1. Title Page

*Turnover of brain DHA in mice is accurately determined by tracer-free natural abundance carbon isotope ratio analysis*

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Running Title: Brain docosahexaenoic acid turnover measured by carbon isotope ratio analysis

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; CSIA, compound specific isotope analysis; DHA, docosahexaenoic acid; EA, elemental analyzer; FAMEs, fatty acid methyl esters; GCC-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry; ICV, intracerebroventricular; VPDB, Vienna Peede Belemnite
2. Abstract

The brain is highly enriched in the long-chain omega-3 (n-3) PUFA DHA. Due to the limited capacity for local DHA synthesis in the brain, it relies on a continual supply from the circulation to replenish metabolized DHA. Previous studies investigating brain DHA turnover and metabolism have relied on isotope-tracers to determine brain fatty acid kinetics, however this approach is cumbersome and costly. We applied natural abundance carbon isotope ratio analysis via high-precision gas chromatography combustion isotope ratio mass spectrometry, without the use of labeled tracers, to determine the half-life of brain DHA in mice following a dietary switch experiment. Mice fed diets containing either α-linolenic acid (ALA) or DHA as the sole dietary n-3 PUFA were switched onto ALA, DHA, or ALA+DHA containing diets at six weeks of age, while control mice were maintained on their respective background diet. We measured brain DHA carbon isotope ratios (reported as $\delta^{13}C_{DHA}$ signature) over a 168-day time course. Brain $\delta^{13}C_{DHA}$ signatures of control mice maintained on background diets over the time course were stable ($p > 0.05$). Brain $\delta^{13}C_{DHA}$ signatures of mice switched to the DHA or ALA+DHA diet from the ALA diet, changed over time, yielding brain incorporation half-lives of 40 and 34 days, respectively. These half-lives determined by natural abundance carbon isotope ratio analysis were consistent with estimates from kinetic isotope tracer studies. Our results demonstrate the feasibility of natural abundance carbon isotope ratio analysis to study fatty acid metabolism without the use of isotopically labelled fatty acid tracers.

Keywords: Brain lipids, Omega-3 fatty acids, fatty acid/metabolism, mass spectrometry, diet and dietary lipids, carbon isotope ratio, natural abundance, docosahexaenoic acid, isotope ratio mass spectrometry, brain fatty acids
3. Introduction

The brain fatty acid composition is unique in comparison with most other tissues in that the brain is highly enriched in the long-chain omega-3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA, 22:6 n-3) (1, 2). Mounting evidence indicates a role of DHA and its bioactive products in the support and regulation of optimal neuronal and synaptic function (reviewed in ref (3)). Furthermore, consistent with the hypothesis that brain DHA homeostasis is critical to support optimal brain function, post-mortem analyses suggest that brain DHA levels may be altered, either as cause or consequence, in many neurological and neuropsychiatric disorders (1). Although it appears to be vital to support brain function, DHA is not efficiently synthesized locally within the brain, and therefore DHA must be incorporated preformed from circulating plasma pools to replenish that which has been metabolized (4, 5).

Rigorous kinetic tracer studies have been conducted to better understand the incorporation and metabolism of DHA in the brain, as well as the factors that may influence its metabolism. Modeling the loss of radioactivity following intracerebroventricular (ICV) injections of radiolabelled DHA, the brain DHA half-life in rats has been determined to range between 30 and 65 days, and is extended to 90 days during n-3 PUFA dietary deprivation (6–8). Acute intravenous infusions of radiolabelled DHA have been used to investigate the effects of mood stabilizing agents on brain DHA uptake and metabolism, including turnover, (9–12) as well as to elucidate the preferred plasma lipid pool supplying the brain (8, 13–15). In addition to the use of radioactive isotopes, stably-labelled DHA tracers have also been applied to probe metabolic questions regarding brain DHA and its origin (16–18). Although these techniques allow for the modeling of brain DHA kinetics, they are often constrained to acute study designs.
and single time point analysis due to the significant expense associated with the custom synthesis of high purity tracer compounds.

Natural abundance isotopic analysis may present an alternative to the use of synthetic isotopic tracers when investigating fatty acid incorporation and metabolism. Since the carbon isotopic makeup of a molecule is conserved following incorporation from the diet, highly precise natural abundance carbon isotope ratio measurements ($^{13}$C/$^{12}$C, expressed as δ$^{13}$C in units mUr) by gas chromatography isotope ratio mass spectrometry can provide insight into the origin of a compound of interest. Importantly, innate differences in the carbon fixation pathways of C3 and C4 photosynthesis result in characteristic average δ$^{13}$C values of -27 mUr and -13 mUr for C3 and C4 plants, respectively (19, 20). Through the use of well controlled diet switch experiments (Figure 1), ecologist have used the natural variation in carbon isotope ratios between C3 and C4 plants to measure tissue turnover rates to better construct models of resource utilization and food web analysis (21, 22). Similarly, previous dietary intervention studies in infants have used variations in natural abundance carbon isotope ratios to investigate the contribution of endogenous synthesis from 18-carbon fatty acids to the long chain PUFA arachidonic acid (ARA, 20:4n-6) and DHA in infants (23, 24).

