esa

ECOSPHERE

SPECIAL FEATURE: BIOMARKERS IN TROPHIC ECOLOGY

Fractionation of stable carbon isotopes of tissue fatty acids in Atlantic pollock (*Pollachius virens*)

Suzanne M. Budge,[1,†] Lacey R. AuCoin,[2] Susan E. Ziegler,[3] and Santosh P. Lall[4]

[1]*Department of Process Engineering and Applied Science, Dalhousie University, Halifax, Nova Scotia B3H 4R2 Canada*
[2]*Department of Biology, Dalhousie University, Halifax, Nova Scotia B3H 4R2 Canada*
[3]*Department of Earth Sciences, Memorial University of Newfoundland, St. John's, Newfoundland A1B 3X5 Canada*
[4]*National Research Council, Institute for Marine Biosciences, Halifax, Nova Scotia B3H 3Z1 Canada*

Citation: Budge, S. M., L. R. AuCoin, S. E. Ziegler, and S. P. Lall. 2016. Fractionation of stable carbon isotopes of tissue fatty acids in Atlantic pollock (*Pollachius virens*). Ecosphere 7(8):e01437. 10.1002/ecs2.1437

Abstract. Reliable estimates of diet to tissue fractionation of fatty acid (FA) stable carbon isotopes are essential for the development of techniques employing these biomarkers. In this work, Atlantic pollock (*Pollachius virens*) was used as a model species to investigate fractionation arising from metabolic processes during assimilation of dietary FA into serum and liver; we also examined fractionation occurring from mobilization of FA from liver during fasting. Pollock were fed diets containing FA of known isotopic composition, and serum and liver were collected postprandially and after fasting. Lipids were isolated from these tissues, and four polyunsaturated FA (PUFA), 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3, within triacylglycerols (TAG), a specific lipid class associated with fat storage, were analyzed for their stable carbon isotope ratios. For 18:2n-6, 20:5n-3, and 22:6n-3, there was no discrimination between diet and serum in postprandially sampled fish, suggesting that fractionation did not occur during hydrolysis and esterification for most PUFA examined here during digestion and transfer into serum. There was a similar lack of fractionation for all four FA between fasted liver and serum, indicating that the assembly of these FA into TAG and their release into serum were not associated with fractionation. However, apparent fractionation was variable and inconsistent for all FA between diet and postprandial liver, indicating a failure of liver TAG to fully reflect the new diet. These data will allow $\delta^{13}C$ values of three PUFA in postprandial serum to be incorporated into mixing models to estimate recent diet in gadoids. Further controlled feeding studies, under conditions that elicit physiological responses that are similar to those of fish in their natural environment, will be necessary before reliable estimates of longer-term diet, derived from $\delta^{13}C$ in liver FA, will be possible.

Key words: assimilation; catabolism; chylomicrons; lipids; liver; mobilization; *Pollachius virens;* serum; Special Feature: Biomarkers in Trophic Ecology.

Received 6 October 2015; revised 7 April 2016; accepted 27 May 2016. Corresponding Editor: D. Soto.

Copyright: © 2016 Budge et al. This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

† **E-mail:** Suzanne.Budge@dal.ca

Introduction

Knowledge of predator diets is essential in understanding predator–prey relationships, but traditional methods of studying diet, such as stomach content and fecal analysis, can be inconclusive and biased toward certain dietary components (McInnis et al. 1983, Yonezaki et al. 2003). Biomarker-based approaches have been developed as alternatives to these techniques. For instance, fatty acid (FA) analysis of animal tissues has been used to investigate spatial and

temporal variation in diets (Wang et al. 2009, Gonçalves et al. 2012) and to establish different feeding niches of predators (Budge et al. 2007). Similarly, the natural abundances of stable isotopes in animal tissues have been used to investigate the reliance of predators on different dietary sources (Tarroux et al. 2012, von Biela et al. 2013) and to track diet changes of consumers through time (Schell 2000, Moody et al. 2012). New technology that allows for accurate compound-specific isotope analysis (CSIA) has expanded the suite of biochemical methods available for studying predator diets (Silfer 1991), particularly in the last 10 years (Evershed et al. 2008), and several studies have used CSIA to reveal information on the origin of individual biomarkers in an animal's diet (Pond et al. 1998, Howland et al. 2003, Budge et al. 2008).

De novo synthesis of protein, carbohydrate, and lipid in a predator results in fractionation of stable C and N isotopes so that the biosynthesized product has a different stable isotope ratio than its precursors (Gannes et al. 1997). This can make it difficult to interpret the results of stable isotope analyses of bulk tissues because $\delta^{13}C$ and $\delta^{15}N$ values will be derived from both endogenous and exogenous C and N sources. Compound-specific isotope analyses allow $\delta^{13}C$ and $\delta^{15}N$ values of single components to be determined and may help avoid some of the ambiguity associated with bulk analyses, particularly when the components analyzed can only be acquired through diet. Compound-specific isotope analysis of FA and amino acids have been recently applied in several studies to examine the diet composition, foraging location, and food web interactions of both marine and terrestrial consumers (Hammer et al. 1998, Howland et al. 2003, Chamberlain et al. 2004, Ruess et al. 2005, Larsen et al. 2013, McMahon et al. 2015). Because of their diversity of structures, FA have particular promise as biomarkers in applications utilizing CSIA.

Fatty acids in a predator's tissues are derived from either diet or de novo synthesis by the animal. Two FA, 18:2n-6 and 18:3n-3, can only be synthesized by plants (Cunnane 2003) and are acquired through diet, while many other long-chain polyunsaturated FA (PUFA), including 20:5n-3 and 22:6n-3, can be produced from those precursors through elongation and desaturation (Fig. 1). Marine fish represent an exception to this, having little or no capacity to carry out those modifications (reviewed in Dalsgaard et al. 2003); thus, 20:5n-3 and 22:6n-3 can also be considered as solely diet derived. As the stable isotopic signatures of long-chain PUFA cannot be influenced by fractionation during synthesis, they are expected to be more similar to that found in the diet. However, all FA, including PUFA, may be catabolized for energy, through a process known as β-oxidation (Fig. 1), where FA are first activated by an acyl CoA enzyme prior to entry into the mitochondria or peroxisomes. Acyl CoA may be more likely to selectively activate FA with lower $\delta^{13}C$ values if a pool of FA is available (Fry 2006), resulting in kinetic fractionation, and an increase in the $\delta^{13}C$ values of FA remaining in the tissue.

To be incorporated into tissues, dietary FA are transported in a number of discreet steps, each enzymatically catalyzed (Tocher 2003), that do not alter FA structure but may also lead to the fractionation of stable C isotopes in FA (summarized in Fig. 2). For instance, FA are normally attached to glycerol by an ester bond, producing triacylglycerols (TAG) and other acylglycerols. To be transported across cell walls in the intestine during digestion, dietary FA must first be hydrolyzed from the glycerol molecules, to release free FA (FFA; Fig. 1) and monoacylglycerol (MAG) which can be absorbed by the enterocytes in the proximal intestine and pyloric ceca (Honkanen et al. 1985, Denstadli et al. 2004). The absorbed FA are then re-esterified to form TAG and subsequently incorporated into serum lipoproteins, known as chylomicrons, to increase solubility for export into blood serum. The FA structure is not modified during this process, but kinetic fractionation may occur, where the isotopically lighter FA are preferentially selected for the hydrolysis and esterification processes. In gadoids, FA bound within TAG in chylomicrons are then transported to either the muscle for catabolism or the liver for storage and/or catabolism. To meet energy needs during fasts, FA may be mobilized from the liver as TAG within serum lipoproteins following similar hydrolysis and esterification mechanisms. Catabolism of liver FA through β-oxidation also helps meet energy requirements while fasting. Knowledge of the fractionation associated with each step in FA

