THE INFLUENCE OF DIETARY FAT CONCENTRATION ON FATTY ACID SIGNATURES AND STABLE CARBON ISOTOPES OF FATYY ACIDS IN LIVER OF ATLANTIC POLLOCK (*POLLACHIUS VIRENS*)

by

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LIST OF ABBREVIATIONS USED

AA	Amino acid
ANOVA	Analysis of variance
CSIA	Compound-specific stable isotope analysis
EAA	Essential amino acid
EFA	Essential fatty acid
ER	Endoplasmic reticulum
FA	Fatty acid
FAME	Fatty acid methyl esters
FFA	Free fatty acid
FID	Flame ionization detector
GC	Gas chromatography
GC-C-IRMS	Gas chromatography-combustion-isotope ratio mass spectrometer
MANOVA	Multivariate analysis of variance
QFASA	Quantitative Fatty Acid Signature Analysis
SI	Stable isotope
SIMPER	Similarity percentage
TAG	Triacylglycerol
TLC	Thin Layer Chromatography
VPDB	Vienna Pee Dee Belemnite

ABSTRACT

A number of biomarker techniques have been employed to investigate animal diets, including analyses of fatty acid (FA) proportions and stable carbon isotopic signatures. Here, the influence of dietary FA concentrations on fish FA signatures and $\delta^{13}C$ values of FA in fish liver was evaluated by feeding Atlantic pollock (*Pollachius virens*; n=108) three diets with different FA concentrations for 20 weeks. Calibration coefficients (CC) and discrimination factors were calculated to compare the FA profile and $\delta^{13}C$ values of FA in liver to those of diet. Most CC showed significant variation across the three diets; however, a number of FA present in smaller proportions had CC that did not vary among the diets. Overall, the two CC sets derived from experiments utilizing feeds with the higher FA concentrations were very similar to each other and showed substantial variation compared to CC derived from feeding the diet with the lowest FA concentrations. Using Quantitative Fatty Acid Signature Analysis (QFASA) and the initial samples of pollock, the three sets of CC derived from fish fed the three treatment diets were applied to determine the influence of these three CC on diet estimates. The results indicated that QFASA may be less sensitive to variation in CC than previously assumed. Discrimination factors calculated using liver and diet $\delta^{13}C$ values varied with dietary FA concentration for five of ten FA. Most discrimination factors for saturated and monounsaturated FA (MUFA) showed significant deviations from zero; in contrast, few discrimination factors for polyunsaturated FA (PUFA), specifically 20:5n-3 and 22:6n-3, were significantly different than zero. These results suggest that $\delta^{13}C$ values for PUFA sampled from a wild fish would closely mirror that of the prey consumed. Combined biomarker techniques, employing both FA proportions and $\delta^{13}C$ values, may prove to be the most reliable in estimating diets of animals in the wild at this time.

CHAPTER 1.0 INTRODUCTION

Knowledge of animal diets is key in examining relationships between predator and prey species. At the most basic level, the examination of predator diets can provide insight into foraging ecology (eg, Polo-Silva et al., 2013) but may also reveal information about migration patterns (Tomiyama et al., 2013) and the effects that predator species have on prey populations (deYoung and Rose, 1993). Gathering information about animal diets is usually the first step in conservation efforts (Hilderbrand et al., 1999). Such interest in diet has led to the development of a number of methods of diet investigation in the marine environment.

Traditional methods of analyzing animal diets include the examination of stomach contents and fecal analysis, both of which have been improved upon by combination with DNA sequencing techniques (Symondson, 2002; Carreon-Martinez et al., 2011; Paquin et al., 2014). However, there are a number of limitations associated with these methods (Furness et al., 1984; Bowen, 2000; Bowen and Iverson, 2012). Some species do not have hard parts or have hard parts that are rapidly digested (e.g. crustacean shells (Bowen, 2000)) and are therefore underestimated in the diet. Overestimation of a particular species can also occur if there is an accumulation of one specific hard part, such as squid beaks (e.g. Furness et al., 1984). With these approaches, only the last meal consumed can be studied, providing a small window into the animal's diet. Due to these limitations, focus has shifted to the development of new biomarker-based approaches using fatty acids (FA) profiles and stable isotopes of carbon and nitrogen and fatty acids (FA).

1.1 FATTY ACIDS

Due to the variety of structures in marine organisms, fatty acids (FA) can serve as biomarkers, indicating which prey a predator has consumed (Copeman et al., 2009; Budge et al., 2011). The structure of marine FA changes very little as they travel through the food web (Budge et al., 2006) because there are biochemical restrictions on the *de novo* synthesis of a number of FA structures (Dalsgaard et al., 2003; Budge et al., 2008). For instance, animals cannot synthesize FA with double bonds at the n-3 and n-6 positions so they rely on organisms at lower trophic levels to provide those essential FA (EFA). Animal FA are defined by the number of carbon atoms and the number and position of double bonds. They can be saturated (no double bonds), monounsaturated (one double bond; MUFA), or polyunsaturated (more than one double bond; PUFA). FA are expressed as *A:Bn-x*, where *A* represents the number of carbon atoms, *B* represents the number of double bonds and *x* indicates the position of the first double bond from the methyl end of the carbon chain.

FA biomarkers have been used to study trophic dynamics of marine ecosystems, and while individual FA biomarkers cannot quantify the dietary components of the consumer, FA that are exclusive to diet can in some cases confirm specific dietary contributions. For instance, FA were used to identify the trophic levels of Antarctic and Arctic krill (Falk-Petersen et al., 2000), to determine dominance of demersal or pelagic fish in herring gull diet (Käkelä et al., 2005), and to identify subtle differences in Arctic pinniped feeding niches (Budge et al., 2007). Subtle patterns, or profiles, of EFA can also be compared among predators to deduce general information about similarity of diets. This approach has been used to study temporal and spatial variation in a number of

species including rainbow trout (Babin, 1987), North Atlantic cod (Alkanani et al., 2005), and bowhead whales (Budge et al., 2008).

More complex methods have been proposed to estimate proportions and types of prey species in individual predator diets using FA profiles. One technique, Quantitative Fatty Acid Signature Analysis (QFASA), uses calibration coefficients (CC; %FA in consumer/%FA in prey) and a statistical model to compare the FA profile of a predator's tissue with combinations of FA signatures of possible prey to calculate the most probable components of a diet profile, while taking into account lipid metabolism in the predator that may cause modifications in the proportions of FA (Budge et al., 2012). QFASA can provide a relatively long-term estimate of animal diets and attempts to identify the relative proportions of individual prey items in a diet (Iverson et al., 2004). When comparing diets of large predators, QFASA has been shown to be quite useful (Iverson et al., 2004). For instance, Beck et al. (2007) used QFASA to determine sex differences in the diets of grey seals, while Iverson et al. (2007) used QFASA to discriminate diet differences in four seabird species.

1.2 STABLE ISOTOPES

Isotopes are forms of the same element that differ from each other in the number of neutrons in the nucleus, and therefore differ in atomic mass. The most commonly used elements in ecological applications are carbon (^{12}C and ^{13}C) and nitrogen (^{14}N and ^{15}N). Like dietary FA, stable carbon isotopes vary in a fixed and predictable manner within a food web (DeNiro and Epstein, 1978) and can be used to gather information about the environmental origin of biological specimens based on their $^{12}C/^{13}C$ ratio (Smith et al., 1996; Pond et al., 1998). Stable isotopes undergo fractionation as they move through the

food web, meaning molecules with the lighter, ^{14}N or ^{12}C, isotope are selectively utilized in biochemical reactions over the heavier, ^{15}N or ^{13}C. Since the lighter isotope, ^{14}N, is preferentially excreted in urea and ammonia, the consumer becomes enriched in the heavier isotope, ^{15}N, by ~3 ‰ (Minigawa and Wada, 1984). This enrichment can be used to estimate predator trophic levels (Gannes et al., 1997). Similarly, ^{13}C isotopes are usually enriched in the predator by ~1 ‰, due to the preferential release of the isotopically lighter ^{12}C during metabolic fractionation, which occurs during catabolism and synthesis of different tissue types (DeNiro and Epstein, 1978).

1.3 LIPID METABOLISM

Lipids and their constituent FA are major components of fish and play a large role as sources of metabolic energy for growth, reproduction, locomotion, and migration (Tocher, 2003). Animal lipids can be categorized into two different groups: polar lipids and neutral lipids. Polar lipids are primarily comprised of phospholipids (PL), while neutral lipids are comprised primarily of triacylglycerols (TAG), which are thought to be better indicators of diet (Tocher, 2003) since excess dietary fat is stored as TAG in most animals.

Fish receive the greater part of their dietary fats in the form of TAG, which are made up of three FA esterified to a glycerol backbone. During digestion in the small intestine, TAG are hydrolyzed by pancreatic enzymes to form free fatty acids (FFA) and monoacylglycerols (MAG), which are then transported across the cell membrane of the intestinal cells (Sheridan, 1988; Olsen and Ringo, 1997). Once in the endoplasmic reticulum (ER) of the intestinal cells, the dietary FFA are re-esterified to form TAG. The TAG then enters a pool of endogenous and dietary TAG, the latter of which is

preferentially transferred to the Golgi apparatus to be assembled into chylomicrons (Sheridan, 1988; Olsen and Ringo, 1997). Chylomicrons are one of several lipoproteins responsible for FA transport in the blood (Chapman, 1985). Chylomicrons are high-density lipoproteins that consist of a TAG core and an exterior PL monolayer.

Chylomicrons are transported in the blood to lipoprotein receptors on adipose cells and are hydrolyzed to FFA via lipoprotein lipase. FFA are then absorbed by the adipose where they are re-esterified into TAG and stored. The liver takes up any remaining TAG in chylomicrons and remnants of chylomicrons, via lipoprotein lipase, where they are oxidized, stored or incorporated into high-density lipoproteins (HDL) (Kwiterovich, 2000; Tocher, 2003). In fatty fish, such as salmon, the lipid is deposited in the muscle, while in lean fish, such as pollock, the lipid is stored in the liver.

In vertebrates, both dietary and endogenous FA can take part in a number of biochemical reactions before or after deposition at the storage sites. For instance, FA may be catabolized for energy through β-oxidation resulting in the breakdown of the FA chain. Desaturation of FA may also occur, where a new double bond is inserted in the carbon chain. The FA chain can also be elongated or shortened. Last, FA may serve as precursors for specific hormone-like molecules known as eicosanoids that have important biological functions such as contraction or relaxation of muscle, stimulation or inhibition of platelet function, activation of leukocytes, and regulation of renal blood flow and mineral metabolism (Marcus, 1984). All of these processes, in addition to hydrolysis and esterification, are enzyme-mediated and result in the modification of the proportions of FA from diet, so that a predator's FA profile is not the same as the diet FA profile. Wherever these reactions occur fractionation of stable carbon isotopes of FA may also

take place due to kinetic isotope effects associated with enzymatic reactions. It has been consistently shown that saturated and MUFA are readily catabolized by β-oxidation; however, there is uncertainty as to the extent that PUFA will undergo β-oxidation (Tocher, 2003). Aquaculture studies have shown preferential catabolism of FA found in the highest proportions (Castledine and Buckley, 1980; dos Santos et al., 1993; Nanton et al., 2003). By maintaining constant proportions of FA in the diet but varying FA concentration, it can be determined whether changes in apparent catabolism of FA are due to dietary concentration rather than proportion.

1.4 OBJECTIVES

My objectives were twofold. First, I determined if dietary fat level, and therefore dietary FA concentration, rather than FA proportion, would influence the modification of liver FA signature in Atlantic pollock (*Pollachius virens*). In other words, I determined if a diet with higher dietary FA concentration results in a greater difference between predator tissue and diet compared to a diet with lower FA concentrations. This was achieved by carrying out a controlled feeding study where pollock were fed three diets of varying fat content, ranging from 4-10 %, while maintaining near constant FA proportions. Calibration coefficients (CC) of all FA in the liver of fish fed different diets will be compared, with the expectation that these will vary with different dietary FA concentration.

Second, I determined whether carbon isotopes of FA in pollock liver changed in relation to differing concentrations of FA in diets by comparing the $\delta^{13}C$ isotopes of specific FA in liver samples to the $\delta^{13}C$ of the same FA after a controlled feeding study. I also determined if the fish preferentially oxidize isotopically lighter FA (those with

greater proportion of ^{12}C), causing an accumulation of ^{13}C in the tissues, when fed a higher fat diet. Thus, I determined if fractionation varies with dietary FA concentration. This was done using compound specific isotope analysis (CSIA), analyzing ten FA including three PUFA (18:2n-6, 20:5n-3, and 22:6n-3).

CHAPTER 2 INFLUENCE OF DIETARY FAT CONCENTRATION ON FATTY ACID PROFILES OF LIVER TRIACYLGLYCEROLS OF ATLANTIC POLLOCK (*POLLACHIUS VIRENS*)

2.1 INTRODUCTION

FA have particular promise as biomarkers because they have a variety of structures with differing numbers and positions of carbon-carbon double bonds, as well as differing chain lengths. Most applications using FA have been qualitative in nature, often examining temporal and spatial variation in a number of species including rainbow trout (Babin, 1987), North Atlantic cod (Alkanani et al., 2005), and bowhead whales (Budge et al., 2008). A number of more quantitative methods employing FA have been developed recently (Galloway et al., 2014; Neubauer and Jensen, 2015) but the technique proposed by Iverson et al. (2004), Quantitative Fatty Acid Analysis (QFASA) remains the most highly used in estimating animal diets using FA biomarkers.

QFASA compares the FA profile of a predator's tissue with combinations of FA signatures of possible prey, while taking into account lipid metabolism in the predator that may cause modifications in the proportions of FA (Iverson et al., 2004). QFASA has four requirements: 1) a library of FA signatures of the prey; 2) a suitable storage tissue where FA are deposited in the predator; 3) calibration coefficients (CC) for each FA found in the predator tissues that account for changes in FA proportions between prey and predator due to the predator's metabolism; and 4) a statistical model to compare the signatures of predator and prey (Iverson et al. 2007). Of these requirements, CC are arguably the most important and difficult to determine. Dietary FA are modified by a variety of biochemical processes, including desaturation, elongation, and β-oxidation

before and after deposition in the tissues (Castledine and Buckley, 1980; dos Santos et al., 1993; Nanton et al., 2003). While initial QFASA applications have assumed that FA metabolism, and therefore CC, is consumer specific, more recent work has suggested it may also be influenced by the FA profile of the diet (Budge et al., 2012).

CC represent simple ratios of the amounts of FA in the consumer's tissues relative to that of the diet (CC = %FA in consumer/%FA in prey) (Budge et al., 2012). They are normally determined by prolonged feeding trials (Iverson et al., 2004) where it is assumed that the consumer's tissue has undergone complete FA turnover, causing the consumer's tissue FA to most closely resemble that of the diet (Wang et al., 2009). For convenience, most studies with captive fish use dry diets with lipid concentrations far exceeding those encountered in the wild. For instance, Budge et al. (2012) used dry diets with a lipid concentration of 16 % to derive CC for Atlantic salmon; typical prey of Atlantic pollock would rarely, if ever, contain lipid proportions > 5 %. The effects of such elevated FA concentrations on fish metabolism are not known. However, aquaculture nutrition studies (Castledine and Buckley, 1980; dos Santos et al., 1993; Nanton et al., 2003) have suggested that in haddock, cod, and rainbow trout, the FA present in larger portions are catabolized more readily than those present in smaller portions. Oxidation also appears to vary with FA structure, with PUFA less readily oxidized via β-oxidation than MUFA (Turchini et al., 2009); therefore, the FA composition of the diet may cause CC to vary, and introduce inaccuracies in QFASA estimates.