Recently, our lab has published on the use of natural abundance carbon isotope ratios measured by compound specific isotope analysis (CSIA) to determine the contribution of retroconversion to plasma DHA levels in rats (25) and in humans (26) and identify the dietary origin of brain DHA (27, 28). By using the naturally occurring variation in carbon isotope ratios between terrestrial plant sourced and marine sourced n-3 PUFA, we previously demonstrated brain DHA, established by feeding either an ALA containing or DHA containing diet, differed by ≈ 6 mUr (27). In the present study, we apply a dietary switch experiment where dietary n-3
PUFA sources and their respective carbon isotope ratios were altered and corresponding changes in brain, liver and adipose DHA carbon isotope ratios (referred to as $\delta^{13}C_{\text{DHA}}$) were measured over a 168-day time course. The resulting changes in $\delta^{13}C_{\text{DHA}}$ signatures were modelled by one-phase decay function to determine experimental incorporation half-lives. Our results indicate that brain incorporation half-lives generated from the diet switch experiment were consistent with those previously determined from radiolabelled kinetic modelling. Additionally, we find that preformed DHA is the preferred source of DHA for the brain, when ALA is provided at equals levels in the diet. These results highlight the utility of natural abundance compound specific isotope analysis to study fatty acid metabolism kinetics in a chronic feeding model without the administration of labelled tracer compounds.
4. Materials and Methods

Materials

All solvents used were American Chemical Society or HPLC grade and were purchased from either Sigma-Aldrich (Mississauga, ON, Canada) or Fischer Scientific (Ottawa, ON, Canada). Fatty acid standards and reference fatty acid methyl ester (FAME) mixtures were purchased from Nu Chek Prep Inc. (Elysian, MN). Isotope reference materials USGS70, USGS71, and USGS72, were obtained from the Reston Stable Isotope Laboratory—United States Geological Survey (Reston, VA).

Animals

Experimental animal protocols were approved by the Animal Ethics Committee of the University of Toronto and conducted in accordance with the policy and guidelines of the Canadian Council on Animal Care and the Regulations of Animals for Research Act in Ontario (protocol # 20011661). All animals were housed held in a temperature-controlled environment under a 14 h / 10 h light/dark cycle, with ad libitum access to food and water.

Two colonies of BALB/c mice were maintained on a diet containing either ALA or DHA as the only n-3 PUFA for two generations. Previously, our laboratory demonstrated that brain $\delta^{13}$C$_\text{DHA}$ signatures are equilibrated with the $\delta^{13}$C signature of dietary n-3 PUFA following two generations of breeding (27). At six weeks of age equilibrated male mice were used for a dietary switch experiment (Figure 2). Mice on the ALA diet were randomly switched onto either the DHA diet or ALA+DHA diet. Conversely, those on the DHA diet were randomly placed onto either the ALA diet or ALA+DHA diet. In addition to the mice undergoing the diet switch, a small subset of mice were maintained on their respective background diet, either the ALA diet or DHA diet, to evaluate the stability of tissue $\delta^{13}$C$_\text{DHA}$ signatures over the course of the experiment. Mice were sacrificed over a time-course of 0, 7, 14, 28, 56, 112, and 168 days.
All mice were sacrificed by cardiac puncture under terminal anesthesia with 0.25 mg/g of body weight of 2,2,2-tribromoethanol (Sigma-Aldrich, Mississauga, ON, Canada). Tissues – brain (left hemisphere), liver (left lateral lobe), and adipose (epididymal) - were collected, flash-frozen in liquid nitrogen, and stored at -80°C until further processing.

Diets

For detailed information on the composition of experimental diet please refer to Lacombe et al. 2017 (29). Briefly, study diets were based on a modified low AIN-93 custom low n-3 PUFA rodent diet (Dyets, Inc., Bethlehem, PA). Background oils were derived from safflower and fully-hydrogenated coconut oils and added fatty acid ethyl esters (32.8, 63.2, and 4.0 % by weight, respectively). For the ALA, DHA, and ALA+DHA diets added oils were 2 % ALA ethyl ester (gift from BASF Pharma, Callanish Ltd., Isle of Lewis UK) and 2 % DHA ethyl ester (gift from BASF Pharm, Callanish Ltd.). Oleate ethyl ester (Nu-Chek Prep, Inc., Elysian, MN, USA) was added to both ALA and DHA diets at 2 % of the fatty acids by weight to maintain equivalent fatty acid content. All added fatty acid ethyl esters were determined to be > 98% pure by gas chromatography – flame ionization detection (GC-FID). The carbon isotope ratios of ALA and DHA from the study diets were determined to be -28.2 ± 0.3 mUr and -21.8 ± 0.2 mUr, respectively (29).

Lipid extraction and transmethylation

Total lipid extracts were isolated from harvested tissues by methodologies adapted from Folch, Lees, and Sloane Stanley (30). Brain and adipose tissue (≈ 200 mg and 50 mg, respectively) were homogenized in 6 mL of a chloroform : methanol solution (2:1; v/v) containing known quantities of docosatrienoic ethyl ester (22:3n-3; Nu-Chek Prep, Inc.) and
either heptadecanoic acid (17:0; Nu-Chek Prep, Inc.) or triheptadecanoin (Nu-Chek Prep, Inc.) for fatty acid quantification. Liver tissue was cryopulverized by mortar and pestle in the presence of liquid nitrogen prior to lipid extraction. For lipid extraction, ≈ 50 mg of pulverized liver was vortexed in 6 mL of a 2:1 chloroform : methanol solution (v/v) containing docosatrienoic acid ethyl ester. All brain, adipose, and liver homogenates were held at 4 °C over night, following which 1.75 mL of 0.88% aqueous KCl was added. Samples were then vortexed vigorously and phase separation was achieved through centrifugation at 500 g for 10 mins. The lower lipid containing chloroform phase was collected into clean glass test tubes and stored at -80 °C until methylation. Transesterification of extracted lipids by 14 % boron trifluoride was carried out following previously published methods (27). The transesterification of lipids in the presence of excess alcohol proceeds quantitatively, therefore no isotopic fractionation effect is expected to be imparted by this process (31).