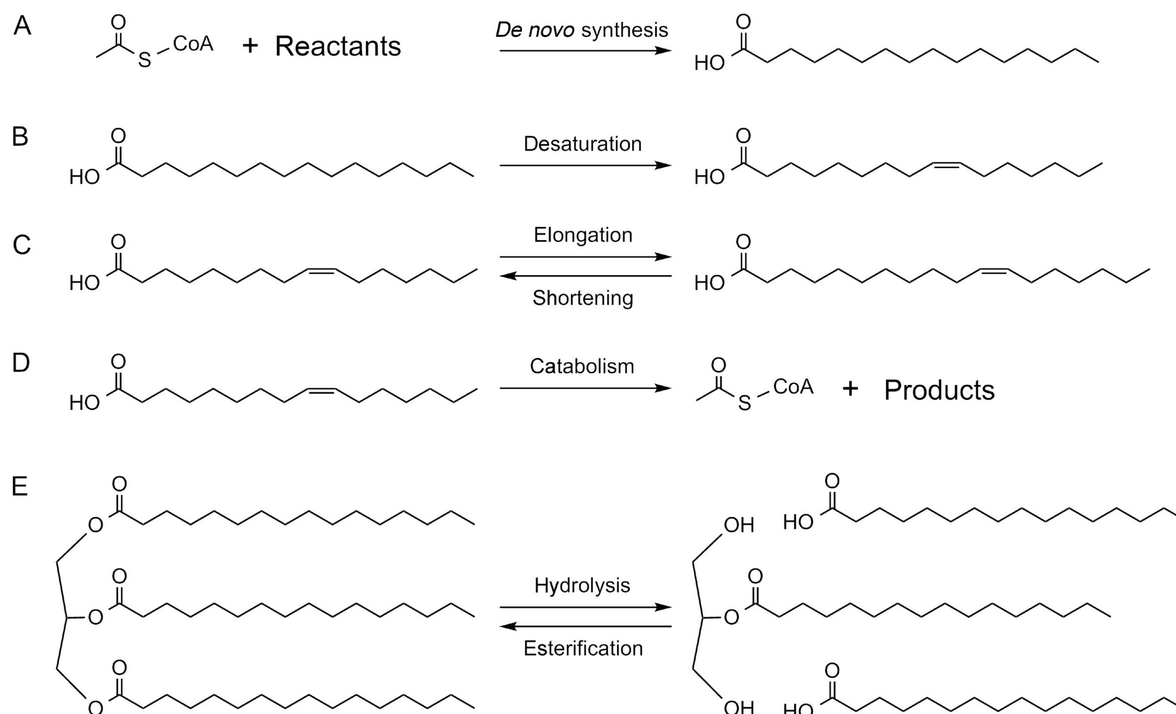

Fig. 1. Biochemical processes that may result in fractionation of fatty acids (FA) in fish liver. FA may be synthesized from acetate in the form of acetyl CoA (A). Saturated FA may be desaturated by the insertion of a C–C double bond (B). The addition of an acetate group to the carboxyl end of the molecule results in elongation, while the reverse process produces a FA chain that is shorter by two carbon atoms (C). Catabolism of FA, or β-oxidation, yields acetyl CoA which can enter the citric acid cycle to generate energy in the form of ATP (D). Hydrolysis of triacylglycerols yields free FA and monoacylglycerol; the reverse process is known as esterification (E).

metabolism is necessary because the $\delta^{13}C$ of FA in the consumer tissue will reflect the influences of all these processes. With that understanding, specific tissues where FA have been incorporated can then be analyzed to make inferences about short- and long-term diets of wild fish based on FA $\delta^{13}C$ data. Chylomicron FA are derived from the most recently consumed meal, while FA in fat storage tissues, such as the liver in gadoids, will represent diet over much longer time frames (weeks to months).

In this study, we have used controlled feeding studies with Atlantic pollock (*Pollachius virens*) to investigate the influence of lipid metabolism on the fractionation of four diet-derived PUFA, 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3. Pollock was chosen as the study species because of ease of husbandry and similarity in physiology to other gadoids, such Atlantic cod and haddock, which are targeted in commercial fisheries. We compared $\delta^{13}C$ of FA in diet and tissues to evaluate fractionation arising from several different processes (Fig. 2). First, we examined fractionation arising from hydrolysis and esterification of FA by determining the difference in $\delta^{13}C$ values of FA in the diet and serum chylomicrons (the discrimination factor). Similarly, we evaluated discrimination factors for the mobilization of FA in liver to serum during a fast; this fractionation would also be principally due to hydrolysis and esterification. Then, we investigated fractionation due to the combined effects of hydrolysis, esterification, β-oxidation, and chain elongation and desaturation by determining discrimination factors for FA in the diet and postprandially sampled liver. Last, fractionation associated primarily with β-oxidation was determined by examining the difference in liver FA $\delta^{13}C$ before and after fasting. To accomplish this, we fed pollock a known diet for 12 weeks and

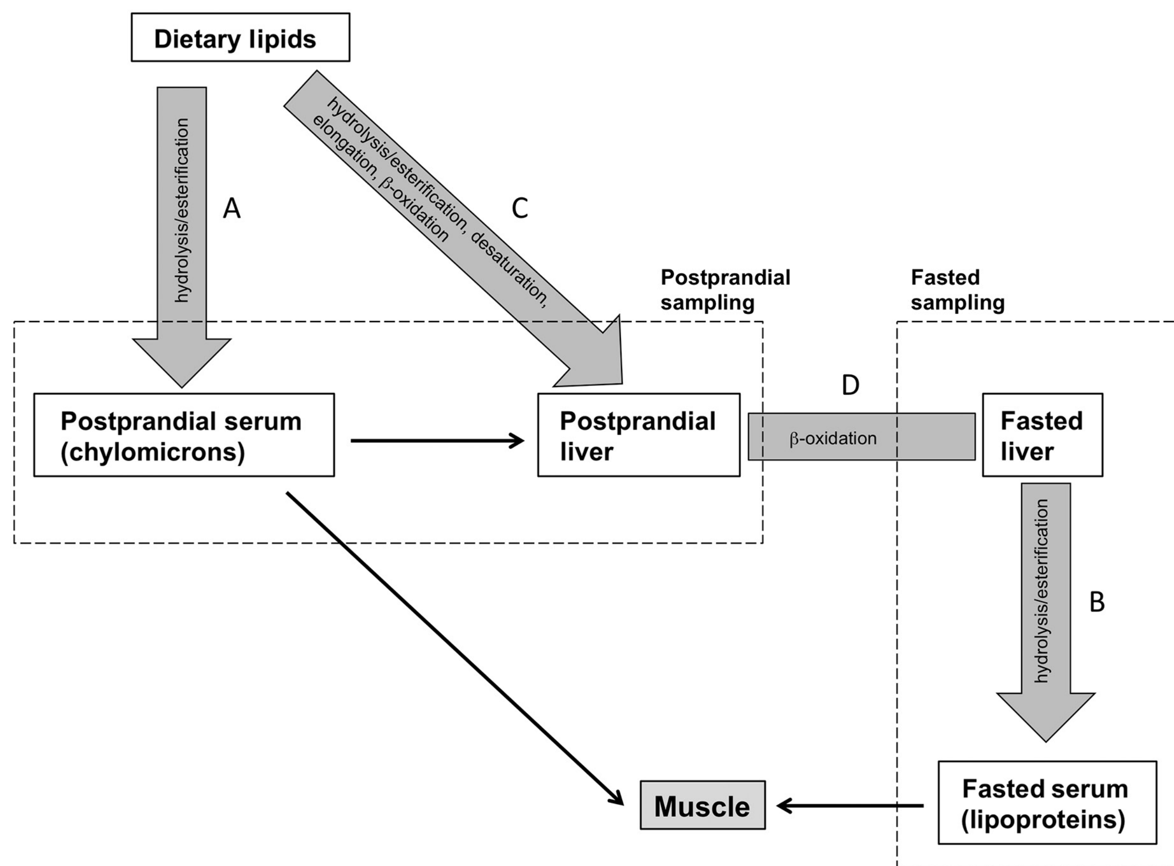

Fig. 2. Conceptual diagram illustrating the transport of polyunsaturated fatty acids (FA) in diet to liver and muscle in fish. The influence of hydrolysis and esterification of triacylglycerols (TAG) on fractionation was assessed by comparing diet to postprandial serum (A); fractionation arising from mobilization of TAG from the liver into fasted serum (B) was also primarily due to hydrolysis and esterification. The difference in FA $\delta^{13}C$ in diet and postprandial liver (C) reflected fractionation from hydrolysis/esterification, elongation, desaturation, and β-oxidation. Fractionation of FA $\delta^{13}C$ between postprandial and fasted liver (D) primarily reflected the influence of β-oxidation. Arrows indicate transport of lipid between diet and tissue or among tissues; thick arrows (A–C) represent the processes investigated in this work. The solid line (D) indicates the relationship between postprandial and fasted liver.

then determined FA proportions and $\delta^{13}C$ values of 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3 in diet and in postprandially sampled serum and liver. The same parameters in serum and liver were measured after a three-week fast, and discrimination factors for FA at each stage in assimilation, mobilization, and catabolism of lipids were calculated. By combining information from all four experiments, we assessed the principle sources of fractionation and their magnitude, allowing for more accurate incorporation of $\delta^{13}C$ of FA biomarkers in mixing models for diet estimation.

Methods

Experimental fish and diets

Wild juvenile Atlantic pollock were caught near Duncan's Cove, Nova Scotia, on 15 July 2009 using a commercial long line baited with mackerel. Pollock (n = 26) were kept in a 684,000-L aerated pool tank with flow-through seawater at ambient temperature from the Northwest Arm of Halifax Harbour, Nova Scotia. From July to December, the temperature of the tank varied according to natural water temperatures of the inlet (6–13°C), and

Table 1. Formulation of the experimental diet†.

Ingredient	Percentage
Herring meal	36.5
Soybean meal	12
Wheat middlings	21.7
CPSP-G‡	3.5
Corn gluten meal	14
Krill meal	2
Choline chloride	0.3
Canola oil	8
Vitamin mixture§	1
Mineral mixture¶	1
Total	100

† Protein and lipid contents of the diet were determined to be 48% and 12.8%, wwb, respectively.

‡ Fish protein hydrolysate, Sopropêche, France.

§ Vitamin mixture: vitamin A, 8000 IU/kg; vitamin D_3, 4500 IU/kg; vitamin E, 300 IU/kg; vitamin K_3, 40 mg/kg; thiamin, 50 mg/kg; riboflavin, 70 mg/kg; pantothenate, 200 mg/kg; biotin, 1.5 mg/kg; folic acid, 20 mg/kg; vitamin B_{12}, 0.15 mg/kg; niacin, 300 mg/kg; pyridoxine, 20 mg/kg; ascorbic acid, 300 mg/kg; inositol, 400 mg/kg; butylated hydroxy toluene, 15 mg/kg; butylated hydroxy anisole, 15 mg/kg.

