidence of a similar general pattern for both these fatty acids and indeed all the analysed fatty acids—where the lowest concentrations were detected in the morning and the highest in the evening—the phase was advanced by three hours for DHA, compared to EPA.

Apart from the obvious sleep-wake cycle, a number of physiological functions including body temperature, blood pressure and metabolism are known to oscillate daily in mammals [22]; therefore, our observation of the presence of diurnal variation in plasma EPA and DHA is somewhat expected. Biological rhythms are controlled via clock genes organized in a transcriptional feedback loop (e.g. Clock, Bmal1, Per and Cry), with the ‘master clock’ located within the superchiasmatic nucleus of the hypothalamus [23]. Clock-controlled genes have also been found...
in most peripheral tissues, with a large number of physiological functions controlled by endogenous circadian clocks that can oscillate independently from sleep and feeding cues [16]. To date, circadian rhythms have been identified at all stages of lipid metabolism including absorption, mitochondrial transport and partitioning [24]. Diurnal variation is therefore evident in tissues such as plasma, and it would appear that different lipid species display distinct rhythms in humans. For example, in one study wherein circadian variation was measured over a 28-h period, the acrophase of the majority of di- and triglycerides occurred in the morning whereas peaks in phosphatidylcholine (PC) species were observed in the evening [25]. Other trials have shown diurnal variation in plasma free fatty acids, with peak concentrations reported in the afternoon and evening [26,27].

Previous contradictory reports of the timing of the peak concentration of n-3 PUFAs specifically indicate that this occurred in the morning [15] or at lunchtime [16], whereas we observed these to occur in the evening. A key factor underpinning this observed difference could be the more naturalistic pattern of eating and sleeping we aimed to achieve with our protocol. Indeed, Singh et al. [28] reported peak total plasma lipids at around 18:00 in 60 healthy young adults (21–40 years) who were synchronized to follow a diurnal pattern of eating and sleep/activity similar to the current study. Given that the standard meals provided to participants throughout the course of the study visit were free from n-3 PUFAs, the observed diurnal rhythms for DHA and EPA most likely reflect daily variations in the transfer of these fatty acids between different lipid pools e.g. plasma phospholipids, triglycerides, and cholesteryl esters, cellular phospholipids, and adipose and other tissues. In plasma, higher levels of EPA and DHA are esterified to phosphatidylcholine (PC) compared to other fractions after repeated dosing [19]. Given that PC species have been shown to peak in the evening [25,29], this suggests that the diurnal variation we observed was probably for EPA and DHA esterified to phospholipids, and possibly PC in particular. It is a limitation of the current study that the concentration of EPA and DHA was measured in total plasma lipids rather than within specific lipid fractions, which would have enabled us to address this issue directly.

In contrast to previous studies, we did not find any evidence of a difference in absolute concentrations of DHA and EPA across the 24-h

**Fig 1.** Diurnal variation of pooled data (N = 21) in plasma concentrations of DHA (a) and EPA (b) expressed as a percentage of their respective mean 24-h value. A significant rhythm is detected for both DHA and 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 ($R^2 \times 100$).
period between males and females. Previously, analysis of plasma metabolites revealed that concentrations of unsaturated fatty acids were higher in females and also more stable across BMI compared to males [30]. In addition, total plasma lipids were reported to be higher in females than males regardless of age between 06:00 and 18:00 [28]. In the current study, the sex differences in plasma concentrations of DHA and EPA were analysed post hoc, and should therefore be interpreted with caution, especially taking into consideration the small number of participants included in this sub-group analysis. However, it is interesting that we did observe a difference of almost three hours in the acrophase of DHA, with females peaking earlier than males. This pattern was also observed for EPA, but to a much lesser extent (~1-h). Sex differences in the timing of melatonin secretion, body temperature, sleep onset and the expression of clock genes in the brain have been observed, with earlier timing consistently demonstrated in females [31]. While hormonal differences such as the circadian rhythm of testosterone and the monthly fluctuations in estradiol and progesterone have been cited as common reasons underpinning any observed differences between males and females with regards lipid metabolism, it has also been argued that the action of insulin, also known to vary by sex, could be key [32]. Therefore, future studies designed specifically to investigate sex differences in diurnal variation of lipid metabolism should seek to control for monthly hormonal fluctuations, menopausal status and insulin- and glucoregulation.