**FAME quantification**

Fatty acid methyl esters were quantified by GC-FID on a Varian 430 gas chromatograph (Bruker, Billerica, MA) equipped with a SP-2560 capillary column (100 m × 0.25 mm i.d. × 0.20 μm d.f.; Supelco by Sigma-Aldrich, Bellefonte, PA, USA). For GC parameters see Lacombe et al 2017 (27). Samples were concentrated to ≈ 1 mg of total FAME per mL of hexane before GC-FID analysis. Fatty acids methyl esters were identified based on comparison of retention time to GLC-569 external reference standard (Nu-Chek Prep, Inc.) and quantified by comparison of peak area to that of docosatrienoic ethyl ester internal standard.

**Adipose FAME fractionation by silver ion chromatography**
Due to the low relative abundance of DHA in adipose fatty acids, DHA and other PUFA were fractionated and concentrated prior to carbon isotope ratio analysis. Silver ion chromatography allows for the separation of FAME by number, configuration and position of double bonds thereby permitting the isolation of low abundant PUFA species from adipose total lipids (32). Separation was carried out using Discovery® Ag-Ion solid phase extraction cartridges (750 mg bed wt., 6 mL volume; Supelco by Sigma-Aldrich) affixed in an Agilent Vac Elut SPS 24 Manifold (Agilent Technologies, Santa Clara, CA, USA). The solvent system for elution of FAME was adapted from the methods of Kramer et al (33). Prior to sample loading, cartridges were washed with 4 mL acetonitrile followed by 4 mL of hexane allowing solvent to flow under gravity. Approximately 1 mg of FAME from adipose TLE was dissolved in 1 mL of hexane and loaded onto the cartridges and run through. Cartridges were then washed sequentially with 6 ml each of hexane : acetone (99:1; v/v), hexane : acetone (90:10, v/v), and acetone (100%) allowing each solvent to flow under gravity with 3 seconds of vacuum applied between each solvent step. Fatty acid methyl esters containing 3 or more double bonds were eluted with 6 mL of acetone : acetonitrile (60:40, v/v). Samples were then evaporated under a gentle stream of nitrogen and reconstituted in 100 µL of heptane for isotopic analysis. A representative chromatogram of adipose sample before and after silver ion fractionation can be found in Supplemental Figure 1.

Carbon isotope ratio analysis

Compound specific isotope analysis of FAME by gas chromatography combustion isotope ratio mass spectrometry (GCC-IRMS) was conducted similarly to methods previously published by our laboratory and described briefly below (27). One µL of FAME were injected onto a 100 m SP-2560 capillary column (Supelco) in a Trace 1310 GC (Thermo Scientific, Bremen, Germany) using a TriPlus RSH autosampler (Thermo Scientific). Analyte peaks were
separated using the same thermal and pneumatic program described in Lacombe et al 2017, yielding base line resolution of analyte peaks of interest. Gas chromatography effluent was swept by He carrier gas to a GC Iso Link II combustion interface (Thermo Scientific) held at 1000 °C featuring Ni and Cu catalysts and interfaced to a Delta V Plus IRMS (Thermo Scientific) via a ConFlo IV (Thermo Scientific) continuous flow interface. Carbon dioxide gas produced through the quantitative combustion of isolated analytes was dried via flowing gas through a Nafion® dryer prior to entering the IMRS ion source (Dupont, Wilmington, DE).

Elemental analysis (EA) was carried out on heptadecanoic acid and methyl heptadecanoate which was derivatized with each batch of samples. This was carried out to determine the carbon isotopic ratio of methyl groups esterified to generate FAME from TLE. Aliquots (≈ 150 µg of total material) were transferred to clean smooth thin walled tin capsules (Elemental Microanalysis, Okehampton, UK) and solvent was evaporated overnight at room temperature. A Zero Blank autosampler (Costech Analytical Technologies Inc., Valencia, CA, USA) was used to introduce capsules into the Flash 2000 Elemental Analyzer (Thermo Scientific). Temperatures were held at 1020 °C and 650 °C for the oxidation and reduction reactors, respectively. Similar to the GCC-IRMS set up, CO₂ gas was introduced to the Delta V Plus IRMS via a ConFlo IV continuous flow interface (Thermo Scientific). Isodat Workplace 3.0 (Thermo Scientific) was used to process all isotopic data.

\textit{Carbon isotope ratio normalization and methyl corrections}

All carbon isotope ratios were normalized using consensus validated 20-carbon FAME reference material USGS70, USGS71, and USGS72 (Reston Stable Isotope Laboratory United States Geological Survey, Reston, VA, USA) using multi-point linear normalization similar to methods previously described (27, 34). Consensus derived carbon isotope ratio values of USGS
reference materials are -30.53 ± 0.04 ‰, -10.50 ± 0.03 ‰, and -1.54 ± 0.03 ‰ for USGS70, USGS71, and USGS72, respectively, and are expressed relative to Vienna Peedee Belemnite on a scale normalized to primary reference materials NBS 19 and LSVEC (35). Reference materials were injected twice throughout each batch of samples analyzed by GCC-IRMS and EA-IRMS under identical analytical conditions to the samples being analyzed. Normalizing equations were generated for each batch of samples from linear regression lines fit through the measured and accepted δ^{13}C values of reference materials. R^2-values for all equations were ≥ 0.9998.

Isotope ratio mass spectrometry measures carbon isotope ratios from CO_2 gas that is generated through the quantitative combustion of sample material or specific organic analytes and therefore does not distinguish from the derivatized carbon units added to fatty acids during transmethylation. To account for the contribution of derivatized carbon units to measured carbon isotope ratios and allow the reporting of true values, methyl corrections were carried using mass balance equations:

\[ n_{FAME} \delta^{13}C_{FAME} = \delta^{13}C_{ME} + n_{FA} \delta^{13}C_{FA} \]  

(equation 1)

where \( n \) refers to the number of moles of carbon in the corresponding FAME and fatty acid and subscripts, \( FAME, ME, \) and \( FA \) refer to the measured carbon isotope ratios of FAME, methyl group and fatty acid respectively. For methyl corrections of measured DHA carbon isotope ratios \( n_{FAME} \) and \( n_{FA} \) equal 23 and 22, respectively. Carbon isotope ratios of the methyl group was determined for each batch of samples by EA analysis of methylated and free heptadecanoic acid standards (Nu-Chek Prep Inc.) as previously described (27). Methyl group carbon isotope signatures ranged from -40.5 mUr to -39.1 mUr.