¶ Mineral mixture: manganous sulfate, 40 mg/kg; ferrous sulfate, 30 mg/kg; copper sulfate, 5 mg/kg; zinc sulfate, 75 mg/kg; sodium selenite, 1 mg/kg; cobalt chloride, 2.5 mg/kg; sodium fluoride, 4 mg/kg.

the water was heated to 8°C upon entry into the facility from January to May. The fish were anesthetized (by immersion, MS220 [Tricaine methanesulfonate], 60 mg/L), weighed, measured, and tagged using a passive integrated transponder tag near the dorsal fin.

Pollock were maintained on a commercial marine oil-based diet consisting of herring meal and a mixture of herring and anchovy fish oil (18% lipid wwb) and were fed to satiation once daily. After 25 weeks, six fish were sampled for liver. The remaining fish ($n = 20$) were switched to a feed formulated using canola oil (Table 1; ~13% lipid wet mass) for 12 weeks. Oil from canola, a C3 plant, was used so that the pollock would adopt a terrestrial signature that was distinct from that of their previously fed marine diet, allowing the changes in FA profiles and $\delta^{13}C$ values to be more easily followed. Approximately half of the fish were sampled following the 12-week feeding period for liver and chylomicrons, while the remaining fish were fasted for 3 weeks prior to sampling serum and liver. Diets were stored at −20°C throughout the study.

Sample collection

Pollock were anesthetized (MS220, 60 mg/L) for blood collection and isolation of chylomicrons and serum, and were euthanized (MS220, 150 mg/L) immediately afterward. When the intent was to isolate chylomicrons, fish were fed 12 h before euthanizing. Blood (5 mL) was retrieved by severing the caudal vein, and fresh blood was transferred to a 7-mL vacutainer without anticoagulant. The more commonly used method of obtaining blood by needle and syringe was avoided to prevent the capillary pressure of the needle from damaging the chylomicrons (Mills et al. 2000). Fresh blood was chilled and centrifuged at 200 *g* for 20 min to separate the serum within 2 h of blood sampling. Whole liver was removed immediately after death, homogenized in a food processor, and stored in 2:1 $CHCl_3$:MeOH solution with 0.01% butylated hydroxytoluene (BHT) at −20°C prior to lipid extraction.

To isolate chylomicrons, postprandial serum was carefully overlain with 0.169 mol/L sodium chloride with 0.01% ethylenediaminetetraacetic acid (EDTA), maintaining a 1:1 volume of salt:serum. Chylomicrons were separated by centrifuging at 25,000 *g* at 15°C for 30 min. Mammalian chylomicrons have been reported to range from 35 to 250 nm in diameter (Davis 1991), and fish chylomicrons are thought to have a corresponding size range (Tocher 2003); therefore, a separation density of 1.006 g/mL that is typically used to isolate mammalian chylomicrons was employed, yielding chylomicrons that were roughly 100 nm in size and minimizing contamination (Mills et al. 2000). Chylomicrons appeared as a white cloudy layer in the top portion of the NaCl solution and were carefully removed using a 1.5-mL fine-tip polyethylene transfer pipette. The chylomicrons were transferred to a 10-mL heavy duty glass test tube and stored in 2:1 $CHCl_3$:MeOH with 0.01% BHT at −20°C until lipid extraction.

Fasting serum was not fractionated into lipoprotein classes. Gadoids mobilize stored FA as TAG from the liver within very low-density lipoproteins (VLDL), but after three-week fasts, high-density lipoproteins, rather than VLDL, have been identified as the major lipoprotein circulating in the blood (Nanton et al. 2006, Kjær et al. 2009). We did not attempt to isolate those two classes because TAG within serum was derived from liver, regardless of lipoprotein carrier, and their FA would continue to bear similarity to the liver profile (Lie et al. 1993, Kjær et al. 2009). We

thus refer to these samples as fasting serum without specifying a particular lipoprotein class.

Lipid extraction and FA analysis

Total lipid was extracted from all samples by a modified Folch et al. (1957) procedure (Budge et al. 2006) using 2:1 $CHCl_3$:MeOH solution. Thin-layer chromatography (TLC) was used to isolate TAG in liver, serum, and chylomicron samples, employing a solvent system of 85:15:1 hexane:diethyl ether:glacial acetic acid. Triacylglycerols were not isolated from the lipid of diet samples because FA assimilated into fish tissues are derived from all lipid classes. All lipid and TAG were then transesterified to fatty acid methyl esters (FAME) using methanol and H_2SO_4 as an acidic catalyst. Fatty acid methyl esters were analyzed using a PerkinElmer Autosystem II (Perkin Elmer, Woodbridge, Canada) capillary gas chromatograph (GC) with a flame ionization detector and a polar DB-23 column (30 m × 0.25 mm ID × 0.25 μm film thickness; Agilent Technologies, Palo Alto, California, USA). Helium was used as the carrier gas, and FAME were analyzed with splitless injection at a concentration of 0.5 mg/mL hexane. Samples were analyzed in duplicate, and 60 FA were identified.

GC–combustion–isotope ratio mass spectrometry

The $\delta^{13}C$ values of 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3 were determined by GC–combustion–isotope ratio mass spectrometry (GC-C-IRMS) at the Core Research Equipment and Instrument Training Network (CREAIT Network) at Memorial University of Newfoundland. Fatty acids were separated by GC (Agilent 6890) using a BPX70 column (50 m × 0.32 mm ID × 0.25 μm film thickness; SGE Analytical Science, Austin, Texas, USA). Samples were applied to the column by splitless injection (at 250°C) with an initial oven temperature of 70°C which was increased at 10°C/min to a temperature of 160°C, held constant for 5 min, then increased at 4°C/min to the final temperature (260°C), which was held for 15 min. Helium was used as the carrier gas at a flow rate of 1.5 mL/min, and column eluent was routed through a CuO/NiO/Pt oxidation reactor in a Thermo Finnigan (Thermo Fisher Scientific, Mississauga, Canada) GC combustion III interface. The generated CO_2 was carried to an IRMS (Finnigan MAT252, Thermo Fisher Scientific, Mississauga, Canada) for analysis of the C isotopes relative to a reference CO_2 gas. Accuracy of the $\delta^{13}C$ FA analyses was assessed by comparison with a commercially available bacterial FA methyl ester standard mixture and a methyl and ethyl ester mixture supplied by Indiana University (F8 standard). The F8 standard was comprised of a mixture of 14:0, 16:0, 18:0, and 20:0 ethyl and methyl esters. Analytical precision, determined by replicate injection of samples and standards, was ≤0.5‰. All $\delta^{13}C$ values are reported relative to the Vienna Peedee Belemnite (VPDB) standard using standard delta notation.

The methanol used for methylation of FA was collected for each batch of samples prepared, and a small aliquot was analyzed for its $\delta^{13}C$ composition on an Aurora 1020 TOC analyzer (O.I. Analytical, College Station, Texas, USA) coupled to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Mississauga, Canada). The average $\delta^{13}C$ of methanol was then used to correct for the additional methyl group added to the FA during transesterification by subtracting the proportional contribution of methanol to the $\delta^{13}C$ values of FAME with the following equation:

$$\delta^{13}C_{methanol} = (n+1)[\delta^{13}C_{FAME}] - n[\delta^{13}C_{FFA}]$$

where n is equal to the number of C atoms in the FFA. Free FA were independently corrected using this equation, as the C in the methyl group varies in its contribution to the total $\delta^{13}C$ value depending on FA structure.

Discrimination factors

Discrimination factors (Δ) were calculated for each FA and tissue by subtracting the $\delta^{13}C$ of diet (D) from each tissue (T) sample, such that $\Delta^{13}C_{T-D} = T - D$. These were determined for both chylomicrons ($\Delta^{13}C_{C-D}$) and postprandial liver ($\Delta^{13}C_{LP-D}$). Discrimination factors were also calculated for the mobilization of FA from liver to serum (S) in the fasted fish ($\Delta^{13}C_{S-LF}$).

Statistical analyses

Individual fish were used as the unit of replication. Previous work in the same facility (Budge et al. 2011) did not find a tank effect with FA proportional data, yielding identical conclusions when tank and individual were used separately as sampling units. FA proportions were normalized to 100% and transformed using a $\log(x + 1)$ function. Bray–Curtis similarity matrices were

generated for the entire data set using PRIMER-E (Primer-E Ltd, Plymouth Marine Laboratory, Plymouth, UK). Permutational multivariate analysis of variance (PERMANOVA in PRIMER-E, v.6+) was used to compare FA signatures among diet and tissues and was followed by pairwise comparisons. Principal components analysis (PCA), using covariance matrices, was used to visualize the variation in the full FA proportional data set after applying the same $\log(x + 1)$ transformation. Multivariate analysis of variance (MANOVA) was used to compare untransformed $\delta^{13}C$ values of the four FA (SPSS 15.0 for Windows; IBM Corporation, Somers, New York, USA). Where overall differences were found, univariate ANOVA, applying a conservative P value of 0.013 (~0.05/4), adjusted for multiple comparisons, was used to determine differences in $\delta^{13}C$ according to sample type. Tukey's test was then used for pairwise comparisons when ANOVA indicated a significant effect.