It has been demonstrated that lipid metabolism in humans is under endogenous circadian clock control [16]. However, although we observed a significant diurnal variation in both EPA and DHA at the group level, our data also revealed a great degree of inter-individual variability as evidenced by the low (<30%) percentage rhythm detected for both fatty acids. This, coupled with the variability in the previously reported rhythmicity of circulating levels of n-3 PUFAs [15,16], would suggest some degree of entrainment with rest/activity and feeding cues. We aimed to mitigate any circadian misalignment due to altered sleep patterns with the exclusion of night shift workers from the study, and all data were collected from April to October during British Summer Time. However, a limitation of the current study is a lack of information regarding participants’ average sleep and eating habits, which could have been used to determine how closely the enforced laboratory schedule actually mimicked their normal day. A seven-day entrainment protocol—wherein participants follow the laboratory schedule at home during the week prior to the study visit—could have also been implemented, which may have helped to reduce inter-individual variability to some degree. However, our study is not the first to report such variability. Indeed, based on evidence showing poor agreement in both the presence and phase of rhythmicity between participants across all lipid species [25], it has been suggested that there may be distinct circadian metabolic phenotypes, which will require further investigation [24]. It must also be noted that although the amplitude of the phase for plasma concentrations of EPA was significant, the percentage rhythm value observed for the variation of EPA was much lower than DHA, and especially so when considering females separately. This finding echoes what has been observed previously [15], and suggests that plasma EPA does not follow as predictable a daily variation as DHA. One reason for this may be differences in fate and function between the two fatty acids with EPA—as an eicosanoid precursor—being released from adipocytes and potentially other tissues in response to spontaneous endogenous changes such as inflammation, for example [33,34].

The observed overnight decrease in DHA, and to a lesser extent EPA, is interesting from a functional perspective. If regarded as a lipid transport pool [19], the nocturnal decline in plasma concentrations of these n-3 PUFAs suggests tissue uptake of these fatty acids during sleep. The aim of the current study was to investigate the diurnal variation in circulating plasma EPA and DHA, in order to inform optimal timing of intake of these n-3 PUFAs in the context of maximising their tissue delivery. The timing of delivery of DHA in particular may be important for brain function, since the brain is especially enriched in this n-3 PUFA [35] that has key structural and functional roles. For example, Umbau et al. [36] suggest that alterations in the specific composition of newly formed neurons or synaptic membranes may underpin changes in neuropsychiatric function following supplementation protocols of just a few weeks that are unlikely to impact the overall concentration of brain DHA. Adult hippocampal neurogenesis is known to occur during sleep [37], and may play an important role in supporting learning and cognitive flexibility [38,39] and memory consolidation [40]. Therefore, we suggest that intake of n-3 PUFAs later in the evening—which would ensure an overnight increase in plasma concentrations of EPA and DHA at a time when background levels are at their daily lowest—presents as a novel strategy to optimise the efficacy of n-3 PUFA supplementation with regards cognitive function. Circadian studies in mice suggest that lipid absorption is lowest at the end of the active/awake phase [41,42], which would need to be taken into account if this dosing protocol was adopted. Of course, testing this hypothesis by observing effects following morning and evening or indeed afternoon dosing protocols would be required. What the data presented herein suggest more concretely, is that the time at which DHA and EPA plasma samples (and other fatty acids more broadly) are collected is an important consideration. Therefore it is recommended that within the same study, samples should be collected at the same time; any conclusions drawn from comparisons to data presented by other studies should be mindful of differences in sampling time points as well.

In conclusion, the current study revealed the presence of diurnal variation in plasma concentrations of EPA and DHA in a sample of healthy young adults with low habitual oily fish consumption. We observed that peak concentrations occur towards the end of the day and fall overnight, with the nadir of each fatty shown to occur in the morning. We suggest that an evening dosing protocol may have implications for efficacy studies with regards cognitive function, which we aim to address in the future. Lastly, given their daily variation, caution should be exercised when comparing absolute concentrations of plasma fatty acid concentrations across studies.

CRediT authorship contribution statement

Philippa A. Jackson: Conceptualization, Methodology, Formal analysis, Writing - original draft, Visualization, Project administration. Cathrine Husberg: Conceptualization, Methodology, Writing - review & editing, Project administration. Svein-Ølav Hustvedt: Conceptualization, Methodology, Supervision, Writing - review & editing. Philip C. Calder: Conceptualization, Methodology, Resources, Writing - review & editing. Julie Khan: Investigation, Methodology, Writing - review & editing. Hannah Avery: Investigation, Methodology, Writing - review & editing. Joanne Forster: Investigation, Methodology, Writing - review & editing. David O. Kennedy: Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

C.H and S.O.H are employees of BASF AS. P.C.C. is an advisor to and has received funding from BASF AS.

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Supplementary materials

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References