**Statistics and Calculations**
All data reported herein are expressed as means ± SD, unless otherwise stated. Sample size for the time course ranged from 3 to 5 mice per time point. Brain, liver, and adipose fatty acid concentrations of mice post dietary switch were compared using two-way ANOVA for the interaction of time and dietary group. As the *a priori* design of this experiment was to assess changes in brain carbon isotope ratios over a 168-day time course for the determination of DHA half-lives, multiple comparisons analysis was not carried out as sample sizes for individual time points were underpowered.

To test for the stability of tissue δ13C_{DHA} signatures over time, carbon isotope ratio values of tissue from the dietary control arms were plotted and slopes of linear regressions were tested for deviation from zero. Statistical significance was determined at *p* < 0.05. Tissue δ13C_{DHA} signatures from mice following the dietary switch were plotted over time and modelled with a one-phase decay function to generate a curve of best-fit:

\[ y = (y_0 - \text{plateau})^{-kx} + \text{plateau} \] (equation 2)

where \( y_0 \) describes the best-fit curve at time zero, \( \text{plateau} \) describes the value of the best-fit curve at an infinite time, and \( k \) defines the decay constant. An exception was made for DHA background mice that were switched onto the ALA+DHA combined diet due to the relatively unchanged δ13C_{DHA} signatures over the time course. Instead, carbon isotope ratios generated from this arm of the diet switch were modeled by linear regression, and slopes were tested for deviation from zero. Incorporation half-lives for tissue δ13C_{DHA} signatures were determined from the best fit curves using the following formula:

\[ t_{1/2} = \frac{\ln(2)}{k} \] (equation 3)
where $k$ is the decay constant from the best fit curve modelled by a one-phase decay function.

When applicable, the rate of loss of DHA from the brain DHA pool, $J_{\text{out}}$ (µmol/g of brain/day), was calculated using the following formula (6, 7, 36):

$$J_{\text{out}} = \frac{0.693 C_{FA}}{t_{1/2}} \text{ (equation 4)}$$

where $C_{FA}$ is the baseline DHA concentration from the given tissue lipid pool, and $t_{1/2}$ is the corresponding experimentally derived half-life. The $J_{\text{out}}$ may be used as an estimate of the net incorporation rate of DHA given that the net rate of loss and the net incorporation rate are assumed to be equal when the brain DHA pool is at steady-state.
5. Results

*Brain DHA concentrations are less influenced by diet than liver and adipose*

Brain, liver and adipose DHA concentrations of ALA and DHA diet control arms are presented in Supplemental Figure 2. With respect to brain DHA concentrations, in the absence of an interaction there were significant main effects observed for diet ($P < 0.0001$) and time ($P = 0.006$) indicating slightly greater (6 to 8%) brain DHA concentrations in mice maintained on the DHA diet compared to those on the ALA diet. Liver DHA concentrations were approximately two-fold higher in mice on the DHA diet compared to mice on the ALA diet across all time points (main effect of diet; $p < 0.0001$). A significant time × diet interaction ($p = 0.0002$) was observed when comparing adipose DHA concentrations between control mice maintained on either the ALA diet or DHA diet. DHA concentrations in adipose total lipids ranged from 3- to 5-fold higher in mice on the DHA diet compared to those on the ALA diet.

Data on DHA concentrations following the dietary switch are presented in Figure 3 and Figure 4 for mice equilibrated to the ALA diet and DHA diet, respectively. With respect to the brain, following the diet switch there was a significant time x diet interaction effect observed in both ALA and DHA background groups ($p = 0.04$ and $p = 0.02$, respectively). Brain DHA concentrations of mice from the ALA background diet switched onto the DHA and ALA+DHA diets increased over time, albeit modestly (mean difference over baseline at 168-days post diet switch: +1.4 µmol and +1.3 µmol, respectively; Figure 3A). In mice equilibrated to the DHA background diet brain DHA concentrations remained relatively stable over the time course in both ALA diet and ALA+DHA diet arms.
Liver and adipose DHA pools responded rapidly to changes in n-3 PUFA intakes. In mice equilibrated to the ALA diet there was a significant main effect of time in both the liver and adipose DHA pools ($p < 0.003$ and $p < 0.0001$, respectively) indicating a trend towards increasing DHA concentrations when switched onto the DHA and ALA+DHA diets (Figure 3B and Figure 3C). In the liver total lipid pool, DHA concentrations doubled over baseline levels after one week of feeding both the DHA and ALA+DHA diets. Adipose DHA concentrations reached a maximal 4-fold increase over baseline concentrations at 56 days and remained $\approx 2.5$-fold higher at 168 days in mice on the DHA and ALA+DHA diets.

Mice equilibrated to the DHA diet experienced a large drop in DHA concentration in both liver and adipose total lipid pools when switched to the ALA diet which was not observed in the ALA+DHA diet arm. In the liver, DHA concentrations decreased by $\approx 50\%$ after 7-days of being on the ALA diet and maintained $\approx 50\%$ lower than mice on the ALA+DHA diet across all time points (Figure 4B). In the adipose, DHA concentrations were 50% lower than baseline levels after 7-days on the ALA diet and plateaued out at $\approx 2\, \mu\text{mol/g}$ at 28-day, $\approx 80\%$ lower than baseline concentrations (Figure 4C). Adipose DHA concentrations remained relatively stable in mice on the ALA+DHA diet over the 168-day time course.