Results

Experimental fish growth

Initial fish had a mean mass of 519 ± 130 g (range: ~300–700 g) and liver lipid proportion of 66% ± 13% wwb. The hepatosomatic index (HSI) was 11.2 ± 1.4. After feeding for 12 weeks, all fish were significantly larger in mass (ANOVA: $F_{1,14} = 16.9$, $P < 0.001$) with a mean mass of 815 ± 217 g (range: 400–1000 g). Liver lipid proportion and HSI did not change over the course of the experiment.

FA proportional data

PERMANOVA showed an overall significant difference in FA profiles of diet and TAG of tissues (pseudo-$F = 25.5$; $P < 0.001$), and pairwise tests indicated differences among all tissue types except liver of fish sampled postprandially and after fasting. The PCA factor plot (Fig. 3) well illustrated the components of the FA profile driving the variation among the diet and TAG of tissues analyzed. PC1 represented 71% of the variation in the data and was mainly influenced by proportions of 18:3n-3, 20:5n-3, 16:1n-7, and 18:0, while PC2 described another 9% of the variation and was mostly driven by proportions of 22:6n-3, 22:1n-11, and 18:2n-6.

The FA profile of the diet (Table 2, Fig. 3) strongly resembled that of canola oil, the principle lipid component, with very high proportions

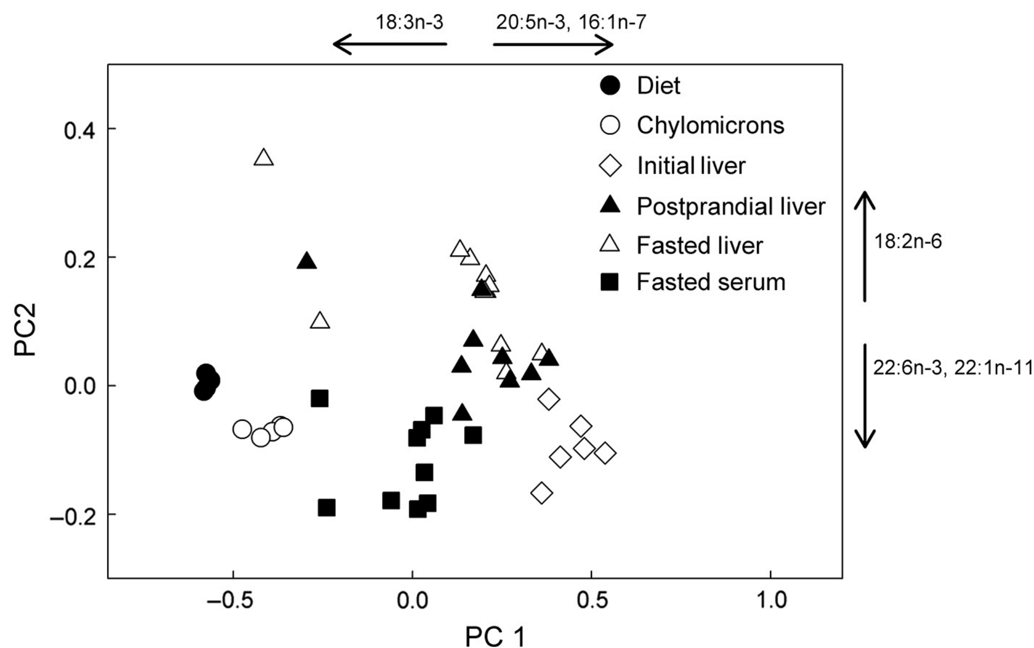

Fig. 3. Principal components analysis of total fatty acids (FA) in diet and triacylglycerol FA in fish tissues. Principal component (PC) 1 and PC2 accounted for 72% and 9% of the variance, respectively. The FA loading highest on each PC are shown with arrows.

Table 2. Fatty acid (FA) composition (mean [SD]) of diet, chylomicrons, liver before diet switch, liver after feeding the experimental diet for 12 weeks, and liver and serum after fasting from weeks 12 to 15.

Fatty acid	Diet (n = 6)	Chylomicrons (n = 6)	Initial liver (n = 6)	Postprandial liver (n = 9)	Fasted liver (n = 11)	Fasted serum (n = 10)
Saturates						
14:0	1.49 (0.03)	1.29 (0.09)	2.27 (0.14)	1.68 (0.29)	1.71 (0.24)	1.28 (0.12)
16:0	8.99 (0.10)	9.00 (0.37)	14.18 (0.35)	11.21 (0.76)	10.86 (0.85)	10.05 (0.76)
18:0	1.82 (0.01)	2.90 (0.44)	5.18 (0.24)	4.27 (0.36)	4.22 (0.48)	3.91 (0.44)
Monounsaturates						
16:1n-9	0.09 (0.00)	0.18 (0.02)	0.43 (0.03)	0.42 (0.06)	0.41 (0.06)	0.34 (0.03)
16:1n-7	1.75 (0.02)	2.01 (0.11)	5.91 (0.18)	4.13 (1.05)	4.14 (0.99)	2.65 (0.52)
18:1n-9	38.72 (0.13)	39.62 (0.93)	28.17 (0.91)	36.05 (4.56)	35.31 (3.40)	37.03 (1.43)
18:1n-7	2.80 (0.09)	3.06 (0.13)	3.99 (0.12)	4.27 (0.54)	4.38 (0.26)	3.71 (0.17)
20:1n-11	0.40 (0.02)	0.63 (0.07)	0.81 (0.12)	0.73 (0.05)	0.67 (0.25)	0.96 (0.89)
20:1n-9	3.58 (0.01)	4.03 (0.32)	3.30 (0.49)	2.97 (0.25)	3.18 (0.44)	3.15 (0.88)
22:1n-11	4.60 (0.05)	5.23 (0.25)	2.74 (0.49)	1.99 (0.23)	2.16 (0.30)	3.14 (0.39)
22:1n-9	0.49 (0.02)	0.53 (0.07)	0.41 (0.09)	0.32 (0.06)	0.31 (0.06)	0.41 (0.06)
Polyunsaturates						
16:2n-4	0.13 (0.00)	0.13 (0.01)	0.34 (0.05)	0.26 (0.08)	0.26 (0.07)	0.15 (0.03)
16:3n-4	0.04 (0.03)	0.11 (0.02)	0.41 (0.11)	0.10 (0.13)	0.27 (0.10)	0.15 (0.08)
16:4n-1	0.14 (0.01)	0.10 (0.01)	0.39 (0.12)	0.26 (0.10)	0.28 (0.09)	0.11 (0.03)
18:2n-6	19.57 (0.13)	16.06 (0.48)	11.22 (0.91)	14.03 (1.44)	13.72 (1.18)	13.38 (0.69)
18:3n-3	5.74 (0.04)	4.07 (0.22)	0.98 (0.03)	2.50 (0.95)	2.29 (0.78)	2.64 (0.47)
18:4n-3	0.16 (0.14)	0.28 (0.01)	0.83 (0.13)	0.57 (0.17)	0.56 (0.20)	0.39 (0.07)
20:4n-6	0.17 (0.01)	0.21 (0.03)	0.50 (0.04)	0.35 (0.11)	0.41 (0.11)	0.44 (0.09)
20:5n-3	2.17 (0.04)	2.50 (0.3)	6.19 (1.11)	4.66 (1.60)	5.10 (1.28)	5.09 (1.18)
21:5n-3	0.07 (0.02)	0.07 (0.04)	0.26 (0.05)	0.18 (0.16)	0.23 (0.08)	0.29 (0.38)
22:5n-3	0.29 (0.02)	0.47 (0.12)	0.96 (0.07)	0.70 (0.24)	0.82 (0.19)	0.83 (0.16)
22:6n-3	3.40 (0.09)	2.69 (0.36)	4.45 (0.52)	3.28 (0.79)	3.76 (0.47)	5.06 (0.83)
Total	96.61	95.14	93.94	94.55	95.04	94.55

Notes: The FA composition of the diet was derived from all lipid classes. For all other sample types, the FA profile was derived from triacylglycerols.

of 18:1n-9 (~39%) and 18:2n-6 (~20%), as well as significant amounts of 18:3n-3 (~6%). The herring and krill meal in the diet contributed typical marine FA, such as 20:5n-3 and 22:6n-3, with levels of ~2% and 3.5%, respectively. The relatively low proportion of 20:5n-3 and high proportion of 18:3n-3, compared to TAG of liver, resulted in the position of diet on the far left of the PCA plot. The FA composition of chylomicron TAG showed a very similar profile to diet, with elevated proportions of 18:1n-9 and 18:2n-6 (Table 2). This similarity of profile resulted in the proximity of diet and chylomicron samples in the PCA factor plot. In contrast, the FA of TAG of the initial liver samples showed more marine lipid characteristics than the diet, with higher levels of 20:5n-3 and 22:6n-3 (~6% and 4.5%, respectively), as well as 21:5n-3, 22:5n-3, and 16 carbon PUFA (Table 2). Proportions of 18:1n-9, 18:2n-6, and 18:3n-3 in initial liver were also much lower than those of diet, resulting in their clear separation from diet and chylomicrons samples and their position at the far right of the PCA plot. Postprandially sampled liver showed the influence of the canola-based diet, with increases in the proportions of the canola associated FA (18:1n-9, 18:2n-6, and 18:3n-3) and decreases in the marine FA (e.g., 20:5n-3 and 22:6n-3). Fatty acids of TAG in liver collected from fasted fish were not significantly different than those of postprandially sampled fish with both plotting together (Table 2). Finally, serum mobilized from liver of fasted fish displayed a FA profile similar to liver TAG, but with increased proportions of 22:1n-11 and 22:6n-3.