Using controlled feeding studies with Atlantic pollock, I investigated variations in patterns of FA deposition in the liver, the principal fat storage site in pollock. I

specifically tested the hypothesis that CC and subsequent QFASA diet estimates will vary with concentration of FA in the diet. The specific objectives were: 1) to determine the effect of dietary FA concentration and profile on CC; and 2) to evaluate the impact of variation in CC on QFASA diet estimates. Reliable CC are needed to use QFASA to estimate diets of wild fish; this work will establish the sensitivity of CC to dietary FA content.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL FISH

Adult wild Atlantic pollock (*Pollachius virens*) were caught on July 31st 2012 and August 3rd 2012. The fish (n=141) were kept in a 1,000 L aerated holding tank aboard the vessel and transferred via truck to the Aquatron Facility at Dalhousie University. An initial sample of 11 fish was euthanized using tricaine methanesulfonate (MS-222) at a concentration of 25 mg L^{-1}. Liver was removed and lipids were extracted as described below. The remaining fish (n = 108) were anaesthetized by brief immersion (MS-222, 25 mg L^{-1}), tagged with a Passive Integrated Transponder (PIT) tag near the dorsal fin, weighed, and length was measured. They were then separated into 9 tanks with 3 tanks for each diet and 12 fish in each tank. The tanks were aerated with flow-through seawater at ambient temperature from the Northwest Arm of Halifax Harbour. Since the water temperature of the inlet varied from July to January, the water was heated upon entry to the tanks and was held at ~13 °C, as tissue turnover rate is dependent on temperature (Hazel, 1984). Tanks were 3 cubic meters, holding 2935 L of seawater each.

Pollock (n = 108) were maintained on one of three experimental diets based on herring oil and each tank of fish was given 1/3 cup (~40 g) twice a day for a period of 20

weeks. This amount was estimated to bring the fish to satiation. At the end of the 20 weeks, all fish were euthanized as described above and liver samples were collected.

2.2.2 EXPERIMENTAL DIETS

Whole flounder (118 kg) was purchased from AC Coverts Distributors in Dartmouth, Nova Scotia. The dry ingredients were mixed at the feed lab at the National Research Council (NRC) at Sandy Cove, NS (Table 2.1). A meat grinder (model 4.532, Hobart Manufacturing Co. Ltd) in the pilot plant at the Canadian Institute of Fisheries Technologies at Dalhousie University, Sexton Campus was used to grind whole flounder. The ground flounder was then mixed in 50:50 proportions by weight with the dry ingredients (Table 2.1) and passed through the grinder again, to make moist pellets of ~4 %, ~7 %, and ~10 % lipid respectively (referred to as Diet 1, 2, and 3). The diets were frozen at -30 °C on metal trays immediately after production. Frozen diets were transferred to Rubbermaid totes where they were stored frozen until consumed by the fish. To ensure consistency, lipid content was measured after each batch of diet was made and after all batches of each diet were combined. In total 96 kg Diet 1, 70 kg of Diet 2 and 60 kg of Diet 3 were produced.

Table 2.1: Moist feed formulation for Diets 1, 2, and 3 containing ~ 4 %, 7 %, and 10 % lipid respectively. Lipid content was determined and is reported as mean (sd).

	Percentage		
Ingredients	**Diet 1**	**Diet 2**	**Diet 3**
Ground fish	50	50	50
Fish meal	26	26	26
Krill meal	1.5	1.5	1.5
Wheat middlings	15.5	12.5	9.5
Corn starch	4	4	4
Vitamin mix	1	1	1
Mineral mix	1	1	1
Fish oil	1	4	7
Choline chloride	0.2	0.2	0.2
Total	100	100	100
Estimated lipid content	**4**	**7**	**10**
Actual FA concentration	**4.9 (0.29)**	**7.5 (0.88)**	**9.1 (0.41)**

2.2.3 LIPID EXTRACTION

Whole liver were removed and homogenized separately in a 1.7 L food processor. Diet samples were ground with a mortar and pestle. Aliquots of liver, and diet (~1.5 g) were placed in 2:1 chloroform: methanol ($CHCl_3$: MeOH) with 0.01% butylated hydroxytoluene (BHT) and stored at -20˚C prior to lipid extraction. Known amounts of the internal standard cholestane (Diet 1 = 100 µL, Diet 2 = 150 µL, and Diet 3 = 200 µL at a concentration of 140 µg/mL) were added to the diet samples to allow for accurate determination of FA concentration. Total lipid was extracted from all diet and tissue samples via a modified Folch et al. (1957) procedure. All lipid extracts were washed with a salt solution (0.088% NaCl) and dehydrated with anhydrous sodium sulfate (Na_2SO_4) before the solvent was evaporated under a stream of nitrogen. Thin-layer chromatography (TLC) was then used to isolate triacylglycerols (TAG) from other lipid classes in the liver samples. The lipid sample (90µL at a concentration of 300 mg/mL) was streaked onto a

silica plate, 250 µm thick, and placed in a glass developing tank containing 85:15:1 (vol/vol/vol) hexane:diethyl ether:glacial acetic acid to separate the lipids based on polarity. Following development, the bands were visualized with UV after spraying with dichlorofluorescein. The TAG band was recovered and TAG were extracted from the silica particles using chloroform.

2.2.4 FATTY ACID ANALYSIS

TAG of the liver samples and total lipids of the diets were transesterified to fatty acid methyl esters (FAME) using methanol and a sulfuric acid catalyst (1.5 % v/v) (Budge et al., 2006), and analyzed using a Bruker 436 capillary gas chromatograph (GC) with flame ionization detection (FID) and a polar column (30 m x 0.25 mm ID Agilent Technologies, DB-23; Palo Alto, CA, USA). The carrier gas was helium, and FAME were analyzed with split injection mode (1:100 split) at a concentration of 50 mg mL^{-1} hexane. The oven temperature program began at 153 ºC and was held for 2 minutes before ramping to 174 ºC where it stayed for 0.2 minutes. It was then ramped to 210 ºC where it was held for 2 minutes. Samples were analyzed in duplicate and >70 FA were identified using authentic standards from Nu-Check Prep and GC-mass spectrometry. Peak identities were assigned and FA mass proportions determined using Compass software package (Bruker Biosciences Corporation, Billerica, MA, USA).

2.2.5 DATA ANALYSIS

FA proportions of the individual tissues and diets were normalized to 100 % and those with means <0.1 % and maximum values <0.3 % were removed from the data set, leaving 51 FA. FA proportions were then renormalized. FA proportions in the diets were

converted to concentration (mg g^{-1}) using the known amount of cholestane added during lipid extraction. Total FA concentrations were tested for normality using the Shapiro Wilks test. ANOVA was used (SPSS Version 20; IBM Corporation; NY, USA) to determine differences in total FA concentrations between diets. When a significant difference was found, Bonferroni pairwise comparisons were conducted to determine which diets had significantly different total FA concentrations. To correct for multiple testing, an adjusted p-value of 0.01 was used. Similar techniques were used to test for differences in length, mass, and liver lipid proportions.

Since tank, rather than individual, represented the sampling unit, fish were grouped according to tank and treatment and means of liver TAG FA of the individuals in each tank were calculated (n = 3, each treatment). FA proportions were transformed using *log (x+1)* and Bray Curtis similarity matrices were generated for the entire transformed data set. Permutational multivariate analysis of variance (PERMANOVA) was carried out using an add-on package of PRIMER-E (Primer-E Ltd, Plymouth Marine Laboratory, UK). This was used to test for differences in liver TAG FA signatures of treatment groups, rather than individual FA proportions. A pseudo-F statistic was determined and reported for all diet and liver comparisons. When differences were found, pairwise comparisons were carried out to determine which treatment groups had significantly different FA profiles. The transformed data were also used to calculate the similarity of percentages (SIMPER) of the sample types using PRIMER-E. This test ranks the FA according to those most likely responsible for the dissimilarity between treatment groups.

Three sets of calibration coefficients (CC), one for each treatment diet, were calculated for each FA using the formula: $CC = FA_{consumer}/FA_{prey,}$ where $FA_{consumer}$

represented the proportion of FA in the consumer and FA_{prey} represented the proportion of the same FA in the diet. The CC were transformed using $log(x)$ but were not normally distributed. Thus, to determine if CC varied by treatment diet for individual FA, Kruskal Wallis tests were carried out for each FA in sequence using SPSS (Version 20; IBM Corporation; NY, USA). Since 50 Kruskal-Wallis tests were conducted an adjusted p-value of 0.001 was used. Pairwise post-hoc tests would normally be carried out to determine which treatment groups were different; however, due to insufficient power, this post-hoc test was not done and results are interpreted in terms of the obvious variation in CC values.

2.2.6 QUANTITATIVE FATTY ACID SIGNATURE ANALYSIS

QFASA is a distance minimizing technique, using the Kullback Liebler (KL) distance measure, that compares FA profiles of the predator with combinations of FA signatures of possible prey while applying CC to correct for lipid metabolism in the predator. Before I used QFASA I used hierarchical clustering (SPSS Version 20; IBM Corporation; NY, USA) to identify groups of initial fish with similar FA profiles (Figure 2.5). Following hierarchical clustering, two groups of fish (Group 1 and 2) consisting of 5 and 3 individuals, respectively, were formed. Three fish did not cluster with any group and remained as individuals (Individual 1, 2, and 3). Group membership was held constant for all QFASA applications using the three sets of CC.

The FA signatures of the initial liver samples were modeled using the FA signatures of potential prey known to pollock including cod, haddock, herring, mackerel, Northern shrimp, Northern sandlance, red crab, red hake, rock crab, silver hake, and white hake (Leim and Scott, 1966; Shearer, 1992; Carruthers et al., 2005). Prey FA

signature were taken from Budge et al. (2002) and were selected based on size; individuals within each species were ranked according to mass and the smallest 20 were used in the modeling. When there were less than 20 individuals from a specific prey available, all individuals from that species were used. Three sets of CC, derived from the feeding experiments, were employed, generating three different diet estimates. The statistical model was written for R (version 2.8.1, Bell Laboratories Inc, NJ, USA) and followed the methods described by Iverson et al. (2004). A number of different FA sets have been used with QFASA (Iverson et al., 2004; Budge et al., 2012), including a full FA list of 51 FA, the 10 FA present in greatest proportions, dietary FA, and an extended dietary FA list. All four sets were used here (Appendix I) but for simplicity, I only report diet estimates generated using the extended dietary FA list which included all dietary FA as well as those that could be biosynthesized from predators, but whose levels in the predator were influenced by the consumption of specific prey.

2.3 RESULTS

2.3.1 EXPERIMENTAL DIETS

Total concentrations of FA (expressed as mg g^{-1} wet weight) differed significantly (ANOVA: $F(2,15) = 76.01$, $p < 0.01$) among the three diets (Appendix II) with total FA concentrations increasing from Diets 1 to 3 (48.7, 75.1, and 90.6 mg g^{-1}), roughly following the proportions formulated for total lipid (4 %, 7 %, and 10 % respectively; Table 2.1). Concentrations of individual FA followed a similar trend (Figure 2.1) as total concentration, except for 18:2n-6 which decreased in concentration from Diets 1 to 3. When the FA data were analyzed as proportions (Appendix III), the diets were much more similar in composition, with several notable exceptions (Figure 2.2). For example,

proportions of 20:1n-9 and 22:1n-11 were lower in Diet 1 compared to Diets 2 and 3, while proportions of 18:2n-6, 18:3n-3, and 22:6n-3 showed the opposite trend. PERMANOVA indicated that, overall, the FA profiles of all three diets were significantly different (Pseudo-F = 262, $p < 0.005$). The FA making the greatest contributions to the differences in profiles were 22:1n-11, 18:2n-6, 20:1n-9, 16:1n-7, 18:1n-9, 22:6n-3, 20:2n-6, 14:0, 18:3n-3, and 20:1n-11, in order of decreasing contribution (SIMPER; Table 2.2).

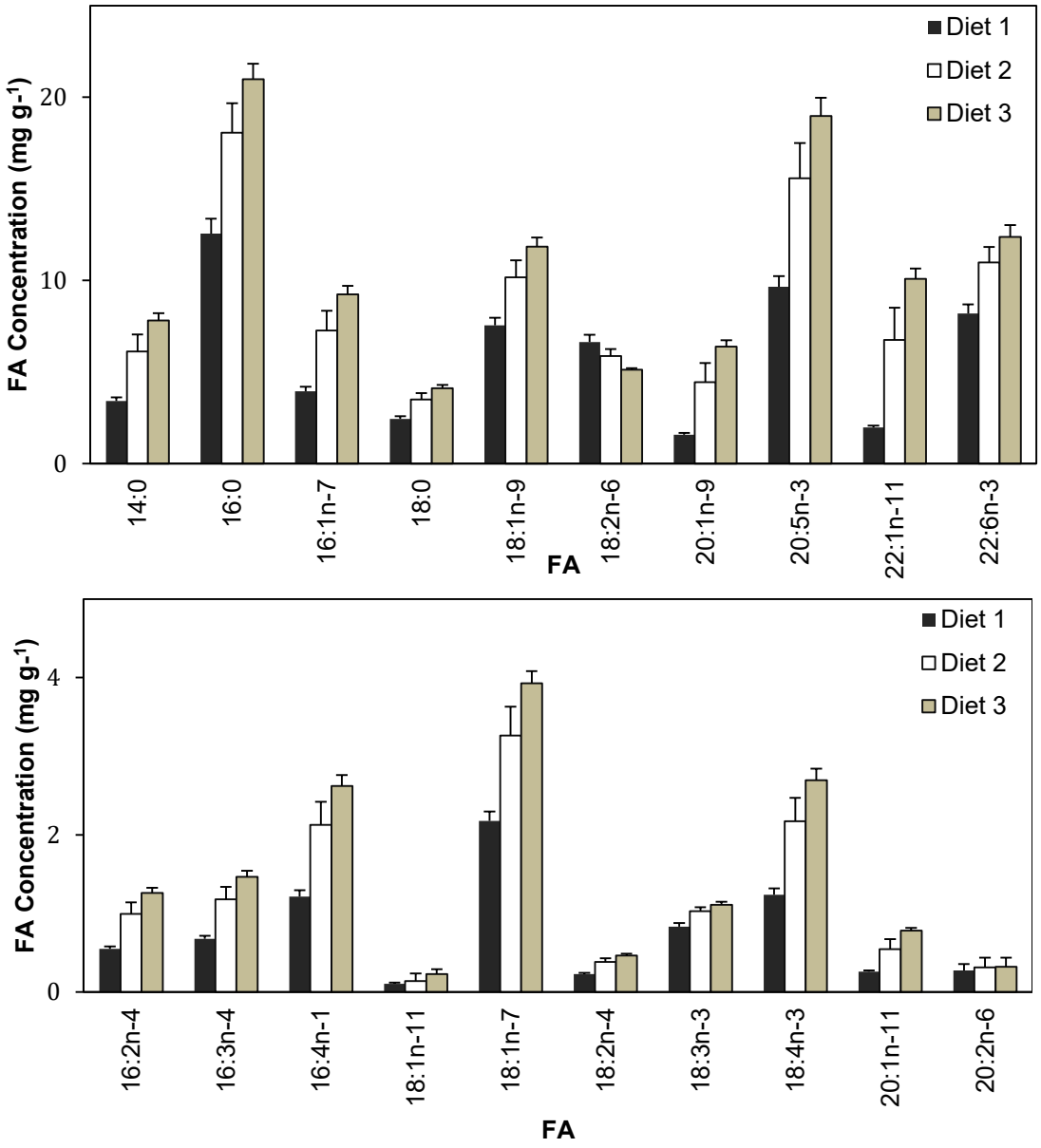


Figure 2.1: Concentrations (mg g^{-1} wet weight; mean +/- sd, n=6 each diet) of selected FA of total lipids in the experimental diets. FA chosen were the 20 that were found to show greatest dissimilarity in SIMPER analysis of proportional data, while ensuring that the 10 FA found in greatest proportions were also shown.