*Tissue $\delta^{13}C$ signatures model the replacement of DHA in tissue total fatty acid pool*

In control mice equilibrated and maintained on their respective ALA and DHA diets over the course of the study brain, and adipose $\delta^{13}C_{\text{DHA}}$ signatures remained stable. Brain $\delta^{13}C_{\text{DHA}}$ signatures over the time course averaged $-26.9 \pm 0.2$ mUr and $-21.8 \pm 0.7$ mUr for ALA and DHA control groups respectively. Adipose $\delta^{13}C_{\text{DHA}}$ signatures averaged $-29.6 \pm 1.1$ mUr and $-23.5 \pm 0.4$ mUr over the time course for ALA and DHA control mice, respectively. Slopes of linear regression lines of $\delta^{13}C_{\text{DHA}}$ signatures in brain and adipose total lipids did not significantly
differ from zero in control mice maintained on either the ALA or DHA diet (Figure 5 and Figure 6). Liver $\delta^{13}$C$_{DHA}$ signatures were stable in control mice maintained on the DHA diet ($p > 0.05$, -23.7 ± 0.5 mUr), however $\delta^{13}$C$_{DHA}$ signatures from ALA became more enriched over the course of the study based on linear regression analysis ($p = 0.004$).

To model DHA turnover, brain, liver and adipose $\delta^{13}$C$_{DHA}$ signatures measured over the 168-day time course were fit with a one-phase decay function (Figure 5 and Figure 6 for ALA and DHA equilibrated mice, respectively). $\delta^{13}$C$_{DHA}$ signatures in brain, liver, and adipose of mice equilibrated on the DHA diet and switched to the ALA+DHA diet did not change over the time-course, therefore carbon isotope ratios from this group were fit with linear regression. Slopes of linear regression lines were determined to not significantly deviate from zero ($p > 0.05$; Figure 6), therefore $\delta^{13}$C$_{DHA}$ signatures were determined to be stable.

Incorporation half-lives were determined for each tissue and diet arm using equation 3 (see methods) from experimentally generated curves and are reported, along with parameters of best-fit curves, for mice equilibrated on the ALA and DHA diet in Table 1. Half-lives and curve parameters were non-determinable for DHA diet equilibrated mice on the ALA+DHA diet as $\delta^{13}$C$_{DHA}$ signatures from this group were not adequately modelled by a one-phase decay function. Liver DHA incorporation half-lives were determined to be between 1 to 2-fold shorter than adipose and between 12 and 15-fold shorter than brain DHA half-lives. Interestingly, brain, liver, and adipose DHA half-lives were longest in mice equilibrated on the DHA diet and switched to the ALA diet. Notably, in ALA equilibrated mice, DHA incorporation half-lives did not appreciably differ between mice on either the DHA or ALA+DHA diet.

Under the assumption that the DHA pool size is stable, estimates of the daily rate of loss ($J_{out}$) can be determined using equation 4 based on the notion that the rate of incorporation of
DHA into a tissue is approximately the rate out (see methods). Given the large and rapid changes in DHA concentrations observed in liver and adipose total lipids resulting from the dietary switch, $J_{out}$ were only calculated for brain total lipids (Table 1). The rate of loss of brain DHA in ALA diet equilibrated mice on the DHA or ALA+DHA diets was found to be ≈ 3-fold faster than DHA diet equilibrated mice on the ALA diet.
6. Discussion

By using a diet switch study, we demonstrated that changes in tissue δ\(^{13}\)C signatures in response to the altered dietary δ\(^{13}\)C signature can be used to adequately estimate brain DHA half-lives. For decades, carbon isotope ratio analysis and diet switch studies have been applied to estimate tissue bulk carbon turnover by ecologists (21, 37, 38), however to the best of our knowledge this is the first time a diet switch study was used in conjunction with natural abundance CSIA for fatty acid specific turnover. By equilibrating the brain δ\(^{13}\)C\textsubscript{DHA} signature with that of the dietary n-3 PUFA (either ALA or DHA) and switching mice to a diet providing n-3 PUFA with a different carbon isotopic signature, changes in brain δ\(^{13}\)C\textsubscript{DHA} signatures were modelled as a function of time allowing for the determination of brain DHA half-lives and turnover.

In mice equilibrated to the ALA diet and switched to the DHA diet or ALA+DHA diet, we determined brain DHA half-lives to be \(\approx 30\) to 40 days (Table 1). These values are consistent with those previously published using ICV administered radio tracers measuring the loss of brain radioactivity over time, which estimate half-lives of between 30 and 65 days in rats (6–8). Previous studies investigating the effects of dietary PUFA on brain DHA turnover used diets with low or non-detectable levels of DHA (6, 7). In the present study diets contained DHA at 2\% of the total dietary fatty acids, equivalent to 0.2\% of the mass consumed. Interestingly, brain DHA turnover values from mice on the ALA background switched to DHA or ALA+DHA diet were similar with the values of rodents not consuming appreciable levels of long chain n-3 PUAFs. This suggests that the addition of DHA to a diet with adequate levels of ALA may not affect brain DHA turnover.
Although, both current and previous methods of determining DHA half-lives yield similar estimates, they operate on different underlying assumptions. The radioactive loss method estimates half-lives based on the metabolic loss of the radiotracer from the brain. In contrast, the current method estimates half-lives based on the incorporation of preformed dietary DHA or newly synthesized DHA into the brain lipid pool, replacing that which has been metabolized. By making the assumption that the brain DHA pool is at steady-state then the net rate of DHA metabolic loss must be equal to the net rate of DHA incorporation (39). Although brain DHA concentrations slightly increased over the time-course in mice from the ALA equilibrated group when switched to either of the DHA containing diets, we anticipate that any influence from the small change in pool size (≈ 6 – 8 %) on the determination of brain DHA half-lives would be minor. Furthermore, calculated $J_{out}$ values in the present study (0.11 – 0.38 µmol / g of brain / day) are broadly similar to the $J_{in}$ of plasma unesterified DHA determined from steady state pulse-labeling studies (0.04 – 1.2 µmol / g of brain / day) (8–12, 40). Collectively the agreement between half-lives and $J_{out}$ values determined by natural abundance carbon isotope ratio analysis with those previously determined increases the confidence of our estimates and demonstrates that this method can adequately model fatty acid turnover. In comparison with mice equilibrated on the ALA diet, the brain DHA half-life determined from the DHA equilibrated mice switched onto the ALA diet was nearly 3-times longer than for either ALA equilibrated diet arm. Peripheral stores of DHA have been shown to be a significant contributor to brain DHA accretion when feeding diets devoid of preformed DHA during brain development (16). Therefore, it is possible that adipose stores could also be a major contributor of DHA for the adult brain in the absence of preformed DHA in the diet (5). The depletion of DHA from liver and adipose in mice equilibrated to the DHA diet and switched to the ALA diet may have
contributed to the observed longer brain DHA half-life in this group. DHA released from these tissues, with an enriched $\delta^{13}$C$_{\text{DHA}}$ signature resembling the dietary DHA, would contribute to maintaining a more isotopically enriched circulating DHA pool available for uptake into the brain thereby decreasing the measured rate of change in brain $\delta^{13}$C$_{\text{DHA}}$ signature effectively lengthening the estimated half-life.