Five outliers were obvious in the PCA plot. All five samples had the highest proportions of 18:3n-3 and lowest proportions of 20:5n-3 within their sample type. Four of these outliers included two pairs of liver and serum samples collected

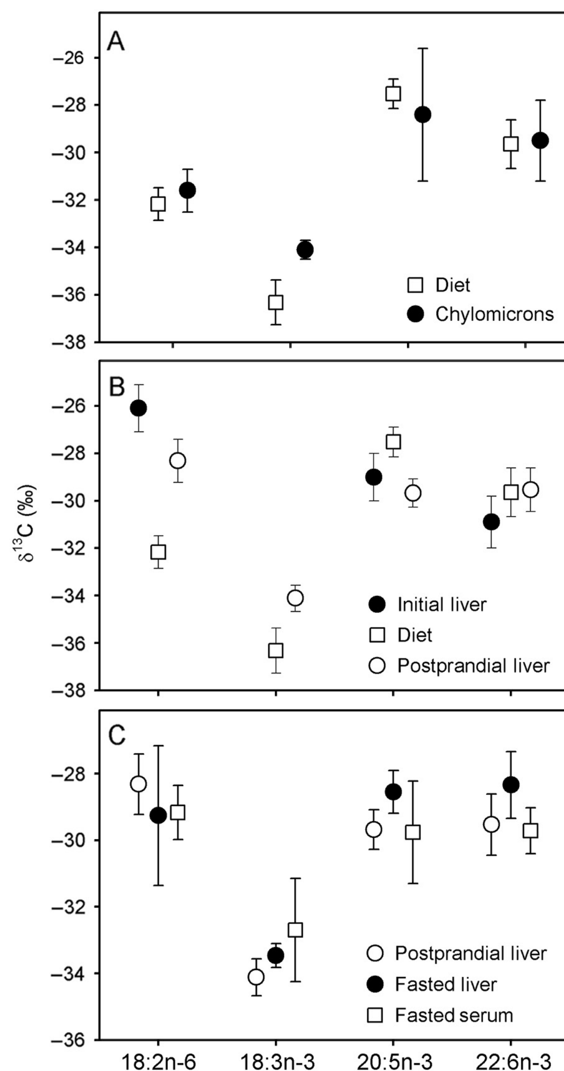

Fig. 4. δ13C values of fatty acids (FA) in diet, chylomicrons, liver (initial, postprandial, and fasted), and fasted serum. Panel A illustrates fractionation principally due to hydrolysis and esterification by examining diet ($n = 6$) and chylomicron triacylglycerols (TAG; $n = 6$). In panel B, the combined effects of FA metabolism on fractionation were investigated by comparing liver TAG of postprandially sampled pollock ($n = 7$) to the experimental diet ($n = 6$) and to liver TAG of initial pollock samples ($n = 6$). Panel C shows fractionation due to two distinct processes. The comparison of liver TAG of postprandially sampled pollock with that of fasted pollock ($n = 5$) examined fractionation due to β-oxidation. Fractionation arising from hydrolysis and esterification was again illustrated in the comparison of TAG FA of mobilized serum ($n = 7$) and of fasted liver.

from the same two fish. These paired samples displayed a similar pattern as noted above, with serum showing a decrease in proportions of 22:1n-11 and 22:6n-3 relative to the liver from which they were mobilized. The single unpaired postprandial sample seemed to represent a true outlier with no obvious explanation.

δ13C of individual FA

δ13C values of 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3 in the TAG of the initial liver ranged from −31‰ to −26‰, while those of diet and treatment tissues were generally lower with δ13C from −37‰ to −28‰ (Fig. 4). There was a significant difference between the δ13C values of FA in diet and TAG chylomicrons (MANOVA: $F_{4,8} = 7.1$, Wilk's $\lambda = 0.22$, $P < 0.01$), but univariate ANOVA showed that only 18:3n-3 differed in the two sample types ($F_{1,11} = 35.2$, $P < 0.001$; Fig. 4A).

To compare δ13C values of FA in diet to those in TAG of initial and postprandial liver, MANOVA was performed on only 18:2n-6, 20:5n-3, and 22:6n-3; 18:3n-3 was excluded because it could not be determined in the initial liver TAG (Fig. 4B). MANOVA did indicate differences in δ13C according to sample type ($F_{6,28} = 21.9$, Wilk's $\lambda = 0.031$, $P < 0.001$); univariate ANOVA, performed on all four FA, followed by Tukey's post hoc tests, revealed a number of differences but few clear patterns. For instance, while δ13C values of FA in diet were significantly different than postprandial liver for 18:2n-6, 18:3n-3, and 20:5n-3, the first two FA showed higher values of δ13C in the liver TAG relative to diet, whereas the δ13C value of 20:5n-3 was lower in liver TAG than diet. The δ13C values of 18:2n-6 and 20:5n-3 in initial liver TAG were both significantly different than those of the diet fed. Further, the δ13C values of 22:6n-3 were equivalent in all three sample types considered.

Although MANOVA indicated a significant difference ($F_{8,24} = 4.7$, Wilk's $\lambda = 0.15$, $P < 0.001$) between δ13C values of FA mobilized from fasted tissues (serum and liver; Fig. 4C) and those of the postprandially sampled liver, univariate ANOVA did not indicate any differences by sample type for any FA ($P > 0.013$, adjusted for multiple comparisons). There was, however, a trend to higher values of δ13C for 20:5n-3 and 22:6n-3 in the fasted liver relative to the serum or postprandial liver.

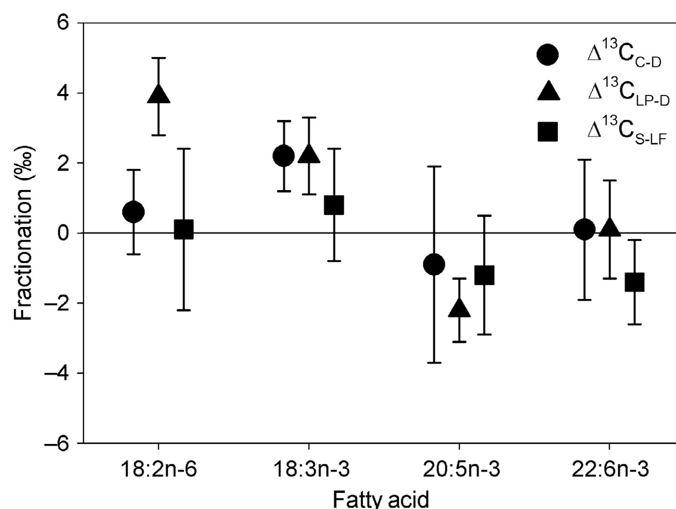

Fig. 5. Discrimination factors showing the fractionation of $\delta^{13}C$ values of fatty acids (FA) between diet and tissue during FA assimilation. Discrimination factors for the mobilization of FA into the serum from liver during fasting are also shown.

Discrimination factors more clearly displayed the patterns in the isotope data (Fig. 5). For instance, all transfers of FA to blood tissues (chylomicrons and fasted serum) yielded discrimination factors ($\Delta^{13}C_{C-D}$ and $\Delta^{13}C_{S-LF}$) of zero for all FA except 18:3n-3. Conversely, only for 22:6n-3 was the discrimination factor ($\Delta^{13}C_{LP-D}$) for postprandial liver to diet equal to zero.

Discussion

Hydrolysis and esterification—uptake of dietary FA by chylomicrons

The absorption and transport of FA from diet to chylomicron TAG primarily involve hydrolysis and esterification. As elongation, desaturation, and β-oxidation cannot occur, there is limited opportunity for modification of FA structure (Olsen and Ringø 1997). Thus, the similarity of the FA profile of chylomicron TAG to that of the diet was expected and much greater than any other tissue examined (Table 2, Fig. 3); however, subtle but significant differences were apparent due to digestion and assimilation. Digestion is principally hydrolysis, involving the action of lipases to cleave FA from intact lipids, while absorption or assimilation refers to the uptake of released FA, MAG, and other smaller molecules in the intestine. Most studies with fish have investigated apparent digestibility, which measures the combined effects of both digestion and absorption and simply represents the difference between the amount of FA ingested and the amount excreted (Doucet and Ball 1994). There is considerable evidence from a number of fish species, including European sea bass (*Dicentrarchus labrax*), rainbow trout (*Oncorhynchus mykiss*), Arctic charr (*Salvelinus alpinus* L.), and Atlantic salmon (*Salmo salar*), that apparent digestibility varies with FA structure (Olsen et al. 1998, Geurden et al. 2009, Eroldoğan et al. 2013, Huguet et al. 2015). The efficiency of digestion appears to be generally dictated by the extent of unsaturation, with the digestibility of PUFA higher than monounsaturated and saturated FA in teleost fish. The chain length of FA also influenced digestibility in rainbow trout (*Oncorhynchus mykiss*), with longer chain FA being more efficiently absorbed (Austreng et al. 1980). Olsen et al. (1998) demonstrated quite clearly that PUFA (18:2n-6 and 18:3n-3) were consistently better assimilated than saturated and monounsaturated FA. This differential digestion and absorption according to FA structure offer a reasonable explanation for the differences in FA profile between diet and chylomicron. In contrast, we observed no difference in $\delta^{13}C$ for 18:2n-6, 20:5n-3, or 22:6n-3 between diet and

chylomicrons, indicating that lipases and esterases involved in hydrolysis and esterification, the principal processes during digestion and assimilation, did not result in a net fractionation of stable carbon isotopes of those FA in this work. Thus, we expect that $\delta^{13}C$ of 18:2n-6, 20:5n-3, and 22:6n-3 in chylomicrons of wild fish will strongly resemble $\delta^{13}C$ of FA in the last meal consumed and will, therefore, provide information about recent diet.