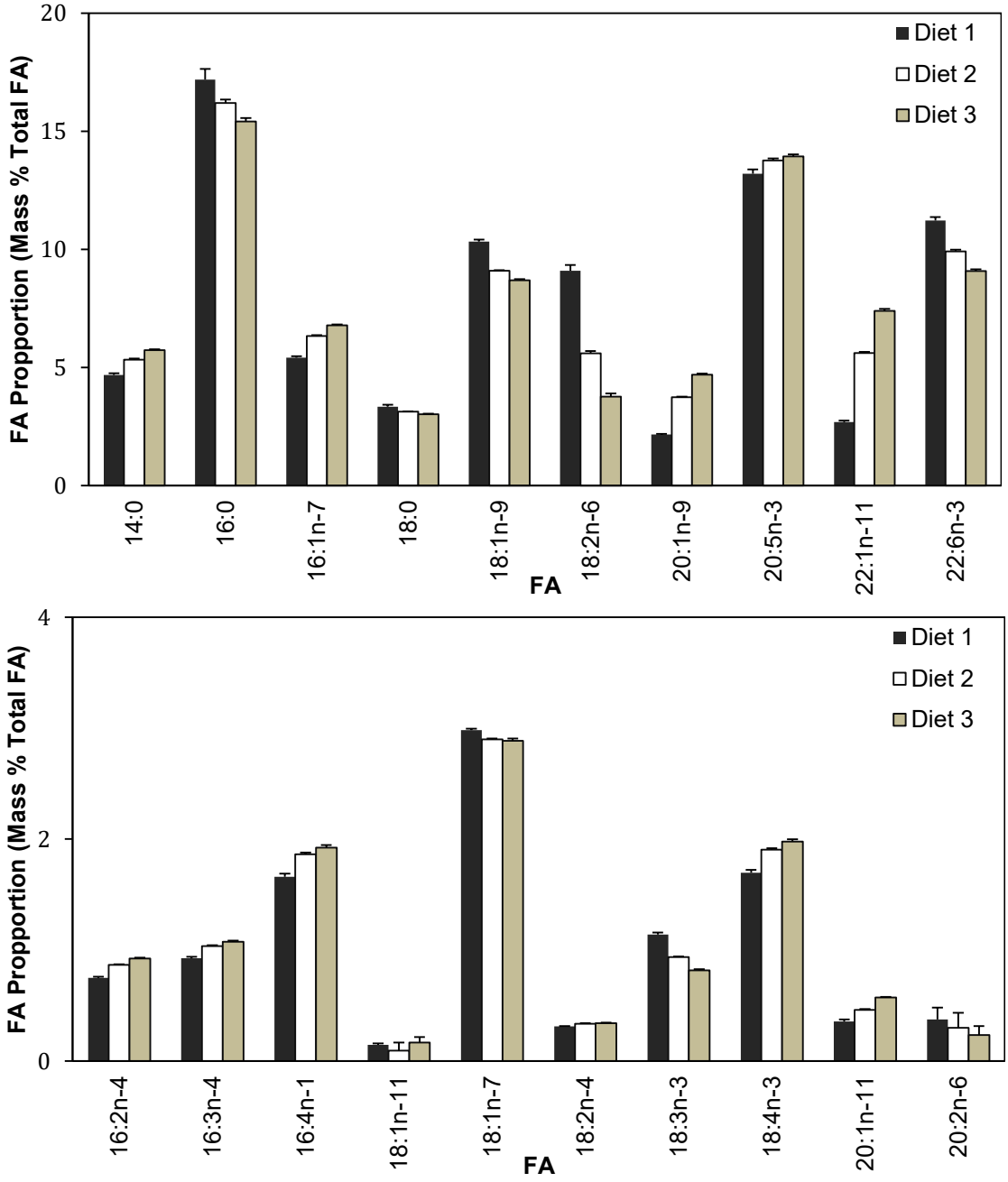


Figure 2.2: Proportions (mass % total FA; mean +/- sd; n=6 each diet) of selected FA of total lipids in the experimental diets. FA chosen were the 20 that were found to show greatest dissimilarity in SIMPER analysis of proportional data, while ensuring that the 10 FA found in greatest proportions were also shown.

Table 2.2: FA making the greatest contributions to dissimilarity identified by SIMPER in pairwise comparisons of FA profiles of the three experimental diets. The pseudo-t statistic from PERMANOVA pairwise comparisons is calculated from the square root of the pseudo-F statistic. P-value < 0.001 for all.

Diet 1 & Diet 2 (t = 13.75)		Diet 1 & Diet 3 (t = 21.78)		Diet 2 & Diet 3 (t = 9.59)	
FA	Contribution to variance (%)	FA	Contribution to variance (%)	FA	Contribution to variance (%)
22:1n-11	17.87	22:1n-11	17.29	18:2n-6	17.57
18:2n-6	13.08	18:2n-6	15.83	22:1n-11	12.91
20:1n-9	12.50	20:1n-9	12.39	20:1n-9	9.77
16:1n-7	4.12	16:1n-7	4.07	20:2n-6	4.34
18:1n-9	3.54	22:6n-3	4.06	18:1n-11	4.32
22:6n-3	3.49	14:0	3.58	22:6n-3	4.27
14:0	3.36	18:3n-3	3.47	20:1n-11	3.94
20:2n-6	3.33	18:1n-9	3.27	18:3n-3	3.48
18:3n-3	3.07	20:1n-11	3.11	14:0	3.29
20:2n-6	2.31	20:2n-6	2.52	16:1n-7	3.19

2.3.2 FISH GROWTH

Overall, the pollock gained mass (ANOVA: $F(2,6) = 7.37$, $p < 0.05$) and increased in length (ANOVA: $F(2,6) = 12.46$, $p < 0.05$) during the course of this experiment. An increase in lipid content (ANOVA: $F(3,16) = 14.80$, $p < 0.05$) in the liver was also noted (Appendix IV). The mass gain for fish fed Diet 1 was significantly less than that of fish fed Diet 2 and 3. Fish fed Diet 3 showed a significantly greater increase in length than fish fed Diet 1. The increase in percent lipid for liver of the fish fed Diet 1 ($Liver_{D1}$), Diet 2 ($Liver_{D2}$), and Diet 3 ($Liver_{D3}$) was higher than the liver of the initial pollock (~50 % vs. 26 %; ANOVA: $F(3,16) = 14.80$, $p < 0.05$); little change occurred between the fish fed the three treatment diets (Appendix IV).

2.3.3 DIET AND TISSUE COMPARISON

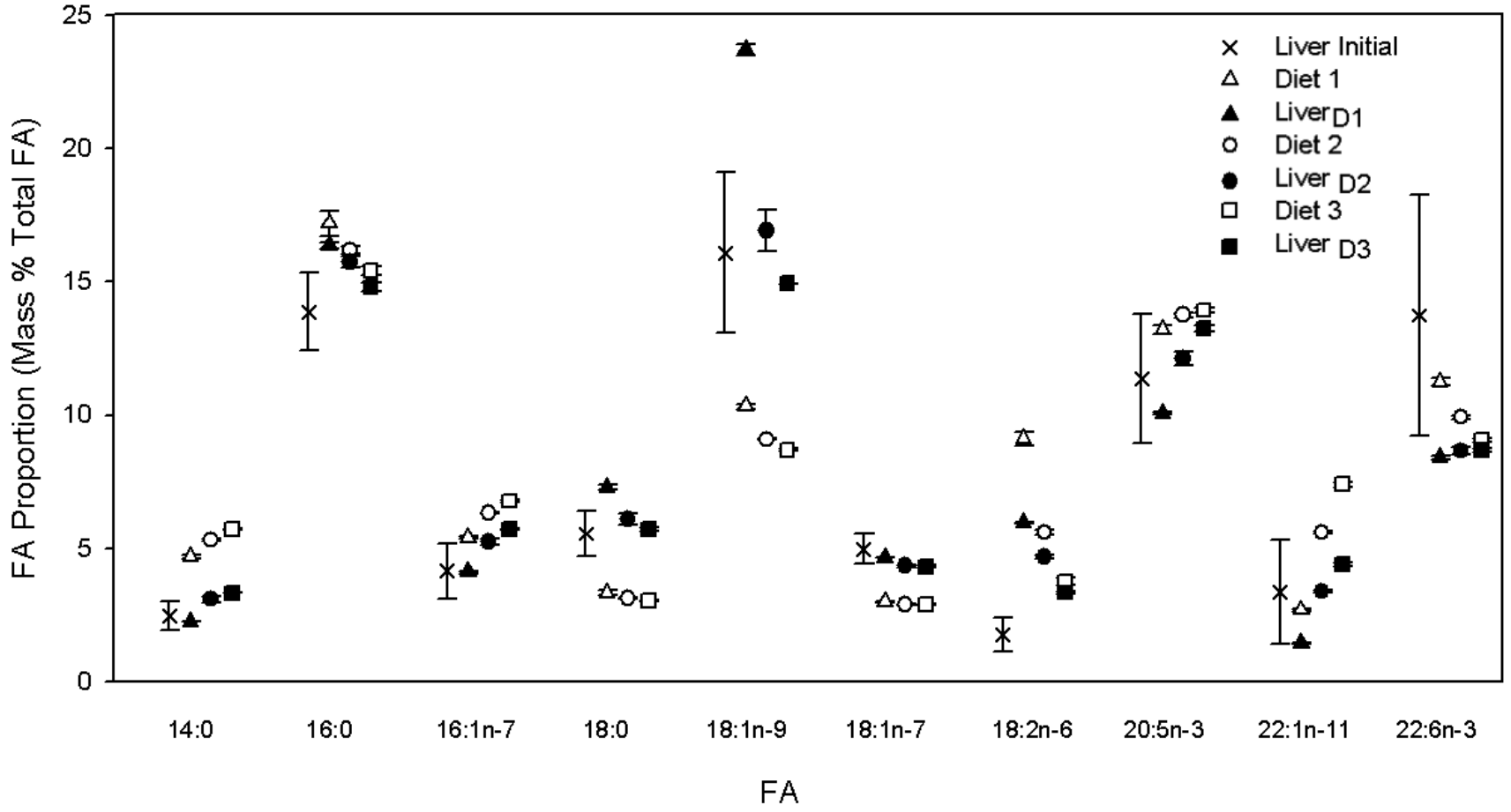
To determine the extent of modification of FA profiles during the 20-week feeding trial, the FA profiles, expressed as mass percent of total FA, of the TAG of liver of initial pollock were compared to those of pollock fed the experimental diets. PERMANOVA indicated significant differences between initial samples and all treatment groups (Pseudo-F = 7.03, $p<0.005$; Figure 2.3). There were distinct differences in proportions of 18:1n-9, 18:1n-11, 18:2n-6, 20:5n-3, 22:1n-11, and 22:6n-3 between the TAG FA of liver of initial fish and those fed the experimental diets. SIMPER supported these differences and indicated that the same six FA, regardless of treatment diet, were responsible for most of the variance (SIMPER; Table 2.3). Other FA identified as being responsible for differences included 16:1n-7, 16:4n-1, 18:2n-4, 20:1n-9, and 20:1n-11.

PERMANOVA indicated significant differences between the FA profiles of diets and of the TAG of liver fed each diet (Pseudo-F = 597.17, $p<0.005$). For instance, there were clear differences in proportions of 16:4n-1, 18:1n-9, and 18:4n-3 between the liver TAG FA and the respective diet fed (Figure 2.4). The FA making the greatest contribution to the difference in profile between FA in liver TAG and the matching diet were 18:1n-9, 18:0, 14:0, 22:1n-11, 16:4n-1, and 18:1n-7 in order of decreasing contribution (SIMPER; Table 2.4). Proportions of many dietary FA, such as 18:2n-6 and 20:5n-3, were higher in the diet than in the liver. However a number of FA, such as 18:2n-4 and 20:1n-11, had the inverse relationship. Interestingly, although the proportion of 20:5n-3 remained near constant among the three diets, 20:5n-3 proportions in the TAG of the liver increased from Diet 1 to 3. In contrast, proportions of 22:6n-3 remained

constant in liver TAG despite a decreasing proportion of 22:6n-3 in the 3 experimental diets.

Figure 2.3: Proportions of FA (mass % total FA; mean +/- sd) in experimental diets (n = 6 for each diet) and TAG of liver samples of initial fish (n = 10) and fish fed Diets 1 (n = 26), 2 (n = 26), and 3 (n = 28). FA chosen were the 20 that were found to show greatest dissimilarity in SIMPER analysis of proportional data, while ensuring that the 10 FA found in greatest proportions in the experimental diets were also included.

a)


FA Proportion (Mass % Total FA)
25
20
15
10
5
0
Liver Initial
Diet 1
Liver D1
Diet 2
Liver D2
Diet 3
Liver D3
14:0
16:0
16:1n-7
18:0
18:1n-9
18:1n-7
18:2n-6
20:5n-3
22:1n-11
22:6n-3
FA

b)

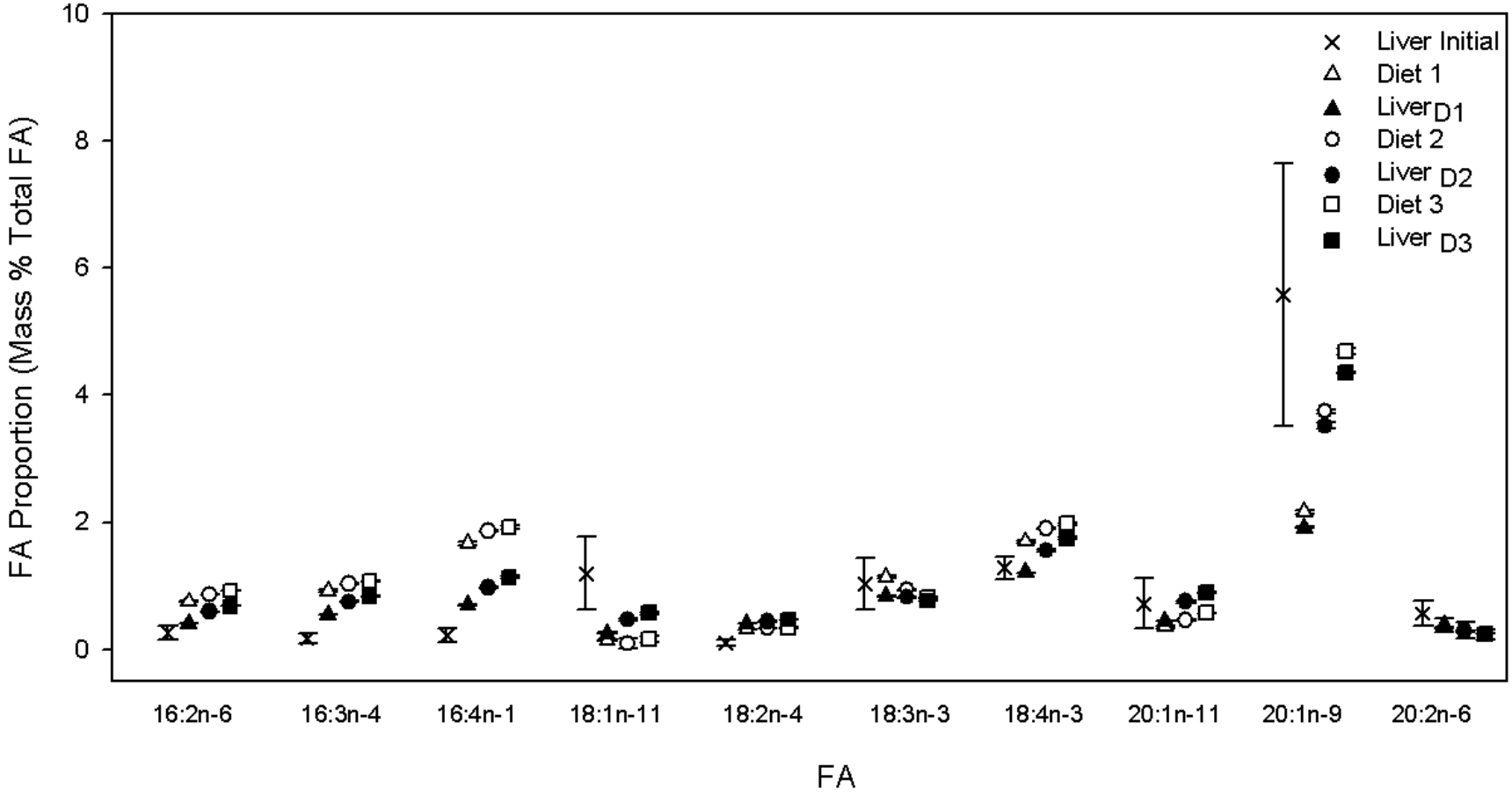

FA Proportion (Mass % Total FA)
10
8
6
4
2
0
Liver Initial
Diet 1
Liver D1
Diet 2
Liver D2
Diet 3
Liver D3
16:2n-6
16:3n-4
16:4n-1
18:1n-11
18:2n-4
18:3n-3
18:4n-3
20:1n-11
20:1n-9
20:2n-6
FA

Table 2.3: FA making the greatest contribution to dissimilarity identified by SIMPER in pairwise comparisons of FA profiles of TAG of initial liver and of post-prandial liver following a 20-week feeding trial. The pseudo-t statistic from PERMANOVA pairwise comparisons was calculated from the square root of the pseudo-F statistic. P-value < 0.001 for all.