The primary focus of the current study was to evaluate the ability of natural abundance CSIA to estimate brain DHA half-lives using a dietary switch study. As a secondary analysis we also examined and modelled the changes in liver and adipose $\delta^{13}$C$_{\text{DHA}}$ signatures following each dietary switch and estimated DHA half-lives in these tissues. Using this method, DHA half-lives were found to be between 2.5 to 17 days in the liver and 5.4 to 30.1 days in the adipose depending on the background equilibrated diet and dietary switch groups. These results were broadly similar to those reported in mice estimated from the depletion of deuterium in labeled liver and adipose lipid pools (41). However, estimates from concentration depletion and accretion studies in pigs and humans suggest adipose half-lives to be much longer (300 – 700 days) (42–44). Discrepancies in estimated half-lives may be attributed to species differences as well as considerable differences in methodological approach. Additionally, it’s important to note that the large accretion of DHA in liver and adipose pools in the ALA equilibrated mice over the course of the study may have contributed to an underestimation of true half-lives of DHA in these tissues.

While few studies have investigated the primary source dietary n-3 PUFA for brain DHA incorporation, there is evidence from well designed isotopic tracer studies to suggest preformed dietary DHA is the major source of DHA for the brain when provided in combination with shorter-chain n-3 PUFA precursors. Previously, in hand-reared rat pups fed a diet providing
isotopically labelled ALA and unlabelled DHA, Lefkowitz et al. showed that \( \approx 90\% \) of brain DHA accumulated over 20-days was from preformed DHA, either from the diet or that which had accumulated in peripheral stores from hepatic synthesis prior to feeding labelled ALA (16). Furthermore, preformed DHA was found to be 7-fold more effective than ALA for brain DHA accretion in newly born baboons (17). In the present study, brain \( \delta^{13}C_{\text{DHA}} \) signatures in DHA equilibrated mice switched to the ALA+DHA diet were not altered with the addition of ALA to the diet. Furthermore, curves of brain \( \delta^{13}C_{\text{DHA}} \) signatures from mice equilibrated to the ALA diet and switched to the DHA diet or the ALA+DHA diet were nearly indistinguishable, and shared similar DHA incorporation half-lives. Therefore, the contribution of DHA synthesized from dietary ALA and incorporated into the brain over the course of the study is minor (\( \approx 4\% \), calculation below) in comparison to the contribution of preformed DHA, similar to the results of Lefkowitz et al. (16).

Because the carbon isotope ratio of fatty acids is more or less conserved following absorption and uptake into tissues, we can use pool mixing models to estimate the contribution of synthesized DHA from ALA in collected tissues. \( \delta^{13}C_{\text{DHA}} \) signatures of brain, liver and adipose \( \delta^{13}C_{\text{DHA}} \) signatures of mice equilibrated to the ALA diet switched to the ALA+DHA diet were 0.8 mUr, 0.6 mUr and 1.1 mUr more deplete than mice switched to the DHA diet when \( \delta^{13}C_{\text{DHA}} \) signatures plateaued. The plateaued values generated from best-fit curves can be considered to represent a steady-state equilibrium between diet and tissue. Based on these values, the contribution of DHA synthesized from ALA to the liver and adipose total DHA pool of mice on the ALA+DHA diet can be estimated using the following mass balance equation (45):

\[
\delta^{13}C_{\text{tissue}} = x \delta^{13}C_{\text{ALA control}} + (1 - x) \delta^{13}C_{\text{DHA control}} \quad (\text{equation 5})
\]
where $\delta^{13}C_{tissue}$ refers to the measured $\delta^{13}C_{DHA}$ signature from the tissue, $x$ refers to the proportional contribution of synthesized DHA to the tissue DHA pool, and $\delta^{13}C_{ALA\_control}$ and $\delta^{13}C_{DHA\_control}$ refer to the $\delta^{13}C_{DHA}$ signatures measured in the final time point of the ALA diet and DHA diet control arms. The purpose of using values measured from the control groups as opposed to the carbon isotope ratios of dietary ALA and DHA is to account for any kinetic isotope effect during the synthesis and assimilation of DHA with the tissue fatty acid pool. By applying equation 5 the contribution of ALA to brain, liver and adipose DHA is estimated to be 4%, 15% and 18% respectively. Broadly similar estimates were reported by Lefkowitz et al. when feeding rats pups a diet providing labelled ALA and unlabelled DHA, ~9%, ~10%, and 8% for brain, liver and white adipose, respectively (16, 46). The small discrepancies between our estimates and those previously obtained through tracer studies could be attributable to differences in life stage between rodent models or the overall length of dietary exposure.