The isotopic results for 18:3n-3 are more difficult to explain. For 18:3n-3, the $\delta^{13}C$ value in the chylomicrons was 2‰ higher than in the diet, a pattern not likely due to selective fractionation of that FA by lipases, considering that the other PUFA did not exhibit a similar fractionation. Further, fractionation due to hydrolysis and esterification would be expected to result in a lower $\delta^{13}C$ value than found in diet due to kinetic fractionation; it is, in fact, difficult to conceive of any mechanism that would result in the selective fractionation of 18:3n-3 seen here. However, in a comprehensive literature review of bulk $\delta^{13}C$ data for animal tissues, including fish, Caut et al. (2009) found an increase in discrimination factor and hence, fractionation, with decreasing dietary $\delta^{13}C$. Here, 18:3n-3 in the diet had the lowest $\delta^{13}C$ value of the four FA measured. It may be that the same relationship exists for FA data, where fractionation between diet and tissue is greatest for FA having the lowest $\delta^{13}C$ values.

Hydrolysis and esterification—mobilization of FA from liver to serum

There were no differences in $\delta^{13}C$ values of any FA in the serum and fasted liver from which they were mobilized, yielding discrimination factors that were equivalent to zero (Fig. 5). As biochemical changes to FA structure cannot occur within the serum, the similarity of serum and liver $\delta^{13}C$ indicates that FA mobilization did not result in a net fractionation of FA C isotopes in our experiments. Mobilization of stored lipid in gadoids occurs through release of VLDL containing esterified TAG (Sheridan 1988, Kjær et al. 2009). Once excreted, the VLDL delivers TAG to extrahepatic tissues, such as the muscle (Fig. 2), where TAG are hydrolyzed by lipoprotein lipases to yield FFA that can be taken up by the tissue and catabolized. Assuming that fish follow the same general processes as mammals, three types of enzymatically controlled processes govern the formation and release of VLDL particles: (1) hydrolysis and esterification of liver lipids at specific organelles to generate TAG and other lipids for incorporation into VLDL (Wei et al. 2007, Markgraf et al. 2014); (2) addition of intact TAG to the VLDL particles; and (3) transfer of VLDL between organelles within the liver (Tiwari and Siddiqi 2012). Of these three processes, it is only hydrolysis and esterification that can form and break bonds involving carbon and that can, in turn, lead to kinetic fractionation. Thus, data from this fasting experiment agree with that from the postprandial chylomicron/diet experiment (Fig. 4A), indicating that hydrolysis and esterification of 18:2n-6, 20:5n-3, and 22:6n-3 did not lead to a net fractionation of C isotopes under the conditions used in this work. Here, we have demonstrated through two completely separate metabolic processes that the enzymatically controlled steps of FA addition to and removal from the glycerol backbone did not lead to a net kinetic fractionation of stable C isotopes for three of four PUFA examined. This consistency in biomarker signal, when used in trophic studies, will be a significant improvement over methods relying only on FA proportions or bulk isotope data.

In contrast to the consistency in FA $\delta^{13}C$ values, the serum FA proportional data were significantly different than those of the liver from which it was derived, suggesting that specific FA structures were preferentially mobilized. Selective mobilization of FA from adipose tissue of birds and mammals is well known, where the most noticeable and consistent features are retention of 20:1n-9 and mobilization of 20:5n-3 (Raclot 2003, Price and Valencak 2012). In this study, those two FA did not change in proportion in fasted liver and serum, likely because mobilization from liver proceeds through release of intact TAG, rather than FFA. The processes involved in the addition of TAG to VLDL and the movement of VLDL particles between organelles are unlikely to be responsible for the increased proportions of 18:1n-9, 22:1n-11, and 22:6n-3 found in the serum relative to liver because that would require that all three of those FA be consistently esterified together within TAG. While there is evidence to show that FA are not arranged randomly within TAG in storage tissues in mammals

(Brockerhoff et al. 1968), there is no literature to support that particular combination of FA in fish. In fact, Nanton et al. (2006) showed that the most common total carbon numbers in VLDL TAG in haddock were 54 and 56; 18:1n-9, 22:1n-11, and 22:6n-3 yields a total carbon number of 62, among the rarest combination found in that work, although the particular TAG species and associated carbon numbers will vary with diet.

Hydrolysis and esterification, involved in TAG synthesis before addition to VLDL, offer a more likely explanation for the selective mobilization observed here because those steps are controlled by enzymes that act on individual FA. Hydrolysis of FA from TAG by lipase during mobilization from adipose tissue is known to be selective in a number of species including fish (reviewed in Price and Valencak 2012) so it is reasonable to assume that the enzymes that control hydrolysis and esterification in other tissues can also target specific FA structures. This agrees with our results for the transfer of dietary FA to chylomicrons, where variation in FA profile between diet and chylomicrons was evident. Preferential hydrolysis of lipoprotein TAG containing specific FA at the destination tissue (muscle in Fig. 2) may also contribute to the variation in TAG FA profile between serum and fasted liver. While the outliers associated with the serum and fasted liver in the PCA plot (Fig. 3) resulted in greater variance in the mean FA proportions (Table 2), they do serve to further illustrate the effect of this consistent selective hydrolysis and esterification. The four outliers are paired samples from only two fish, showing that the FA profile of the serum continues to have increased proportions of 22:1n-11 and 22:6n-3, arising from selective hydrolysis and/or esterification, despite the unusually elevated amounts of 18:3n-3 in the liver from which the VLDL are assembled. Kjær et al. (2009) also found a similar selective transfer of 22:6n-3 to TAG serum in Atlantic cod, suggesting that it may be an important mechanism to deliver that PUFA to tissues.

Integrated FA metabolism—uptake and modification of dietary FA in the liver

The FA profile of the postprandial liver TAG reflects modification arising from a number of metabolic processes (i.e., elongation, desaturation, β-oxidation of FA), in addition to hydrolysis and esterification associated with digestion and assimilation; however, we still expected to find similarity in liver TAG and diet FA profiles, as seen in other studies (Nanton et al. 2003, Torstensen et al. 2004). The proportions of several FA in the postprandial liver TAG did show obvious changes in response to the diet (i.e., 18:1n-9, 18:2n-6, 18:3n-3, and 20:5n-3; Table 2, Fig. 3). Despite this, the PCA plot (Fig. 3) indicates that, overall, the FA profile of the liver of most postprandial fish remained much more similar to that of the initial fish than to the diet fed. Similarly, we had anticipated that β-oxidation would lead to higher $\delta^{13}C$ values of FA in the liver, due to preferential catabolism of isotopically lighter FA, following the mechanisms originally proposed by DeNiro and Epstein (1978). However, here, we found fractionation varying from +4‰ for 18:2n-6 to −2‰ for 20:5n-3 (Fig. 5), while none was found for 22:6n-3. Taken together, the data indicate that the feeding trial was not conducted for a sufficient period of time to allow the liver FA to fully exchange with those of the new diet.

The initiation of the experiment coincided with a drop in ambient water temperature from 13° to 8°C that may have led to a reduction in metabolic activity (Johnston and Dunn 1987, Sandblom et al. 2014). While the fish did increase in mass during the experiment, it was noted that the pollock showed reduced appetite and interest in the new diet. These factors, combined with a decrease in the lipid content of the diet (from 18% in the commercial maintenance diet to 13% in the experimental diet) and the very high liver lipid content in the initial fish of ~66%, may have created a situation where the fish liver TAG still contained a substantial contribution from the FA signature of the commercial diet. The very similar FA profiles of the initial and postprandial liver TAG (Fig. 3) certainly support this. The $\delta^{13}C$ values measured for 18:2n-6, 18:3n-3, 20:5n-3, and 22:6n-3 in the postprandial liver would also be influenced by FA acquired from the commercial diet used to feed fish prior to the initiation of the experiment and, thus, discrimination factors calculated using these data are not likely accurate nor representative of the fractionation that would truly be found between diet and liver. Future work to replicate these experiments must place more emphasis on the use of realistic diets

and growth conditions that elicit physiological responses that are similar to those of fish in their natural environment.