Initial & $Liver_{D1}$ (t = 2.95)		Initial & $Liver_{D2}$ (t = 2.44)		Initial & $Liver_{D3}$ (t = 2.45)	
FA	Contribution to variance (%)	FA	Contribution to variance (%)	FA	Contribution to variance (%)
18:2n-6	9.95	18:2n-6	8.68	16:4n-1	6.49
20:1n-9	8.02	16:4n-1	5.68	18:2n-6	5.56
22:1n-11	6.09	22:6n-3	4.98	16:3n-4	5.25
18:1n-11	5.55	16:3n-4	4.68	22:6n-3	4.89
22:6n-3	4.63	20:1n-9	4.62	22:1n-11	4.71
18:1n-9	4.01	18:1n-11	4.45	18:1n-11	3.78
16:4n-1	3.50	22:1n-11	3.62	16:1n-7	3.47
16:3n-4	2.97	18:2n-4	3.22	18:2n-4	3.41
20:4n-6	2.94	20:4n-6	3.17	16:2n-4	3.36
18:2n-4	2.57	16:1n-7	2.92	20:1n-9	3.34

$Liver_{D1}$ & $Liver_{D2}$ (t = 16.75)		$Liver_{D1}$ & $Liver_{D3}$ (t = 36.40)		$Liver_{D2}$ & $Liver_{D3}$ (t = 7.10)	
FA	Contribution to variance (%)	FA	Contribution to variance (%)	FA	Contribution to variance (%)
22:1n-11	13.79	22:1n-11	12.93	18:2n-6	13.44
20:1n-9	10.16	20:1n-9	9.80	22:1n-11	10.46
18:1n-9	7.46	18:2n-6	7.62	20:1n-9	8.59
14:0	5.35	18:1n-9	7.09	18:1n-9	5.98
18:2n-6	4.76	14:0	4.66	20:5n-3	4.15
16:1n-7	4.61	16:1n-7	4.42	20:1n-11	3.86
20:1n-11	4.43	20:1n-11	4.32	16:1n-7	3.81
20:5n-3	3.94	20:5n-3	4.07	16:4n-1	3.67
18:1n-11	3.67	18:1n-11	3.68	18:4n-3	3.60
18:0	3.59	16:4n-1	3.65	18:1n-11	3.53

Table 2.4: FA making the greatest contribution to dissimilarity identified by SIMPER in pairwise comparisons of FA profiles of the experimental diets and their corresponding liver TAG. The pseudo-t statistic from pairwise comparisons was calculated from the square root of pseudo-F. P-value < 0.001 for all.

Diet 1 & $Liver_{D1}$ (t = 28.33)		Diet 2 & $Liver_{D2}$ (t = 21.84)		Diet 3 & $Liver_{D3}$ (t = 26.48)	
FA	Contribution to variance (%)	FA	Contribution to variance (%)	FA	Contribution to variance (%)
18:1n-9	10.36	18:1n-9	9.94	18:0	9.97
18:0	8.62	18:0	9.37	18:1n-9	9.60
14:0	7.39	14:0	7.54	22:1n-11	8.49
16:4n-1	5.95	22:1n-11	7.05	14:0	8.48
22:1n-11	5.54	16:4n-1	6.38	16:4n-1	6.12
18:2n-6	4.93	18:1n-7	5.46	18:1n-7	6.06
18:1n-7	4.65	18:1n-11	5.10	18:1n-11	5.77
22:6n-3	3.46	20:1n-11	3.14	20:1n-11	3.58
20:5n-3	3.32	16:1n-7	2.81	16:1n-7	2.83
16:1n-7	3.00	16:2n-4	2.74	20:0	2.75

2.3.4 CALIBRATION COEFFICIENTS

CC_{D1}, CC_{D2}, and CC_{D3} derived from fish fed Diets 1, 2, and 3 were broadly similar, although clear variation was apparent in a number of FA (Figure 2.4; a complete list of CC can be found in Appendix V). In Figure 2.4, logarithm of CC has been plotted, so that a CC of zero results from a consumer:prey FA ratio of 1:1. Correspondingly, when a CC>0, that FA was present in higher proportions in the consumer than in the prey. Prominent differences were apparent in the CC of 18:1n-11, 18:2n-6, 18:3n-3, 18:4n-3, 20:5n-3, and 22:6n-3 among the three diets. Multiple ANOVA supported this indicating significant differences between the three CC for the 6 FA listed above ($p<0.001$) and many others not included in Figure 2.4 (Appendix V).

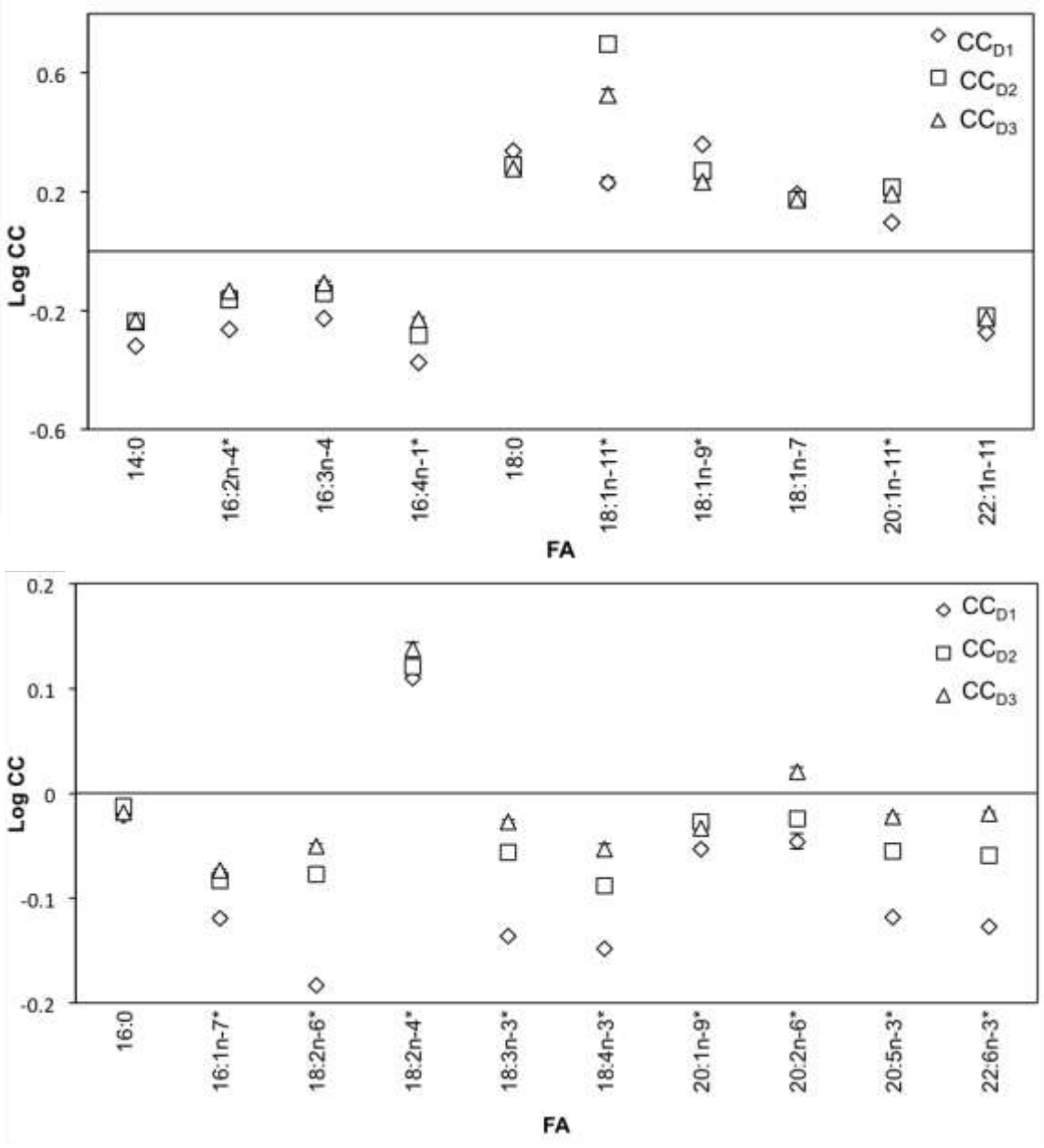


Figure 2.4: Calibration coefficients (CC; log mean +/- se) of TAG FA of post-prandial liver of fish fed the experimental diets. To maintain consistency, the FA displayed here are the same as in earlier plots but are grouped according to magnitude of log CC. Asterisks denote significantly different CC between treatment diets.

2.3.5 INFLUENCE OF CC ON DIET ESTIMATES DERIVED FROM QFASA

Overall, variation in CC did not have extreme effects on diet estimates of groups or individuals (Appendix VI; Figures 2.6a - 2.6d). For example, regardless of CC applied, Northern sandlance dominated the diet estimates of Group 2 (55 % - 69 %; Figure 2.6b); contributions of herring, red crab, and rock crab did vary but their proportions remained low regardless of CC used. Similar effects were seen with Group 1 (Figure 2.6a) where the dominant contributors to diet remained consistent for all three CC, with Northern sandlance the major diet item. For Individuals 1 and 2 (Figures 2.6c and 2.6d), herring and rock crab, respectively, were the principal contributors to diet estimates, but Northern sandlance continued to be an important prey item, varying from 19 – 50 %. However, substantial variation was evident in Individual 3 (Figure 2.6e), where cod dominated the estimate for CC_{D2} and CC_{D3} with minor contributions from Northern sandlance and none from haddock. When CC_{D1} were applied, cod, haddock and Northern shrimp contributed similar proportions.

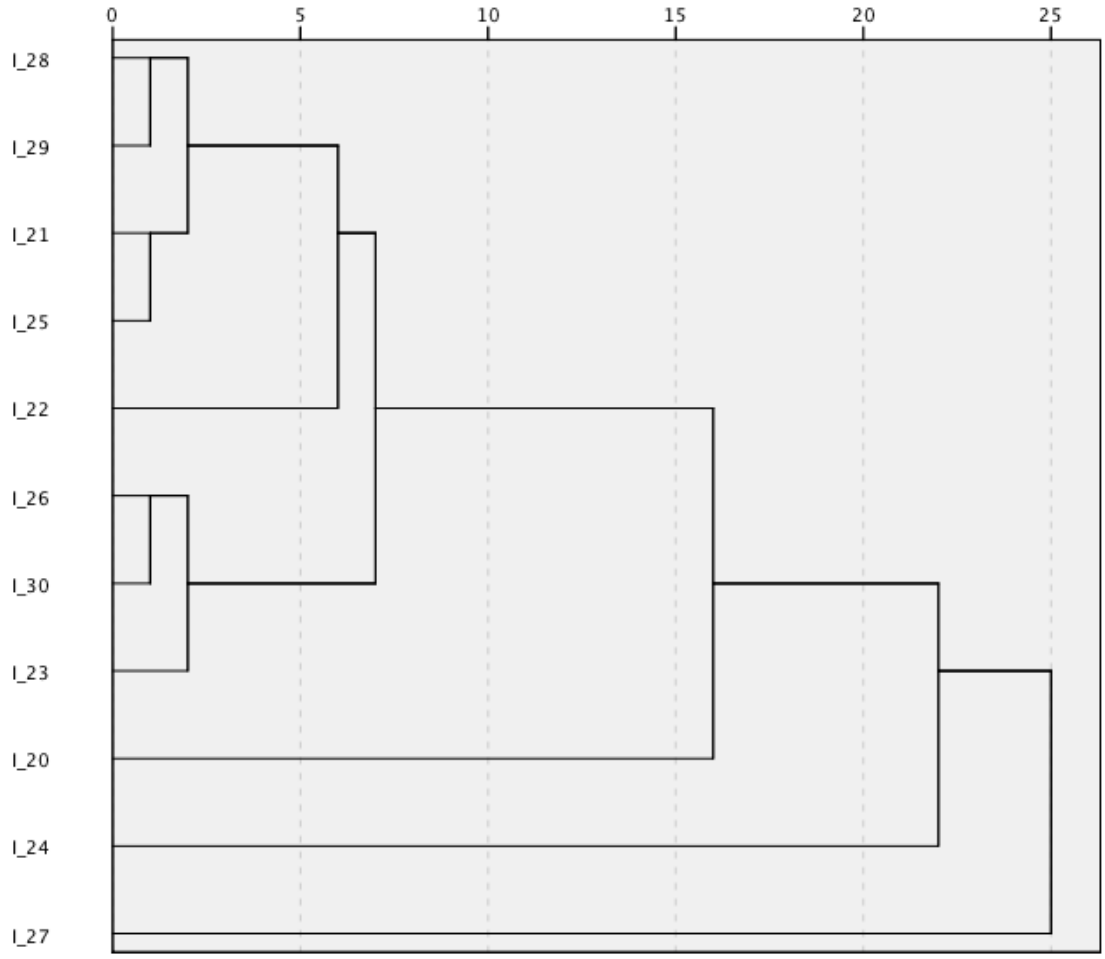
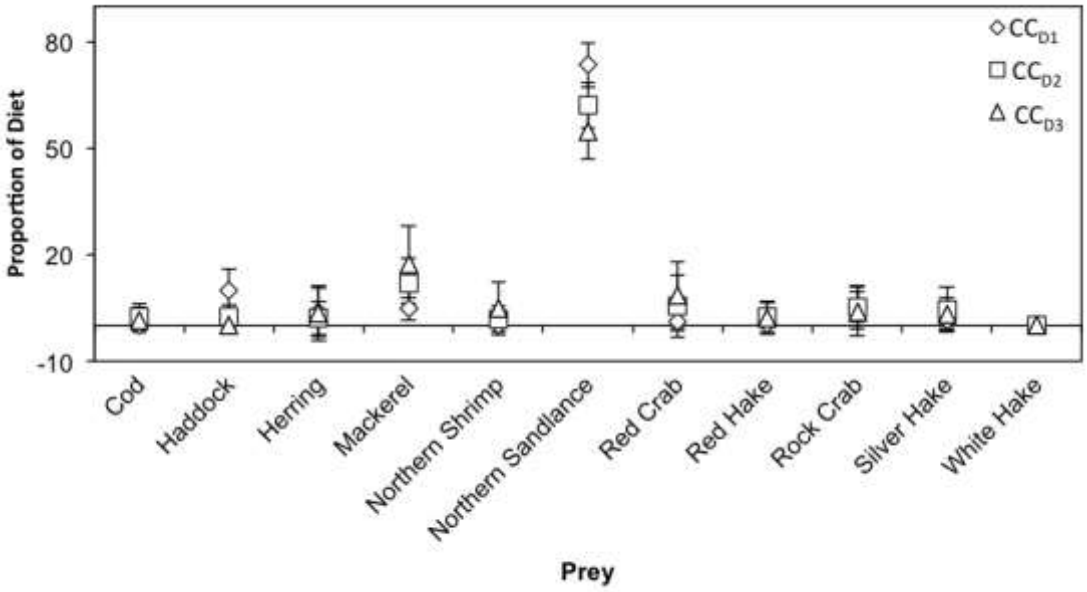
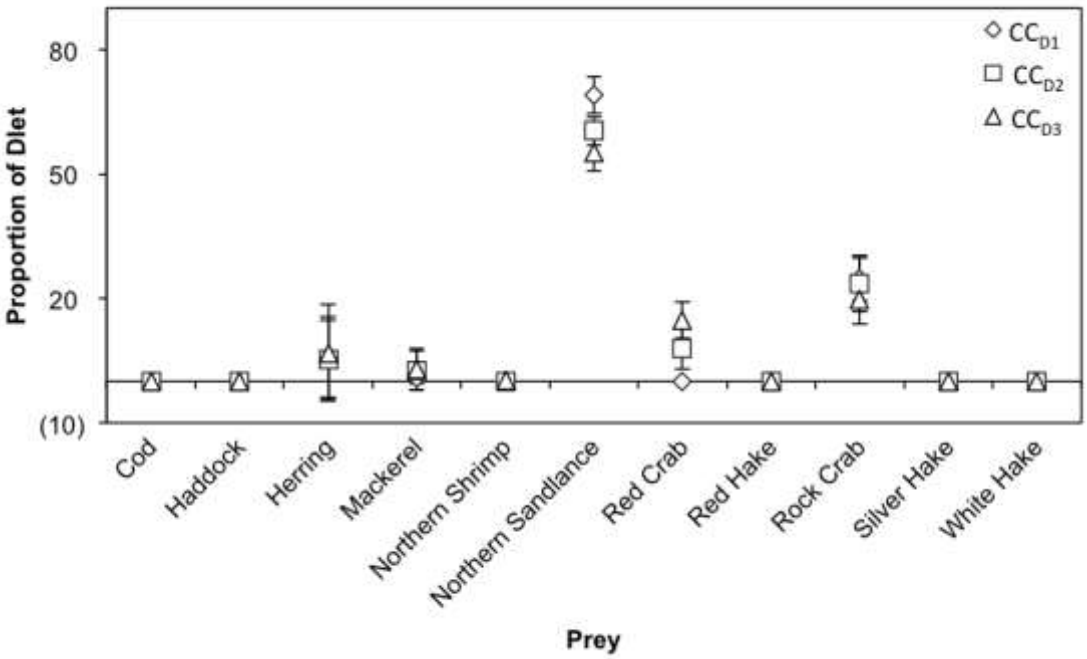