The novel approach to measure brain DHA half-lives in the current work presents some benefits over previous methods (6–8), namely the lack of expensive and potentially hazardous radiolabelled tracers, but is not without limitations. By avoiding the use of expensive tracers, studies can be carried out more cost-effectively, however natural abundance CSIA necessitates the use of high-precision GCC-IRMS instruments which pose a sizable upfront expense. Furthermore, as experienced in the current work and briefly discussed above, switching diets may contribute altering the concentration of the analyte of interest within a specific tissue pool which complicates the interpretation of calculated half-lives as the tissue pool may not be a steady state. This can be addressed by taking measures to only manipulate the carbon isotope ratio of the compound of interest in the diet while maintaining the same quantity in the diet. In the case of DHA, this could be carried out by switching between a marine sourced product and a
more isotopically enriched algal product such as DHASCO® (24). Additionally, to ensure the quality of CSIA measurements effort must be taken to ensure sample processing does not introduce a kinetic isotope effect which would erode the validity of measured carbon isotope ratios. However, if these concerns are appropriately addressed during the initiation of a study, the application of natural abundance CSIA can be applied as a useful tool to study complex areas of metabolism.

The natural $^{13}$C enrichment of marine sourced fatty acids, the predominant source of long chain n-3 PUFA, compared to relatively $^{13}$C deplete dietary sources of ALA (e.g. canola, soy, flax) presents an opportunity to study the nuances of long-chain PUFA metabolism in humans, without tracer compounds. Recently our group has used natural abundance carbon isotope signatures of plasma long-chain n-3 PUFA following 12 weeks of supplementation with fish oil concentrates to fingerprint the flux of PUFA through the endogenous biosynthetic pathway (26). In doing so, we were able to demonstrate that increases in plasma EPA pool size following DHA supplementation are not the result of apparent retro-conversion, confirming earlier observations made in rodents similarly using natural abundance isotope ratio measurements (25, 26).

Additionally, commercially available of algal oils with unique $^{13}$C enrichment in long-chain PUFA, even greater than those of marine sources, are favorable candidates to study PUFA metabolism in clinical applications. For example, Carnielli et al. used ARASCO® and DHASCO® fortified infant formula coupled with natural abundance carbon isotope ratio analysis to generate estimates of ARA and DHA synthesis in infants (24). Due to the variation in carbon isotope ratios of n-3 PUFA in the environment, natural abundance carbon isotope ratio analysis is well suited for the investigation of n-3 PUFA metabolism in humans and offers benefits over tracer studies, namely the wide availability of supplemental sources and lower cost.
To conclude, this study determined brain DHA half-lives to be ≈ 30 to 135 days in mice depending on the diet group, using natural abundance CSIA. Estimates from ALA equilibrated mice switched to a DHA containing diet matched those previously determined through the use of radiolabel decay models and calculated via pulse-labeling with unesterified DHA (6–8). The agreement of these findings provides confidence to the estimate that brain DHA in rodents has a half-life of ≈ 30 days. Furthermore, the ability of peripheral stores to supply the brain with DHA when it is not present in the diet was indicated by the longer half-life of DHA equilibrated mice switched to the ALA diet. These results aptly highlight the utility of natural abundance carbon isotope ratio analysis in a diet switch experiment to study the metabolism and turnover of specific fatty acids and presents the basis for the greater application of natural abundance isotope measurements for the investigation of fatty acid and lipid metabolism in future studies.
7. Acknowledgments / grant support

RJSL and RPB conceive and designed the study. RJSL and C-CL conducted the experimental work. RJSL wrote the first draft of the manuscript and was responsible for the final content of the manuscript. All authors read and approved the final version of the manuscript. The authors wish to thank Ashley St. Pierre of The Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto, Canada for assistance with IRMS analysis. This study was funded by the Natural Sciences and Engineering Research Council of Canada (grant #482597). RPB is supported by grant funding through the Canadian Institutes of Health Research and the Natural Sciences and Engineering Research Council of Canada and holds a Canada Research Chair in Brain Lipid Metabolism. RJSL holds an Ontario Graduate Scholarship and receives funding support through the Peterborough KM Hunter Charitable Foundation.

8. Conflicts Statement

RPB has received industrial grants, including those matched by the Canadian government, and/or travel support related to work on brain fatty acid uptake from Arctic Nutrition, Bunge Ltd., DSM, Fonterra, Mead Johnson and Nestec Inc., Moreover, RPB is on the executive of the International Society for the Study of Fatty Acids and Lipids and held a meeting on behalf of Fatty Acids and Cell Signaling, both of which rely on corporate sponsorship. RPB has given expert testimony in relation to supplements and the brain. RJSL and C-CL do not have any conflicts to declare.
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### 9. Tables