β-Oxidation—comparison of postprandial and fasted liver

De novo synthesis of long-chain PUFA in the fasted liver is impossible, and elongation/desaturation were likely minimal. In particular, the very similar FA profiles of the fed and fasted liver TAG (Fig. 3, Table 2) indicate that elongation and desaturation of FA were not occurring at detectable levels; not all FA are equivalent candidates for elongation and desaturation (Price and Valencak 2012) so the similar profiles in fed and fasted fish would not be possible if those processes were occurring. Mobilization, involving principally hydrolysis and esterification, and investigated above, did not lead to fractionation in our study. Thus, we can interpret the differences between fed and fasted liver data as primarily due to β-oxidation. Oxidation, like hydrolysis and esterification, would be expected to preferentially utilize FA depleted in ^{13}C, resulting in FA with higher $\delta^{13}C$ accumulating in the liver. However, the differences in FA $\delta^{13}C$ values in fed and fasted liver were not significant, indicating that β-oxidation did not fractionate C isotopes in this experiment. This also supports our assertion that the postprandial data suffered from incomplete turnover of FA; because β-oxidation and mobilization did not fractionate C isotopes of FA, incomplete turnover is the only reasonable explanation for the apparent fractionation observed between diet and postprandially sampled liver.

The relatively high fat diet fed to fish may have also influenced the patterns seen in FA proportions and $\delta^{13}C$ values. Wild pollock typically have a liver lipid content of ~25% (Townsend 2015), compared to 66% found here, suggesting that unusually large amounts of lipid had accumulated in our experimental fish. In this situation, oxidation and mobilization of FA may not have occurred to sufficient extent to cause a noticeable change in the FA proportions or $\delta^{13}C$ values. This potential masking of the effects of oxidation and mobilization would not be expected in wild fish with lower lipid levels and again points to the importance of conducting feeding studies that use lipid and FA concentrations similar to those encountered in the natural environment.

Applications to trophic studies

Combined biomarker approaches, coupling stable isotope analyses of total organic material with FA analysis, have become common in studies of trophic ecology, ranging in complexity from qualitative techniques, correlating changes in FA profiles and $\delta^{13}C$ data with spatial and temporal variation in predator foraging (Galloway et al. 2013, Browning et al. 2014), to more quantitative applications, making use of mixing models applied to prey stable isotope or FA signatures (Iverson et al. 2004, Parnell et al. 2010, Galloway et al. 2014*a*, *b*, Neubauer and Jensen 2015). The primary focus of this work was to investigate the modifications that lean fish make to dietary FA $\delta^{13}C$ values during and after assimilation into their tissues so that $\delta^{13}C$ of individual FA can also be incorporated into such diet models.

By comparison among tissues and to diet, we have shown that hydrolysis, esterification, and β-oxidation did not result in a net fractionation of stable C isotopes of FA under the conditions used in these experiments. However, the dissimilar FA profiles of diet and postprandial liver indicated insufficient turnover of lipid in that tissue, likely due to the relatively high fat content of the initial diet fed, and prevented us from evaluating the integrated result of FA metabolism in fish liver. Thus, we consider the discrimination factors derived for the transfer of FA between diet and postprandial liver TAG to be unreliable, and our data cannot be applied to study long-term dietary records preserved in liver FA. More controlled feeding studies of sufficient duration, using realistic growth conditions and diets with lower lipid content, are needed to better estimate fractionation arising from the combined metabolic processes that liver FA experience. Such experiments could also be used to validate the discrimination factors reported here for a diet with a relatively high dietary fat content of 13%.

In contrast to the comparison of diet and postprandial liver data, we found strong similarity in FA proportions in diet and chylomicron TAG and in fasted liver and serum TAG. This similarity suggests that the discrimination factors associated with FA transfer between diet and chylomicrons, and fasted liver and serum are reliable. Therefore, our results support the use of chylomicron TAG in serum as indicators of dietary FA

$\delta^{13}C$ values, providing information about recent diet. Replication of these results using different species, diets, and conditions is an important next step to confirm their applicability to wild populations. As our understanding of the metabolic processes causing fractionation increases, the accuracy of discrimination factors will also increase, allowing greater application of $\delta^{13}C$ values of FA in quantitative models to address general questions in trophic ecology.

Acknowledgments

We thank Sean Tibbetts at NRC, Sandy Cove, Nova Scotia, for assistance in producing the experimental diets and the staff of the Aquatron, Dalhousie University, for their help in rearing the salmon. We also thank Geert Van Biesen at the CREAIT Network at Memorial University of Newfoundland and Anne Timmins at the Canadian Institute of Fisheries Technology at Dalhousie University for assistance with sample analysis. Financial support was provided by the Natural Sciences and Engineering Research Council of Canada.

Literature Cited

Austreng, E., A. Skrede, and Å. Eldegard. 1980. Digestibility of fat and fatty acids in rainbow trout and mink. Aquaculture 19:93.

Brockerhoff, H., R. J. Hoyle, P. C. Hwang, and C. Litchfield. 1968. Positional distribution of fatty acids in depot triglycerides of aquatic animals. Lipids 3:24–29.

Browning, N. E., S. D. McCulloch, G. D. Bossart, and G. A. J. Worthy. 2014. Fine-scale population structure of estuarine bottlenose dolphins (*Tursiops truncatus*) assessed using stable isotope ratios and fatty acid signature analyses. Marine Biology 161:1307–1317.

Budge, S. M., S. J. Iverson, and H. N. Koopman. 2006. Studying trophic ecology in marine ecosystems using fatty acids: a primer on analysis and interpretation. Marine Mammal Science 22:759–801.

Budge, S. M., S. N. Penney, and S. P. Lall. 2011. Response of tissue lipids to diet variation in Atlantic salmon (*Salmo salar*): implications for estimating diets with fatty acid analysis. Journal of Experimental Marine Biology and Ecology 409:267–274.

Budge, S. M., A. M. Springer, S. J. Iverson, and G. Sheffield. 2007. Fatty acid biomarkers reveal niche separation in an Arctic benthic food web. Marine Ecology Progress Series 336:305–309.

Budge, S. M., M. J. Wooller, A. M. Springer, S. J. Iverson, C. P. McRoy, and G. J. Divoky. 2008. Tracing carbon flow in an arctic marine food web using fatty acid-stable isotope analysis. Oecologia 157:117–129.

Caut, S., E. Angulo, and F. Courchamp. 2009. Variation in discrimination factors ($\delta^{15}N$ and $\delta^{13}C$): the effect of diet isotopic values and applications for diet reconstruction. Journal of Applied Ecology 46:443–453.

Chamberlain, P., I. Bull, H. J. Black, P. Ineson, and R. Evershed. 2004. Lipid content and carbon assimilation in Collembola: implications for the use of compound-specific carbon isotope analysis in animal dietary studies. Oecologia 139:325–335.

Cunnane, S. C. 2003. Problems with essential fatty acids: Time for a new paradigm? Progress in Lipid Research 42:544–568.

Dalsgaard, J., M. St John, G. Kattner, D. Muller-Navarra, and W. Hagen. 2003. Fatty acid trophic markers in the pelagic marine environment. Advances in Marine Biology 46:225–340.

Davis, R. A. 1991. Lipoprotein structure and secretion. Pages 403–426 *in* J. E. Vance and D. E. Vance, editors. Biochemistry of lipids, lipoproteins and membranes. Second edition. Elsevier Science, Amsterdam, The Netherlands.

DeNiro, M. J., and S. Epstein. 1978. Influence of diet on the distribution of carbon isotopes in animals. Geochimica et Cosmochimica Acta 42:495–506.

Denstadli, V., A. Vegusdal, Å. Krogdahl, A. M. Bakke-McKellep, G. M. Berge, H. Holm, M. Hillestad, and B. Ruyter. 2004. Lipid absorption in different segments of the gastrointestinal tract of Atlantic salmon (*Salmo salar* L.). Aquaculture 240:385–398.

Doucet, C. M., and J. P. Ball. 1994. Analysis of digestion data: apparent and true digestibilities of foods eaten by beavers. American Midland Naturalist 132:239–247.

Eroldoğan, T. O., A. H. Yilmaz, G. M. Turchini, M. Arslan, N. A. Sirkecioğlu, K. Engin, I. Özçahinoğlu, and P. Mumoğullarinda. 2013. Fatty acid metabolism in European sea bass (*Dicentrarchus labrax*): effects of n-6 PUFA and MUFA in fish oil replaced diets. Fish Physiology and Biochemistry 39:941–955.

Evershed, R. P., I. D. Bull, L. T. Corr, Z. M. Crossman, B. E. van Dongen, C. J. Evans, S. Jim, H. R. Mottram, A. J. Mukherjee, and R. D. Pancost. 2008. Compound-specific stable isotope analysis in ecology and paleoecology. Pages 480–540 *in* M. Michener and K. Lajtha, editors. Stable isotopes in ecology and environmental science. Blackwell, Oxford, UK.

Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry 226:497–509.

Fry, B. 2006. Stable isotope ecology. Springer, New York, New York, USA.

Galloway, A. W. E., A. T. Lowe, E. A. Sosik, J. S. Yeung, and D. O. Duggins. 2013. Fatty acid and stable isotope biomarkers suggest microbe-induced differences in benthic food webs between depths. Limnology and Oceanography 58:1451–1462.

Galloway, A. W. E., M. E. Eisenlord, M. N. Dethier, G. Holtgrieve, and M. T. Brett. 2014*a*. Quantitative estimates of isopod resource utilization using a Bayesian fatty acid mixing model. Marine Ecology Progress Series 507:219–232.