Figure 2.5. Hierarchical cluster analysis using the extended FA set of liver TAG of initial fish to identify groups with similar FA components, where I=Initial fish. Group 1: I_21, I_22, I_25, I_28, and I_29; Group 2: I_23, I_26, and I_30; Individual 1: I_20; Individual 2: I_24; Individual 3: I_27.

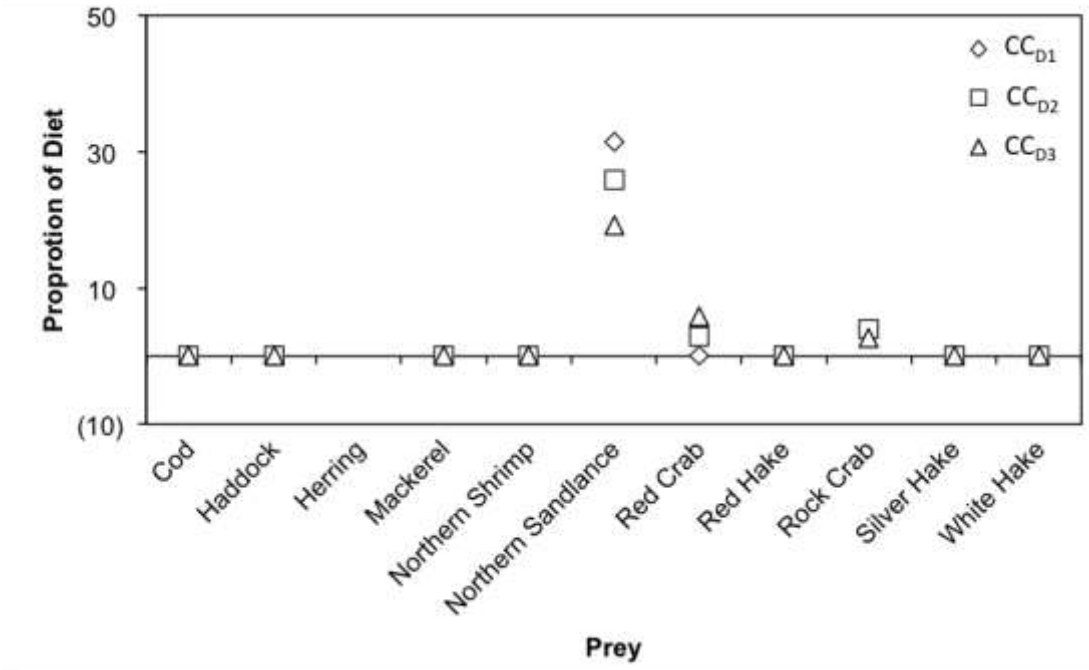
a)


Proportion of Diet
80
50
20
-10
CC_D1
CC_D2
CC_D3
Cod
Haddock
Herring
Mackerel
Northern Shrimp
Northern Sandlance
Red Crab
Red Hake
Rock Crab
Silver Hake
White Hake
Prey

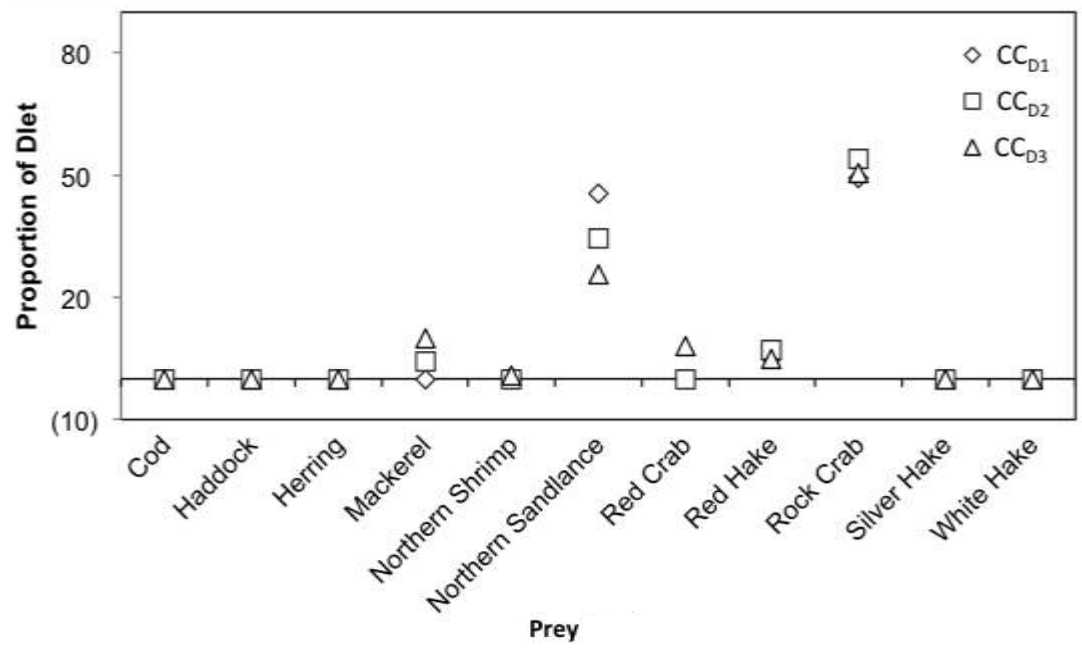
b)


Proportion of Diet
80
50
20
(10)
CC_D1
CC_D2
CC_D3
Cod
Haddock
Herring
Mackerel
Northern Shrimp
Northern Sandlance
Red Crab
Red Hake
Rock Crab
Silver Hake
White Hake
Prey

c)


50
30
10
(10)
Proprotion of Diet
CC_D1
CC_D2
CC_D3
Cod
Haddock
Herring
Mackerel
Northern Shrimp
Northern Sandlance
Red Crab
Red Hake
Rock Crab
Silver Hake
White Hake
Prey

d)


80
50
20
(10)
Proportion of Diet
CC_D1
CC_D2
CC_D3
Cod
Haddock
Herring
Mackerel
Northern Shrimp
Northern Sandlance
Red Crab
Red Hake
Rock Crab
Silver Hake
White Hake
Prey

e)

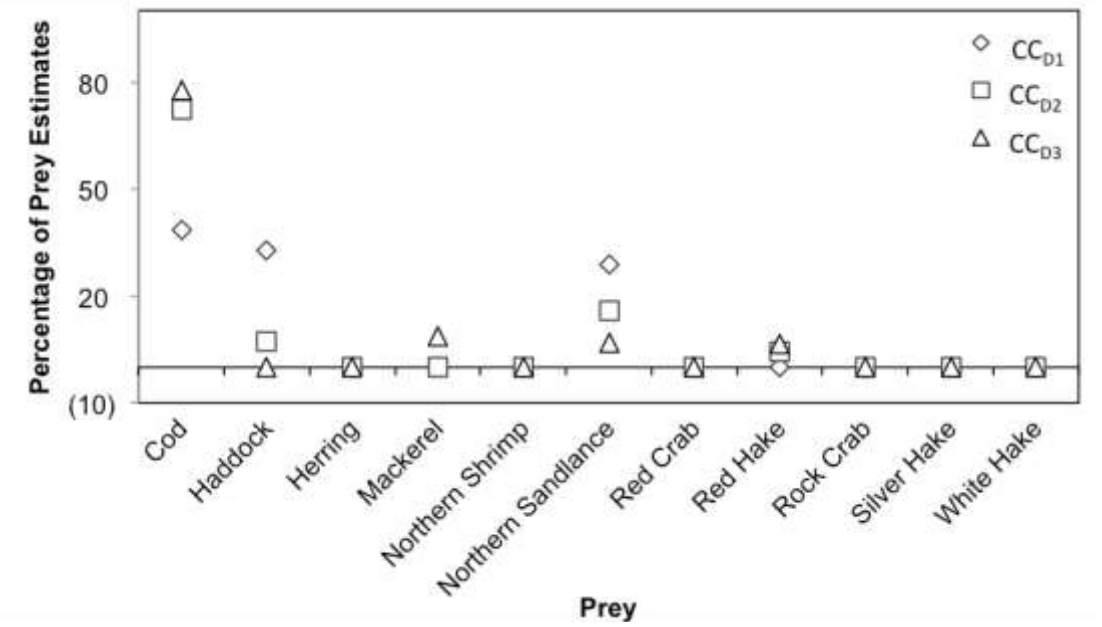


Figure 2.6: Variation in QFASA estimates of diet using all 3 CC applied to TAG FA of initial pollock liver in: a) Group 1; b) Group 2; c) Individual 1; d) Individual 2; and e) Individual 3.

2.4 DISCUSSION

2.4.1 INFLUENCE OF DIET ON LIVER TAG FA PROPORTIONS

The experimental diets were formulated with FA concentrations increasing from Diet 1 to Diet 3 and the results clearly supported this pattern (Figure 2.1). To accurately test the hypothesis that FA concentrations, rather than FA proportions, influence metabolism of FA and deposition in the liver, I attempted to formulate diets with identical FA proportions. Formulating diets in this way proved difficult, as protein was needed as a base, but available protein, whether in low fat fish like flounder, or the fish or krill meal, contains small amounts of lipid (~2 %, ~10 %, and ~18 % wet weight respectively, USDA Nutrient Database; Roncarati et al., 2011; García-Romero et al.,

2014). While the amount of lipids, and therefore FA, contributed from these sources remained constant in all diets, the added oil content varied (Table 2.1). Thus, it was impossible to avoid slight variations in FA proportions when diets were formulated in this manner as moist pellets. However, compared to the FA concentrations, the differences in FA proportions were much more subtle (Figure 2.2).

A number of investigations have used dry feeds, either commercially available or lab-formulated, to examine FA deposition in fish tissues (Budge et al., 2011a; Budge et al., 2012; Trushenski et al., 2012). In the majority of those studies, the feeds included a number of ingredients, such as corn gluten meal, soybean meal, dried whey, wheat middlings, and pregelatinized starch, that are not normally found in wild fish diets. These ingredients are added as sources of protein and carbohydrates, and to supply the essential nutrients and energy required by growing animals to meet their physiological needs (Lall and Tibbetts, 2009). However, these ingredients do contain small amounts of lipid and therefore may also affect FA proportions and possibly metabolism. In addition, diets used in controlled feeding studies often have lipid proportions much greater than those found in natural diets of fish. For instance, Budge et al. (2011a and 2012) used diets formulated to contain 14 % lipid wet-weight basis (WWB) (16 % dry-weight basis (DWB)) in studies with juvenile salmon, while diets of 15 % lipid DWB were fed to juvenile Atlantic spadefish (Trushenski et al., 2012). Diets of wild Atlantic salmon consist of Arctic squid, sandlance, Arctic shrimp, and herring (Leim and Scott, 1966; Shearer, 1992) and would be expected to have a lipid content < 6 % WWB. Moist diets, with a base of ground flounder, were formulated for this study to more closely resemble those of wild fish in lipid content and composition (Table 2.1). Previous work from the marine

lipids lab showed unusual patterns of FA deposition in the belly flap of Atlantic salmon after feeding dried pellets containing 14 % lipid WWB for 22 weeks (Budge et al., 2011a). For example, proportions of several FA, including 20:5n-3 and 22:6n-3, in the belly flap did not mirror the changes in dietary FA proportions. It was thought that this variation was caused by the high lipid content in the diets, thereby making it difficult to apply the results to wild salmon consuming a diet with lower lipid levels. The diet of wild pollock, which is composed primarily of mackerel, herring, and sandlance (Leim and Scott, 1966; Carruthers et al., 2005), is ~5 % lipid WWB. Thus, diets more closely resembling prey found in the wild were formulated for this experiment.

After fish were fed the experimental diets for 20 weeks, clear changes in TAG FA profiles were apparent in the liver of the treatment fish (Figure 2.3) when compared to those of the initial liver, indicating that sufficient dietary intake of FA had occurred to modify the original signatures. These changes observed in FA profiles of liver TAG followed three discreet patterns. The first pattern was evident in most of the FA, including 16:0, 18:2n-6, 20:1n-11, 20:1n-9, and 22:1n-11, where the proportions of FA in the liver seemed to follow the changes in proportion of FA in the diet (Figure 2.3). This same pattern has been observed in many other studies (Elsdon, 2010; Budge et al., 2011a; Fallah et al., 2011) and is further indication of the clear influence of dietary FA proportion on tissue FA profiles.

A second prominent pattern is illustrated by 18:1n-9, where proportions remained quite similar in the diets while the tissue FA proportions showed a substantial decrease within a FA. This pattern supports the hypothesis that FA concentrations (rather than just proportions) in diet influence deposition and metabolism of FA in storage tissue as has

been observed in other studies on Atlantic salmon (Jobling et al., 2002; Jobling and Bendiksen, 2003; Budge et al., 2011a). Aucoin (2011) showed that there was little difference between dietary FA proportions and blood chylomicrons in Atlantic pollock, indicating that only minor changes in FA composition occur during digestion and absorption into the blood stream. While it is possible that lipoproteins responsible for transfer of FA from chylomicrons into the liver of Atlantic pollock discriminate against 18:1n-9, it seems unlikely given the lack of similar discrimination in the action of other digestive lipases. Thus, metabolic processes within the liver seem to be more likely responsible for the changes seen here (Figure 2.3).

The concentration of 18:1n-9 increased from 7.5 to 11.8 mg g^{-1} from Diet 1 to Diet 3 (Appendix II), and may have promoted the use of that FA as a substrate for metabolic processes such as oxidation, elongation, chain-shortening or desaturation within the liver. Substantial elongation or chain-shortening of 18:1n-9 would result in correspondingly large increases in proportions of 20:1n-9 and 16:1n-9, respectively; however, there was no evidence of such patterns in the data. Similarly, desaturation of 18:1n-9, potentially coupled with elongation, would result in unusual PUFA with n-9 structures because of the inability of animals to insert double bonds between the n-9 and terminal methyl positions. Thus, oxidation of 18:1n-9 is the only likely mechanism to explain the results, corresponding with a number of other studies (Olsen and Henderson, 1997; Bell et al., 2003; Tocher, 2003). The proportions of 18:1n-9 in TAG liver of fish fed Diet 2 and Diet 3 were much more similar than that of fish fed Diet 1. These similarities may suggest that there is some critical concentration, likely close to the concentration found in Diet 2 (6.5 mg g^{-1}), above which excess 18:1n-9 is directed to

oxidation. Unfortunately, with the three diets formulated here, it is impossible to determine the minimum concentration at which 18:1n-9 reaches excess and oxidation begins. To determine that, further captive feeding experiments would be necessary, using diets with FA concentrations between those of Diet 1 and Diet 2.