#### Table 1: Kinetic parameters from best-fit curves of $\delta^{13}$C\textsubscript{DHA} signature response to diet switch\textsuperscript{a}

| Tissue | Diet       | $Y_0$\textsuperscript{b} (SEM) | Plateau\textsuperscript{b} (SEM) | $k$\textsuperscript{c} (SEM) | $t_{1/2}$\textsuperscript{d} (95% CI) | $J_{out}$\textsuperscript{e} |
|--------|------------|-------------------------------|---------------------------------|-------------------------------|------------------------------------|-------------------|
|        | Equilibrated to ALA diet |                  |                                 |                               |                                    |                       |
| Brain  | DHA        | -27.2 (0.2)                  | -21.0 (0.4)                      | 177.8 (3.4)                   | 39.0 (27.9, 65.0)                  | 0.32               |
|        | ALA+DHA    | -27.1 (0.2)                  | -21.8 (0.3)                      | 205.5 (3.5)                   | 33.7 (24.9, 52.2)                  | 0.38               |
| Liver  | DHA        | -30.2 (0.2)                  | -23.9 (0.1)                      | 277.1 (37.8)                  | 2.5 (2.0, 3.5)                     | ND                 |
|        | ALA+DHA    | -30.2 (0.2)                  | -24.5 (0.1)                      | 252.5 (35.0)                  | 2.7 (2.1, 3.8)                     | ND                 |
| Adipose| DHA        | -30.1 (0.3)                  | -23.3 (0.2)                      | 128.3 (21.8)                  | 7.9 (6.5, 10.0)                    | ND                 |
|        | ALA+DHA    | -30.1 (0.4)                  | -24.4 (0.2)                      | 87.6 (9.0)                    | 5.4 (4.0, 8.3)                     | ND                 |
|        | Equilibrated to DHA diet |                  |                                 |                               |                                    | ND                 |
| Brain  | ALA        | -21.7 (0.2)                  | -29.3 (2.8)                      | 5.6 (3.2)                     | 124.4 (57.1, $\infty$)            | 0.11               |
|        | ALA+DHA    | ND                           | ND                              | ND                            | ND                                 | ND                 |
| Liver  | ALA        | -24.0 (0.2)                  | -30.0 (0.1)                      | 41.0 (3.7)                    | 16.9 (14.2, 20.8)                  | ND                 |
|        | ALA+DHA    | ND                           | ND                              | ND                            | ND                                 | ND                 |
| Adipose| ALA        | -23.3 (0.4)                  | -28.9 (0.5)                      | 23.0 (6.6)                    | 30.1 (19.0, 72.5)                  | ND                 |
|        | ALA+DHA    | ND                           | ND                              | ND                            | ND                                 | ND                 |

\textsuperscript{a} Kinetic parameters were generated from best-fit curves plotted through $\delta^{13}$C\textsubscript{DHA} signatures using a one-phase decay function.

\textsuperscript{b} Units in mUr.

\textsuperscript{c} Decay constant generated from best-fit curves; units in days$^{-1} \times 10^{-3}$.

\textsuperscript{d} Generated from best-fit curves using equation 1; units in days.

\textsuperscript{e} Generated from best-fit curves using equation 2 and baseline DHA concentrations; units in $\mu$mol/g brain/ day.

Abbreviations: ALA, $\alpha$-linolenic acid; DHA, docosahexaenoic acid; ND, non-determinable; SEM, standard error of the mean.
10. Figures and figure legend

Figure 1. Graphical representation of a diet switch experiment. The top panel is an example of the isotopic dietary parameters of a diet switch experiment. Animals are equilibrated to study diet X with an isotope ratio of -21.5 mUr, and then switched to diet Y with a isotope ratio of -28 mUr at time 0. The bottom panel depicts the resulting changes in tissue carbon isotope ratios in response to the dietary switch. As the compound of interest is metabolized it is replaced with that from the diet which has a different δ^{13}C value. By measuring and plotting changes in tissue δ^{13}C signatures over a time-course, tissue specific turnover can be modelled.
Figure 2. Study design schematic for the dietary switch experiment. Prior to the dietary switch male BALB/c mice were equilibrated to either the ALA diet or DHA diet by breeding over two generations while maintained on the respective study diet. From the DHA equilibrated group, mice were randomly placed onto either the ALA diet or the ALA+DHA diet at 6 weeks of age. Similarly, mice from the ALA diet were switched onto either the DHA diet or the ALA+DHA diet. Control arms from both equilibrated groups remained on their respective diets over the study time-course. Mice were sacrificed at 7, 14, 28, 56, 112, and 168-days post diet switch. Control mice were sacrificed at 28 and 112-days. A group of mice from both equilibrated diet groups was sacrificed at 6-weeks of age for baseline measurements. Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid.
Figure 3. Brain (A), liver (B) and adipose (C) DHA concentrations following the diet switch from mice equilibrated to the ALA diet. Data are shown as means ± SD and compared by two-
way ANOVA for the interaction of time and diet (n= 3 to 5 per time point); significance at $p < 0.05$. Baseline DHA concentrations are represented by the dashed line with the grey band indicating ± SD. Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; NS, not significant.
Figure 4. Brain (A), liver (B) and adipose (C) DHA concentrations following the diet switch from mice equilibrated to the DHA diet. Data are shown as means ± SD and compared by two-
way ANOVA for the interaction of time and diet (n= 3 to 5 per time point); significance at \( p < 0.05 \). Baseline DHA concentrations are represented by the dashed line with the grey band indicating ± SD. Abbreviations: ALA, \( \alpha \)-linolenic acid; DHA, docosahexaenoic acid; NS, not significant.
Figure 5. Change in Brain (A), liver (B), and adipose (C) $\delta^{13}C_{\text{DHA}}$ signatures following the diet switch from mice equilibrated to the ALA diet. Changes in carbon isotope ratios were fit with a
one-phase decay function on untransformed data (n = 3 to 5 per time point). Abbreviations: ALA, \( \alpha \)-linolenic acid; DHA, docosahexaenoic acid; VPDB, Vienna Peedee Belemnite.
Figure 6. Change in Brain (A), liver (B), and adipose (C) $\delta^{13}C_{\text{DHA}}$ signatures following the diet switch from mice equilibrated to the DHA diet. Changes in carbon isotope ratios for the ALA diet group were fit with a one-phase decay function on untransformed data ($n = 3$ to $5$ per time point).
Carbon isotope ratios from the ALA+DHA group were not adequately modelled by a one-phase decay function and were instead fit by linear regression; slopes from linear regression analysis from all tissues did not deviate from zero ($p > 0.05$). Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; VPDB, Vienna Peedee Belemnite.