Galloway, A. W. E., S. Taipale, M. Hultunen, E. Peltomaa, U. Strandberg, M. T. Brett, and P. Kankaala. 2014*b*. Diet specific biomarkers show that high quality phytoplankton fuels herbivorous zooplankton in large boreal lakes. Freshwater Biology 59:1902–1915.

Gannes, L. Z., D. M. O'Brien, and C. M. del Rio. 1997. Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 78:1271–1276.

Geurden, I., F. Jutfelt, R. Olsen, and K. S. Sundell. 2009. A vegetable oil feeding history affects digestibility and intestinal fatty acid uptake in juvenile rainbow trout *Oncorhynchus mykiss*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 152:552–559.

Gonçalves, A. M. M., U. M. Azeiteiro, M. A. Pardal, and M. De Troch. 2012. Fatty acid profiling reveals seasonal and spatial shifts in zooplankton diet in a temperate estuary. Estuarine, Coastal and Shelf Science 109:70–80.

Hammer, B. T., M. L. Fogel, and T. C. Hoering. 1998. Stable carbon isotope ratios of fatty acids in seagrass and redhead ducks. Chemical Geology 152:29–41.

Honkanen, R., M. Rigler, and J. Patton. 1985. Dietary fat assimilation and bile salt absorption in the killifish intestine. American Journal of Physiology 249:G399–G407.

Howland, M. R., L. T. Corr, S. M. M. Young, V. Jones, S. Jim, N. J. Van Der Merwe, A. D. Mitchell, and R. P. Evershed. 2003. Expression of the dietary isotope signal in the compound-specific d^{13}C values of pig bone lipids and amino acids. International Journal of Osteoarchaeology 13:54–65.

Huguet, C. T., F. Norambuena, J. A. Emery, K. Hermon, and G. M. Turchini. 2015. Dietary n-6/n-3 LC-PUFA ratio, temperature and time interactions on nutrients and fatty acids digestibility in Atlantic salmon. Aquaculture 436:160–166.

Iverson, S. J., C. Field, D. W. Bowen, and W. Blanchard. 2004. Quantitative fatty acid signature analysis: a new method of estimating predator diets. Ecological Monographs 74:211–235.

Johnston, I. A., and J. Dunn. 1987. Temperature acclimation and metabolism in ectotherms with particular reference to teleost fish. Symposia of the Society for Experimental Biology 41:67–93.

Kjær, M. A., A. Vegusdal, G. M. Berge, T. F. Galloway, M. Hillestad, Å. Krogdahl, H. Holm, and B. Ruyter. 2009. Characterisation of lipid transport in Atlantic cod (*Gadus morhua*) when fasted and fed high or low fat diets. Aquaculture 288:325–336.

Larsen, T., M. Ventura, N. Andersen, D. M. O'Brien, U. Piatkowski, and M. D. McCarthy. 2013. Tracing carbon sources through aquatic and terrestrial food webs using amino acid stable isotope fingerprinting. PLoS ONE 8:e73441.

Lie, Ø., A. Sandvin, and R. Waagbø. 1993. Influence of dietary fatty acids on the lipid composition of lipoproteins in farmed Atlantic salmon (*Salmo salar*). Fish Physiology and Biochemistry 12:249–260.

Markgraf, D. F., R. W. Klemm, M. Junker, H. Hannibal-Bach, C. Ejsing, and T. Rapoport. 2014. An ER protein functionally couples neutral lipid metabolism on lipid droplets to membrane lipid synthesis in the ER. Cell Reports 6:44–55.

McInnis, M. L., M. Vavra, and W. C. Krueger. 1983. A comparison of four methods used to determine the diets of large herbivores. Journal of Range Management 36:302–306.

McMahon, K. W., M. J. Polito, S. Abel, M. D. McCarthy, and S. R. Thorrold. 2015. Carbon and nitrogen isotope fractionation of amino acids in an avian marine predator, the gentoo penguin (*Pygoscelis papua*). Ecology and Evolution 5:1278–1290.

Mills, G. L., P. A. Lane, and P. K. Weech. 2000. A guidebook to lipoprotein techniques. Elsevier Science, New York, New York, USA.

Moody, A., K. Hobson, and A. Gaston. 2012. High-arctic seabird trophic variation revealed through long-term isotopic monitoring. Journal of Ornithology 153:1067–1078.

Nanton, D. A., S. P. Lall, N. W. Ross, and M. A. McNiven. 2003. Effect of dietary lipid level on fatty acid b-oxidation and lipid composition in various tissues of haddock, *Melanogrammus aeglefinus* L. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 135:95–108.

Nanton, D., M. A. McNiven, and S. Lall. 2006. Serum lipoproteins in haddock, *Melanogrammus aeglefinus* L. Aquaculture Nutrition 12:363–371.

Neubauer, P., and O. P. Jensen. 2015. Bayesian estimation of predator diet composition from fatty acids and stable isotopes. PeerJ 3:e920.

Olsen, R. E., R. J. Henderson, and E. Ringø. 1998. The digestion and selective absorption of dietary fatty acids in Arctic charr, *Salvelinus alpinus*. Aquaculture Nutrition 4:13–21.

Olsen, R. E., and E. Ringø. 1997. Lipid digestibility in fish: a review. Pages 199–265 *in* S. G. Pandalai, editor.

Recent research developments in lipid research. Volume 1. Transworld Research Network, Trivandrum, India.

Parnell, A. C., R. Inger, S. Bearhop, and A. L. Jackson. 2010. Source partitioning using stable isotopes: coping with too much variation. PLoS ONE 5:e9672.

Pond, D. W., M. V. Bell, D. R. Dixon, A. E. Fallick, M. Segonzac, and J. R. Sargent. 1998. Stable-carbon-isotope composition of fatty acids in hydrothermal vent mussels containing methanotrophic and thiotrophic bacterial endosymbionts. Applied and Environmental Microbiology 64:370–375.

Price, E. R., and T. G. Valencak. 2012. Changes in fatty acid composition during starvation in vertebrates: mechanisms and questions. Pages 237–255 *in* M. D. McCue, editor. Comparative physiology of fasting, starvation, and food limitation. Springer, New York, New York, USA.

Raclot, T. 2003. Selective mobilization of fatty acids from adipose tissue triacylglycerols. Progress in Lipid Research 42:257–288.

Ruess, L., A. Tiunov, D. Haubert, H. H. Richnow, M. M. Häggblom, and S. Scheu. 2005. Carbon stable isotope fractionation and trophic transfer of fatty acids in fungal based soil food chains. Soil Biology and Biochemistry 37:945–953.

Sandblom, E., A. Gräns, M. Axelsson, and H. Seth. 2014. Temperature acclimation rate of aerobic scope and feeding metabolism in fishes: implications in a thermally extreme future. Proceedings of the Royal Society of London B: Biological Sciences 281:20141490.

Schell, D. M. 2000. Declining carrying capacity in the Bering Sea: isotopic evidence from whale baleen. Limnology and Oceanography 45:459–462.

Sheridan, M. A. 1988. Lipid dynamics in fish: aspects of absorption, transportation, deposition and mobilization. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 90:679–690.

Silfer, J. A. 1991. Stable carbon and nitrogen isotope signatures of amino acids as molecular probes in geologic systems. Dissertation. University of Oklahoma, Norman, Oklahoma, USA.

Tarroux, A., J. Bêty, G. Gauthier, and D. Berteaux. 2012. The marine side of a terrestrial carnivore: intra-population variation in use of allochthonous resources by Arctic foxes. PLoS ONE 7:e42427.

Tiwari, S., and S. A. Siddiqi. 2012. Intracellular trafficking and secretion of VLDL. Arteriosclerosis, Thrombosis, and Vascular Biology 32:1079–1086.

Tocher, D. 2003. Metabolism and functions of lipids and fatty acids in teleost fish. Reviews in Fisheries Science 11:107–184.

Torstensen, B. E., L. Frøyland, and Ø. Lie. 2004. Replacing dietary fish oil with increasing levels of rapeseed oil and olive oil—effects on Atlantic salmon (*Salmo salar* L.) tissue and lipoprotein lipid composition and lipogenic enzyme activities. Aquaculture Nutrition 10:175–192.

Townsend, K. 2015. The influence of dietary fat concentration on fatty signatures and stable carbon isotopes in liver tissue of Atlantic pollock (*Pollachius virens*). Thesis. Dalhousie University, Halifax, Nova Scotia, Canada.

von Biela, V., C. Zimmerman, B. Cohn, and J. Welker. 2013. Terrestrial and marine trophic pathways support young-of-year growth in a nearshore Arctic fish. Polar Biology 36:137–146.

Wang, S. W., S. J. Iverson, A. M. Springer, and S. A. Hatch. 2009. Spatial and temporal diet segregation in northern fulmars *Fulmarus glacialis* breeding in Alaska: insights from fatty acid signatures. Marine Ecology Progress Series 377:299–307.

Wei, E., M. Alam, F. Sun, L. B. Agellon, D. E. Vance, and R. Lehner. 2007. Apolipoprotein B and triacylglycerol secretion in human triacylglycerol hydrolase transgenic mice. Journal of Lipid Research 48:2597–2606.

Yonezaki, S., M. Kiyota, N. Baba, T. Koido, and A. Takemura. 2003. Size distribution of the hard remains of prey in the digestive tract of northern fur seal (*Callorhinus ursinus*) and related biases in diet estimation by scat analysis. Mammal Study 28:97–102.