While it might be presumed that oxidation is simply associated with FA found in the largest proportions in the diet, such as 18:1n-9 (Tocher, 2003; Budge et al., 2012), this is not the case here. Other FA, such as 16:0 and 20:5n-3, were present at levels greater than that of 18:1n-9 but did not display any evidence to suggest oxidation. A number of other studies with Atlantic salmon have reported a similar FA-specific catabolism, showing preferential oxidation of FA that are present at high concentrations in diets (Bell et al., 2004; Torstensen et al., 2004a,b). In fact, 20:5n-3 showed a moderate increase in liver proportions with increasing concentrations in the diet and constant diet proportions (Figure 2.1). This pattern seems more of a plateau effect, where the FA proportion in the tissue is approaching some constant proportion, as was observed when studying spectacled and Steller's eiders, and Atlantic salmon (Wang et al., 2010; Budge et al., 2011a).

A third pattern was observed with FA such as 18:3n-3 and 22:6n-3 (Figure 2.3) where constant liver FA proportions were found with changing dietary proportions. This same effect was seen in 22:6n-3 in muscle and adipose of Atlantic salmon (Bell et al., 2003; Budge et al., 2011a). Budge et al (2011a) suggested that 22:6n-3 was influenced more by physiological processes rather than diet, as 22:6n-3 has important roles in maintaining membrane fluidity in response to temperature fluctuations. (Tocher, 2003; Arts et al., 2009). A corresponding explanation for the response of 18:3n-3 is difficult to

formulate but it is known to be a precursor in the formation of 22:6n-3. While it is unlikely that elongation and desaturation of 18:3n-3 was occurring in relevant amounts in fish consuming a diet high in long chain n-3 PUFA, the response seen in 18:3n-3 may also be tied to membrane fluidity.

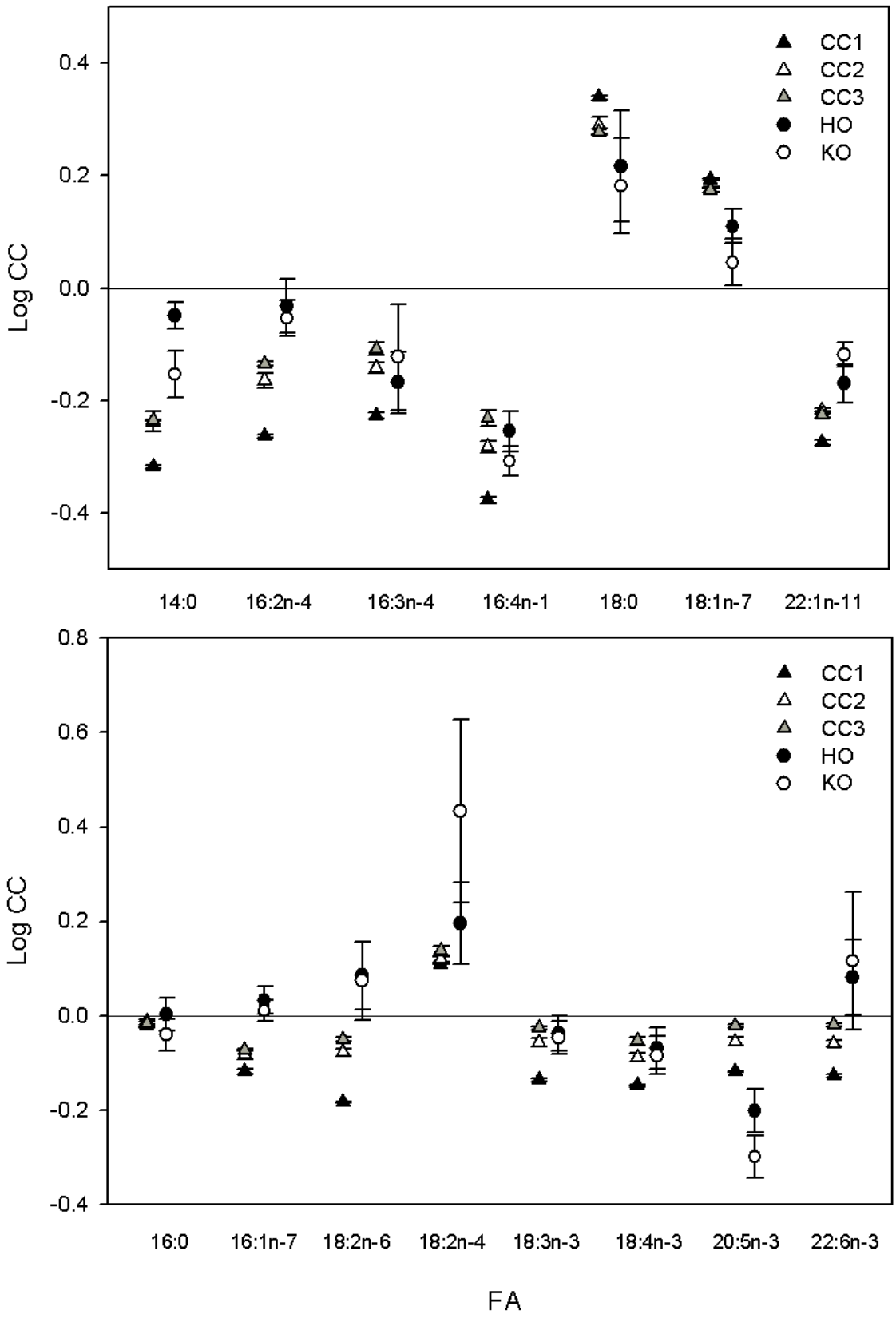
2.4.2 VARIATION IN CC

The majority of CC in Figure 2.4 showed significant variation across the three diets, with the exception of those for 16:0, 18:0, 18:1n-7, 20:1n-9, and 22:1n-11. A number of FA present at smaller proportions (i.e.,<1.4 %; Appendix V), including *i*-17:0, *ai*-17:0, 17:1(b), 17:1, 18:3n-6, 18:4n-1, 20:1n-7, 22:1n-7, and 24:1n-9, also had CC that did not vary among the three diets. For all FA, CC_{D2} and CC_{D3} were very similar; differences became apparent only when CC_{D1} were compared to the other two sets. For instance, the EFA 20:5n-3 and 22:6n-3 had log CC_{D2} and log CC_{D3} that were closer to zero than those for CC_{D1}. The influence of other ingredients, such as protein and carbohydrate content, can be eliminated as a source of this variation, as the diets were formulated so that they varied only in FA concentration and signature. Thus, the differences observed in CC_{D1} for those FA might offer support for the hypothesis that Diet 1 contained FA below some critical concentration at which a FA is directed to oxidation, indicating that FA concentration does affect CC. This may be a plausible explanation for 18:2n-6, 20:5n-3 and 22:6n-3 that are present in concentrations >5 % in the tissue; however, 16:0 was among the most concentrated FA in the diet but did not show variation in CC. Similarly, 18:3n-3 and 18:4n-3 show variation in CC were present only at modest levels in the tissue and diet (<2 %). Therefore, while FA concentration

may influence deposition pattern and the resulting CC, its effect is not equivalent for all FA.

Pollock CC from this experiment were generally similar to those of Atlantic salmon (Budge et al., 2012; Figure 2.7) with most FA having log CC values that were consistently greater or less than zero for both species; only a few FA, including 18:2n-6 and 22:6n-3, did not follow this trend. These subtle variations found between pollock and salmon could be due to the different FA content in the diets, as well as the different tissue analyzed (belly flap versus liver). Salmon CC were derived from analysis of total lipids, not a specific lipid class as done here, which would be expected to also contribute to differences between the sets. Further, the CC of the salmon more resembled those of Diets 2 and 3 for pollock. This resemblance of salmon CC and pollock CC derived from Diets 2 and 3 may be because the diets fed to the salmon had similar lipid proportions (~14 %) as those of Diets 2 and 3. Based on this data, CC may depend more on the diet consumed than the metabolism of the predator.

Figure 2.7: Calibration coefficients (log CC; mean +/- sd) of FA of post-prandial liver in pollock fed the experimental diets of the current study compared to CC derived from FA of post-prandial belly flap in Atlantic salmon fed diets based on herring oil (HO) and krill oil (KO) from Budge et al. (2012). FA that could not be compared were 18:1n-9, 18:1n-11, 20:1n-9, and 20:1n-11, due to co-elution of isomers in Budge et al. (2012). Since the logarithm of CC has been plotted, a CC of zero results from a consumer:prey FA ratio of 1:1. Correspondingly, when a CC>0, that FA was present in higher proportions in the consumer than in the prey.


CC1
CC2
CC3
HO
KO
Log CC
0.4
0.2
0.0
-0.2
-0.4
14:0
16:2n-4
16:3n-4
16:4n-1
18:0
18:1n-7
22:1n-11
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
16:0
16:1n-7
18:2n-6
18:2n-4
18:3n-3
18:4n-3
20:5n-3
22:6n-3
FA

While numerous controlled feeding studies have taken place to determine CC (i.e., Iverson et al., 2004; Iverson et al., 2007; Wang et al., 2010), they are unlikely to be applicable to pollock as they involve species in different classes of vertebrates (birds and mammals) and were sometimes fed diets with compositions very different than those consumed in the wild. However, there does seem to be similar trends among pollock, sea birds, and marine mammals. For example, for all species listed above, the CC of 16 carbon PUFA (16:2n-4, 16:3n-4, 16:4n-1), 18 carbon MUFA (18:1n-11, 18:1n-9), and 20:5:n-3 followed a similar pattern, where the CC was above or below zero for all species. These similarities in CC may point to commonalities in physiology and lipid metabolism in these diverse species.

A similarity found among all controlled feeding studies for pollock, sea birds, and sea mammals was the large variation associated with 18:1n-11. Since 18:1n-11 elutes on the shoulder of 18:1n-9, proportions of 18:1n-11 may be inconsistent; therefore, it was removed from QFASA diet estimates in all studies. Because the Kullback-Liebler (KL) distance gives more weight to rare or trace FA, albeit more conservatively and proportionally than other distances, small errors in minor FA with large and varying CC might have substantial effects on diet estimates (Iverson et al., 2004).

2.4.3 QFASA DIET ESTIMATES

The QFASA exercise was used to determine if small changes in CC have an effect on the diet estimates from QFASA; it was not for the purpose of estimating actual pollock diets. This was principally due to limitations associated with the prey library. Most importantly, the pollock used in this experiment were juveniles that live mostly inshore and would therefore feed on planktonic organisms such as copepods and

zooplankton, as well as juvenile fish such as herring, sandlance, cod, hake, and capelin (< 15 cm in length), and crustaceans including shrimp and crabs (< 6.5 cm in length) (Leim and Scott, 1966; Carruthers et al., 2005). The prey library used here contained much larger fish (10 – 32 cm length; 5 – 355 g weight), and since fish FA profiles change with size (Lilly 1986; Canalejo et al. 1989; Martell and McClelland 1994; Iverson et al., 1997), the available prey did not have FA signatures representative of the smaller fish likely to be consumed by these pollock. Furthermore, a full prey database was not used. For instance, the prey FA signatures taken from Budge et al. (2002) did not include euphausiids and other planktonic organisms, which are known to be consumed by juvenile pollock (Cargnelli et al., 1999) Thus, diet estimates produced here using QFASA are very likely to be inaccurate.

Overall, there were subtle differences in diet estimates for most individuals and groups. For example, Northern sandlance dominated estimates for most groups and individuals when CC_{D1} was applied (Figure 2.6); however when CC_{D3} were used the contribution of sandlance decreased and contribution from minor prey items increased. This pattern suggested that overall, QFASA may be less sensitive to the variation in CC than previously assumed. The lack sensitivity to variation in CC could be a positive finding, indicating that concerns of accurate CC are not as critical as previously thought (Budge et al., 2012). However, it may also indicate an unsuitable prey library was contributing to the effect of insensitivity seen here. The QFASA model attempts to minimize the distance between the predator's FA profile and that of the prey, but produces an estimate of diet regardless of the minimum distance achieved. When an incomplete or inappropriate prey library is used, it may be that the minimum distance

achieved does not represent a good match between predator and prey FA profiles. Thus, in addition to possibly generating an unreliable diet estimate, use of an incomplete prey library might also fail to demonstrate the true impact of CC variation. To summarize, it is possible that the model was performing so poorly in this situation that the true effect of variation in CC cannot be assessed.

Lipid proportions in liver of fish fed the experimental diets increased from ~26 % in wild (Initial) fish, to ~50 % in fish fed Diet 1, 3, and 2 (respectively). The treatment fish, particularly those consuming Diets 2 and 3, were fed higher fat diets than normally encountered in the wild and they may also have been consuming more food than wild fish, since they were fed to satiation each day. Either of these conditions would cause fish to consume more fat than encountered in the wild and may have led to modifications in metabolism. Changes in fat metabolism would, in turn, cause inaccurate CC. While studies of β-oxidation in fish suggest a substrate preference for saturated FA and MUFA over PUFA (Kiessling and Kiessling, 1993; Henderson, 1996), these preferences appear to be minimized when FA are in dietary surplus (Stubhaug et al., 2007). When dietary levels are high, 20:5n-3 and 22:6n-3 are highly β-oxidized in Atlantic salmon tissue; however, when dietary levels are low, the preferred FA substrate for β-oxidation appears to switch and 20:5n-3 and 22:6n-3 are stored rather than catabolized for energy (Stubhaug et al., 2007). Since the increase in lipid proportions in liver of fish fed Diet 1 was lower than those fed Diets 2 and 3, fish fed Diet 1 are more likely metabolize FA in a similar manner to wild fish and therefore CC_{D1} would be the most appropriate of the three CC to use. However, the lipid content of liver in fish fed Diet 1 was much greater than that of the initial fish, suggesting that even those fish fed the diets with lowest lipid

content may still have been consuming a relatively more lipid enriched diet than naturally encountered by pollock. While the CC generated from Diet 1 are likely to be the lowest that can be formulated using regular diets, no data are available to show that lower fat diets (< 4.9 %) might continue to generate different CC.

Atlantic pollock was used as a model species in this work for two main reasons: 1) the ease of maintenance in a captive setting, and 2) the physiological similarities to cod (*Gadus morhua*) and haddock (*Melanogrammus aneglefinus*) in that all store lipids in the liver. Therefore, the CC developed during this project, particularly those from Diet 1, which contained the lowest lipid levels, may in time be applied to study diets of wild gadoids that are important in commercial fisheries. While the impact of the different CC developed here could be established in a controlled feeding study where diets are known, the influence of incomplete prey libraries on diet estimates is an important area for further investigation. Future work should investigate features of the existing QFASA model (Bromaghin et al., 2015), including the importance of a complete prey library, as well as *in vivo* feeding experiments with captive fish. Use of additional biomarkers, such as stable isotopes, in combination with FA profiles may also represent another means to achieve reliable estimates of diet.

CHAPTER 3 INFLUENCE OF DIETARY FAT CONCENTRATION ON STABLE CARBON ISOTOPE RATIOS OF FATTY ACIDS IN LIVER TRIACYLGLYCEROLS IN ATLANTIC POLLOCK (*POLLACHIUS VIRENS*)

3.1 INTRODUCTION

Bulk stable isotope analysis has been used in numerous applications to provide information about trophic interactions, foraging ecology, and predator diets (Hobson and Clark, 1992; Gannes et al., 1997; Pond and Gilmour, 1997; Bloomfield et al., 2011). The use of naturally occurring stable isotopes in ecological applications relies on the premise that their concentrations are predictably changed as they are transferred to consumers at higher trophic levels. Thus, if prey items, or groups of prey, have distinct isotopic signatures, those signatures can be recognized in the predators' tissues. With representative sampling of predator and potential prey, mixing models (e.g., Parnell et al., 2010) can then be used to determine likely contributions of prey that would yield the isotopic patterns found in the predators' tissues. Carbon and nitrogen are the elements most used in ecological applications; they are normally measured in a specific tissue type, without regard to the particular nutrient that they are derived from. This approach has been applied to a wide range of organisms from top predators, such as sharks and seals (Rau et al., 1992; Tucker et al., 2007; Carlisle et al., 2012) to herbivores utilizing different plant sources (Boutton et al., 1983; Codron et al., 2006; Radloff et al., 2013).

Despite their widespread application, there are two inherent difficulties associated with the use of bulk stable isotopes: isotopic routing and fractionation. Stable isotopes undergo isotopic routing during deposition in the tissue, when the isotopes of different dietary nutrients are routed to specific tissues (Gannes et al., 1997). For instance, carbon

from dietary protein is much more likely to be incorporated into muscle than carbon from dietary carbohydrate. Thus, if muscle containing protein is sampled in an omnivore, the contribution of plants (mainly carbohydrate) in the diet may be underestimated. With bulk isotopes, appropriate selection of sampling tissue may avoid some of the problems associated with routing (Gannes et al., 1997) but it is often an unacknowledged issue.

Isotopic fractionation may occur when a reaction involves forming or breaking a bond that contains the elements of interest; chemical kinetics dictate that the molecule with the lighter isotope participating in the breaking or formation of a bond will react at a faster rate than one with a heavier isotope at that position so that the products of the reaction have a different distribution of heavy and light isotopes than the reactants (Fry, 2006) When considering isotopes of the same element, the potential energy barrier for reactions involving, for instance, molecules containing ^{12}C is less than those containing ^{13}C, leading to the faster reaction rate (Bigeleisen and Mayer, 1947; Bigeleisen and Wolfsberg, 1958). In animals, predator tissues are usually enriched in ^{13}C, showing an increase in $\delta^{13}C$ by ~1 ‰ (typically expressed as a discrimination factor, Δ) relative to diet, due to the preferential release of the light CO_2 during catabolism of different tissue types (DeNiro and Epstein, 1978). A similar effect is observed with nitrogen. The lighter isotope, ^{14}N, is preferentially excreted in urea and ammonia so that the consumer becomes enriched in the heavier isotope ^{15}N by ~3 ‰ (Minigawa and Wada, 1984). These predictable enrichments can be incorporated into mixing models and used to estimate predator diets and trophic levels (Gannes et al., 1997).

The difficulty with fractionation arises with the assumption that enrichment of ^{13}C and ^{15}N in the predator is fixed. Discrimination factors, and hence fractionation, vary

among species, tissues within a single species, and diets (DeNiro and Epstein, 1978; McCutchan et al., 2003; Caut et al., 2009; Hussey et al., 2014). Further, there is no consensus on the impact of this variation in discrimination factors on results from mixing models (Phillips and Gregg, 2003; Martinez del Rio, 2009; Phillips, 2012).

Compound-specific isotope analysis (CSIA) has been suggested as an alternative to avoid the aforementioned limitations associated with bulk isotope analysis (Chamberlain et al., 2006; Dungait et al., 2008; Sauheitl et al., 2009; McMahon et al., 2010). The primary advantage of CSIA of FA over bulk stable isotope analysis is the elimination of issues associated with isotopic routing because the same diet-derived compounds are compared in predator tissue and diet, thus avoiding the problem of differential assimilation of dietary components. By focusing on PUFA that vertebrates cannot synthesize, a compound-specific approach can actually take advantage of isotopic routing because dietary PUFA are routed and stored in adipose in the predator, where they can be easily sampled.

Although CSIA does target specific FA, fractionation remains an issue. In animal tissues, fractionation of FA stable isotopes is primarily due to enzyme-catalyzed reactions, which can be classified as those arising from assimilation and deposition of FA in adipose or from modification of the FA structure after deposition. During assimilation, dietary FA are esterified to a glycerol backbone in the form of triacylglycerols (TAG). These are large molecules and to pass through the intestine and adipose cell walls, they must be hydrolyzed during digestion to form smaller molecules of free FA and monoacylglycerols (a TAG derivative). These molecules pass through the intestinal wall where they are then reassembled into TAG form and transported in the blood and stored

in tissues such as adipose and liver in TAG form, after again being hydrolyzed and reassembled into TAG. Thus, FA undergo several cycles of hydrolysis and re-esterification before deposition in the storage tissue, all of which may lead to fractionation. However, AuCoin (2011) compared the $\delta^{13}C$ of FA in blood chylomicrons and diet in Atlantic pollock and found no change in 18:2n-6, 20:5n-3 and 22:6n-3, thus eliminating digestion as a major point of carbon isotope fractionation during FA metabolism.

Once deposited in the adipose, FA may undergo a number of processes that may cause fractionation, including desaturation, elongation, chain shortening, and β-oxidation; FA may also be created through *de novo* synthesis. However, the influence of many of these processes on fractionation can be eliminated if dietary PUFA are the target compounds, simply because they must be directly derived from diet and cannot arise from these processes. It is only β-oxidation, or breakdown of FA for energy, that may apply to PUFA because this process can make use of any FA substrate. Budge et al. (2011b) suggested that such catabolism might explain the consistent enrichment of $\delta^{13}C$ in linoleic acid in captive eiders fed a diet rich in that same FA. Other work supports this idea. For instance, Nanton et al. (2003) found significantly higher β-oxidation in liver of Atlantic haddock after feeding a diet composed of 18 % lipid compared to 12 % lipid. Similarly, Bell et al (2003) noted that FA present in high concentrations in dietary lipid were preferentially utilized for metabolism in Atlantic salmon. Thus, β-oxidation might lead to selective fractionation of FA present in the largest proportions in the diet.

The overall objective of this project was to determine the influence of FA concentration in the diet on the extent of fractionation of carbon stable isotopes between

tissue and diet in Atlantic pollock, with the assumption that any change would be due to β-oxidation. I anticipated that FA present in diets at high concentrations would be more likely to undergo β-oxidation than those present at low concentrations; therefore, I expected discrimination factors ($\Delta = \delta^{13}C_{consumer} - \delta^{13}C_{prey}$) to be higher in fish fed high concentrations of FA. To investigate the effect that FA concentration had on $\delta^{13}C$ values of liver TAG FA, I used a controlled feeding study arranged with three diets of different FA concentrations, but very similar FA $\delta^{13}C$ values. Using CSIA, I determined if there was an increase in $\delta^{13}C$ of liver TAG FA when the fish were fed a higher fat diet. The specific objectives were 1) to evaluate the influence of FA concentration of diet on $\delta^{13}C$ of FA in the liver, the primary storage tissue of pollock; and 2) to calculate discrimination factors for essential and non-essential FA.

3.2 MATERIAL AND METHODS

3.2.1 EXPERIMENTAL FISH AND DIETS

See Sections 2.2.1 and 2.2.2 for description of study design, fish husbandry and formulation of diets.

3.2.2 LIPID EXTRACTION AND FAME PREPARATION

Lipids were extracted and FAME prepared following the same processes as described in Section 2.2.3. FAME from all diet samples (n = 18) and liver from initial fish (n = 11), as well as liver samples from each fish fed a treatment diet ($Liver_{D1}$ n = 13; $Liver_{D2}$ n = 14; $Liver_{D3}$ n = 13) were made up to concentrations of 50 mg/mL using dichloromethane. The liver samples were chosen for CSIA based on the amount of FAME remaining after analysis by GC-FID, rather than equivalent sample dispersion

among tanks. Therefore, tank could not be used as the sampling unit and each fish was treated as an individual sample.

3.2.3 ISOTOPE ANALYSIS

The $\delta^{13}C$ values of FAME from liver TAG, isolated by TLC as described in Section 2.2.4, were analyzed on an Agilent 6890N GC coupled to a Thermo Scientific Delta V+ isotope ratio mass spectrometer (IRMS) via a ConFlo III interface at the Core Research Equipment and Instrument Training Network (CREAIT Network) at Memorial University in Newfoundland. The inlet was held at 250 ˚C and 1 mL of sample was injected in splitless mode (purge flow on at 0.5 min) using a GC-PAL A200S autosampler on a BPX-70 column (SGE Analytical Science; 50 m x 0.32 mm x 0.25 um) with helium as carrier gas at 1.5 mL/min. The initial column temperature was 70 °C, and the temperature was ramped at 10 ˚C/min to 160 ˚C, held for 5 min and then ramped at 4 °C/min to 260 °C with a 10 min hold. The oxidation reactor was held at 940 °C and was regenerated daily for 10 min.

$\delta^{13}C$ measurements specifically targeted the long-chain PUFA that cannot be synthesized by fish (18:2n-6, 20:5n-3, and 22:6n-3) and FA that were present in proportion > 3 % mass of total FA (14:0, 16:0, 16:1n-7, 18:0, 18:1n-9, 18:1n-7, 20:1n-9). FAME were separated by GC and combusted to CO_2 by a CuO/NiO/Pt oxidation reactor in a Thermo Finnigan GC combustion III interface. Water molecules were removed, and CO_2 was carried to an IRMS for analysis of the C isotopes relative to a reference CO_2 gas calibrated against an OzTech CO_2 standard of -40.63 ‰ vs. Vienna Pee Dee Belemnite (VPDB) using the dual inlet method, and had a value of -40.97 ‰. Results were expressed as:

$$\delta^{13}C = [(R_{sample}/R_{standard}) - 1] \times 1000$$

where R_{sample} and $R_{standard}$ are the ratios of $^{13}C/^{12}C$ for the sample and standard. A standard containing 4 FAMEs and 4 fatty acid ethyl esters (FAEEs) of 14:0, 16:0, 18:0, and 20:0 was analyzed several times a day. The certified $\delta^{13}C$ values were plotted against the measured values, and the equation of the linear regression line was used to make a small daily correction to the measured $\delta^{13}C$ values of the samples.

The methanol used for methylation of FA was isotopically analyzed (G.G. Hatch Laboratories, University of Ottawa) to ensure that the thermodynamic isotope effects were minimal in the methanol during the extraction stage of the experiment. The methanol was dropped into smooth-walled tin capsules via a syringe. Helium was used to gently flush the capsule before it was cold sealed with a special press and loaded into an elemental analyzer (Isotope Cube manufactured by Elementar, Germany) interfaced to an IRMS. The methanol was flash combusted at 1800 °C and the gas products carried by helium through columns of oxidizing/reducing chemicals optimized for CO_2 and N_2. The gases were separated by a purge and trap absorption column and sent to the IRMS interface (Conflo III manufactured by Thermo, Germany), and then to the IRMS (Delta Advantage manufactured by Thermo, Germany). All $\delta^{13}C$ values are reported as ‰ vs. V-PDB and normalized to internal standards calibrated to International standards IAEA-CH-6(-10.4 ‰), NBS-22(-29.91 ‰), USGS-40(-26.24 ‰) and USGS-41(37.76 ‰). The internal standards used were C-51 Nicotiamide (0.07,-22.95), C-52 mix of ammonium sulphate + sucrose (16.58,-11.94), C-54 caffeine (-16.61,-34.46), blind std C-55: glutamic acid (-3.98, -28.53).

3.2.4 DATA AND STATISTICAL ANALYSIS

$\delta^{13}C$ data of FAME was corrected for the addition of methanol to the FA when transesterifying lipids to produce FAME. $\delta^{13}C$ values for the free FA (FFA) were calculated for each FAME using the equation:

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

where n is the number of carbons in the FFA. The $\delta^{13}C_{FFA}$ was used for the remaining experiment.

A Shapiro Wilks test was used to assess normality of the $\delta^{13}C_{FFA}$ data of diets, initial liver, and treatment liver. For treatment diets, when normality was confirmed, multivariate analysis of variance (MANOVA) was used to determine if $\delta^{13}C$ of FA in fish liver varied according to diet. When MANOVA indicated an overall significant result, ANOVA was performed on each FA variable with a Tukey post-hoc test to determine the similarity of $\delta^{13}C$ among diets. For the ANOVA, an adjusted p-value of 0.005 was used to compensate for multiple testing. Similarly, a MANOVA followed by ANOVA with Tukey post-hoc tests was also used to determine differences in $\delta^{13}C$ within FA of the initial and three treatment livers, using an adjusted p-value of 0.005 for the ANOVA.

To determine the extent of modification of $\delta^{13}C$ of FA in the liver during the 20-week feeding trial, the $\delta^{13}C$ values of the liver of fish fed the treatment diets were compared to the treatment diets. Discrimination factors (Δ) were calculated for each FA by subtracting the mean $\delta^{13}C$ of diet (D) from the mean of the treatment liver (T) sample, such that $\Delta_{T-D} = \delta^{13}C_T - \delta^{13}C_D$. To determine if the discrimination factors were significantly different from zero, MANOVA, followed by ANOVA and Tukey post-hoc tests, as appropriate, was used to compare the liver and treatment diet for each FA, again

using an adjusted p-value of < 0.005 for the ANOVA. SPSS (Version 20; IBM Corporation; NY, USA) was used for all statistical analysis.

3.3 RESULTS

3.3.1 TREATMENT DIETS

The majority of FA fell into two groups, with $\delta^{13}C$ values that ranged from -25 to -22 ‰ and -28 to -26 ‰; 18:2n-6 did not fall into either group, with $\delta^{13}C$ values at ~ -30‰ (Figure 3.1). While MANOVA indicated an overall significant multivariate effect of treatment diet on FA $\delta^{13}C$ (Wilks $\lambda = 0.006$, $F_{20,8} = 4.9$, $p < 0.05$), univariate tests did not identify any effect of diet type by individual FA ($p < 0.005$; Figure 3.1). Comparing mean $\delta^{13}C$ values across FA, a number were significantly different from each other (ANOVA: $F(9,168) = 356.8$, $p < 0.005$). For example, the mean $\delta^{13}C$ value for 20:1n-9 was significantly different than all other FA. Similarly, while mean $\delta^{13}C$ values for 20:5n-3 and 22:6n-3 were equivalent, they were both significantly different than all other FA. The FA 18:2n-6 was obviously significantly different than all others. However, 14:0, 16:0, and 18:1n-9 were equivalent in $\delta^{13}C$ values; 16:1n-7 and 18:0 were also the same.

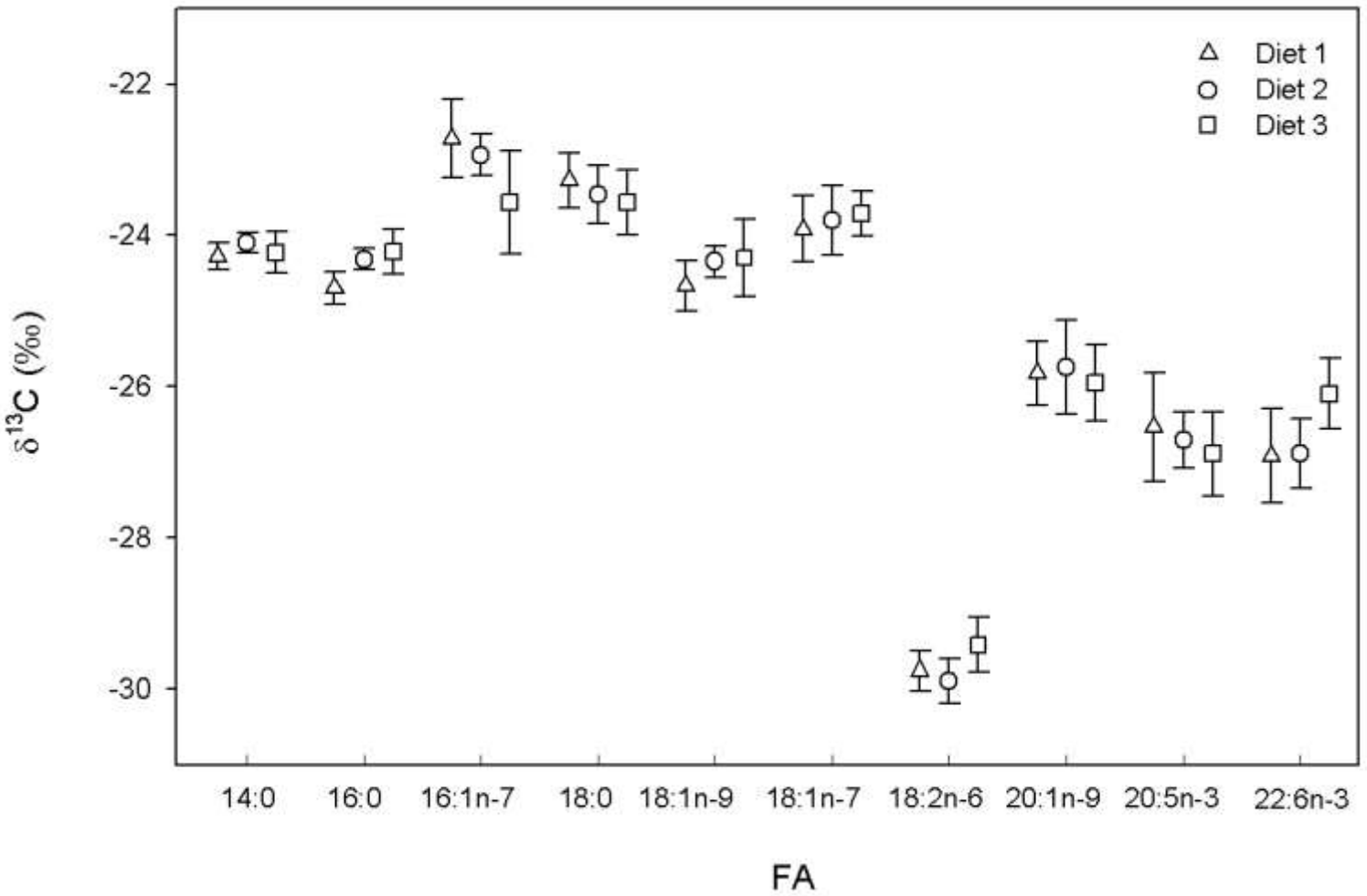


Figure 3.1: $\delta^{13}C$ (‰; mean +/- sd, n=6 for each diet) of selected FA in the experimental diets. Data for all FA with sufficient peak size to allow reproducible measurements are shown.

3.3.2 LIVER TAG

Initial liver FA had $\delta^{13}C$ ranging from -31 to -25 ‰, with mean values of 20:5n-3 and 22:6n-3 most depleted (ANOVA: $F(9,99) = 99.60$, $p < 0.005$), while the FA of treatment liver were generally more enriched, with the exception of 18:2n-6. In the treatment liver, $\delta^{13}C$ values followed a trend similar to that of diet where most FA had values that fell into two groups ranging from -25 to -22 ‰ or -28 to -25 ‰. Also, similar to diets, 18:2n-6 was depleted relative to other FA in the treatment livers. $\delta^{13}C$ values of all FA in the treatment liver differed significantly from the $\delta^{13}C$ values of initial liver tissues (MANOVA: Wilks $\lambda = 0.006$, $F(30,109) = 17.16$, $p < 0.001$; all univariate ANOVAs: $p < 0.005$; Figure 3.2). Within a FA, diet did not have an effect on liver $\delta^{13}C$ values for 16:1n-7, 18:1n-9, 18:1n-7, 18:2n-6, and 20:1n-9. For all other, feeding Diet 1 resulted in enriched $\delta^{13}C$ values in the liver relative to Diet 2 or 3 (Tukey's test: $p < 0.05$).

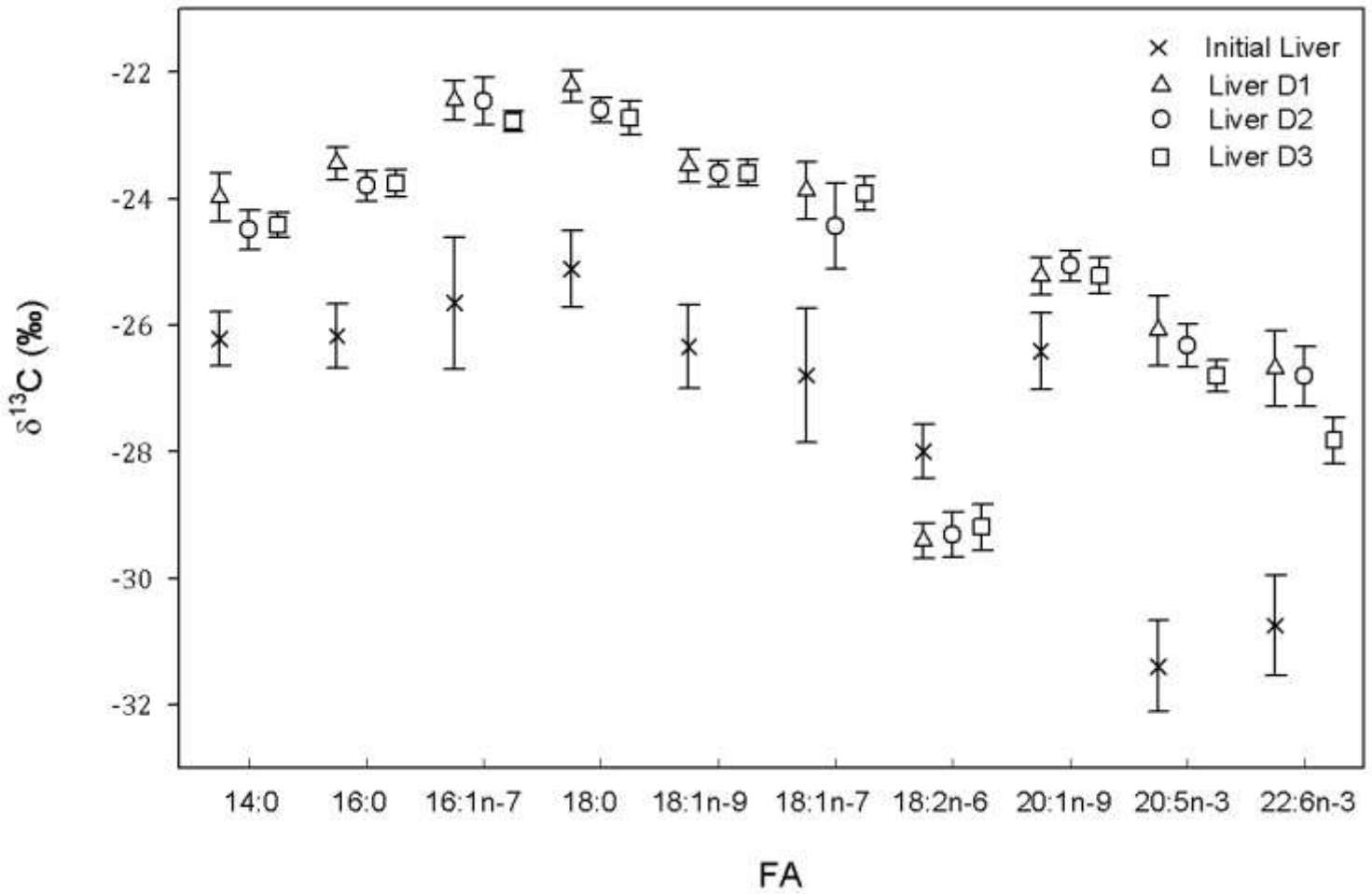


Figure 3.2: $\delta^{13}C$ (‰; mean +/- sd) for TAG FA of the liver of initial pollock (n=11) and the liver of fish fed treatment Diets 1, 2, and 3 (n=14, 13, and 14 respectively). Data for all FA with sufficient peak size to allow reproducible measurements are shown.

3.3.3 DISCRIMINATION FACTORS

Discrimination factors varied between 1.2 and -1.2 (Figure 3.3), with the most extreme values seen in 18:0 and 22:6n-3. MANOVA indicated that discrimination factors were significantly different than zero ($F(10,44) = 23.99$, $p < 0.001$, Wilks $\lambda = 0.155$, partial $\eta^2 = 0.845$); univariate analysis (ANOVA) demonstrated that this effect varied with FA and dietary treatment. There was some consistency in 16:0, 18:0, 18:1n-9, and 20:1n-9, with all having discrimination factors that were significantly greater than zero for all dietary treatments. For all other FA, there were no clear patterns according to dietary treatment (Table 3.1). For instance, for 16:1n-7, the discrimination factor calculated from feeding Diet 3 was not different from zero, but for 22:6n-3, Diet 3 was the only treatment that produced a discrimination factor that was significantly less than zero. Conversely, for 18:2n-6, Diet 3 generated a discrimination factor that was significantly greater than zero. For 20:5n-3, it was Diet 1 that gave a discrimination factor significantly greater than zero, while Diet 2 produced a discrimination factor less than zero for 18:1n-7.

When FA were grouped according to those that are more likely to be synthesized or undergo biosynthetic modification, a clearer pattern emerges. Regardless of diet fed, most discrimination factors for saturated and MUFA showed significant deviations from zero; in contrast, few discrimination factors for PUFA were significantly different than zero (Table 3.1).

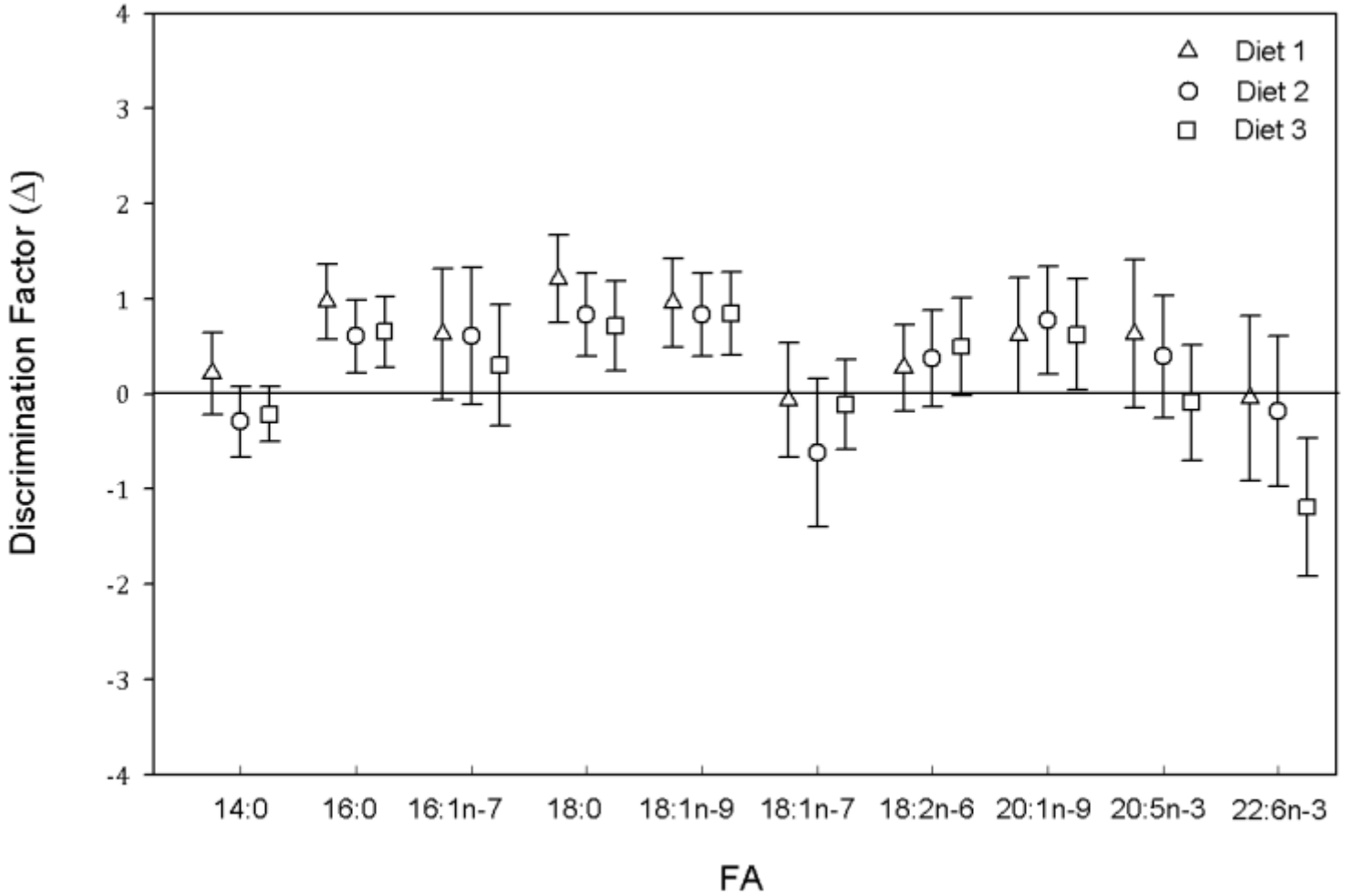


Figure: 3.3: Discrimination factors ($\Delta = \delta^{13}C_T - \delta^{13}C_D$; mean +/- sd) of FA for liver of pollock fed Diets 1 (n=14), 2 (n=13), and 3 (n=14). Data for all FA with sufficient peak size to allow reproducible measurements are shown.

Table 3.1: Discrimination factors (mean +/- sd) for liver of pollock fed Diets 1, 2, and 3. Asterisks denote FA with discrimination factors that are significantly different than zero.

FA	Diet 1		Diet 2		Diet 3	
	Mean	SD	Mean	SD	Mean	SD
Saturated						
14:0	0.22	0.41	-0.29	0.34	-0.21	0.34
16:0	0.97*	0.33	0.61*	0.28	0.65*	0.37
18:0	1.21*	0.44	0.83*	0.43	0.71*	0.50
Monounsaturated						
16:1n-7	0.63*	0.61	0.61*	0.46	0.30	0.71
18:1n-9	0.96*	0.42	0.83*	0.29	0.84*	0.55
18:1n-7	-0.06	0.64	-0.62*	0.81	-0.11	0.40
20:1n-9	0.62*	0.54	0.78*	0.67	0.63*	0.58
Polyunsaturated						
18:2n-6	0.28	0.37	0.37	0.46	0.50*	0.52
20:5n-3	0.63*	0.90	0.39	0.50	-0.09	0.61
22:6n-3	-0.04	0.87	-0.18	0.66	-1.19*	0.59

3.4 DISCUSSION

3.4.1 TREATMENT DIETS

The three treatment diets were isotopically equivalent, likely because the major source of FA in the diet was the herring oil. This equivalency eliminated any bias from isotopic composition, and allowed the assessment of differential FA concentrations on metabolism and subsequent influence on $\delta^{13}C$ of FA. There was significant variation among the FA, with $\delta^{13}C$ values ranging from -27 to -22 ‰; 18:2n-6 was an exception and had much more depleted $\delta^{13}C$ values then all other FA at -30 ‰. This FA is often cited as a marker of terrestrial material (Bell et al., 1994; Blanchard et al., 2008) and C_3 plants are isotopically depleted in ^{13}C relative to marine or C_4 plants (Chisholm et al., 1982). Thus, the depleted values for 18:2n-6 may point to a terrestrial source, specifically a C_3 plant, of this FA in the diet. Wheat middlings, which comprise from 9 – 16 % of the experimental diets, are a C_3 plant high in 18:2n-6 (Laudadio and Tufarelli, 2011) so it can

be assumed that much of the 18:2n-6 seen in the diet (Figure 2.1) was derived from wheat middlings.

3.4.2 LIVER TAG AND DISCRIMINATION FACTORS

The liver of the initial fish, reflecting diets of wild fish, showed lower $\delta^{13}C$ values for 20:5n-3 and 22:6n-3 compared to the other FA (Figure 3.2). The literature is conflicting as to whether this depletion in ^{13}C is normal in marine systems. Murphy and Abrajano (1994) saw similarities between two sampling sites (Conception Bay and Trinity Bay) for horse mussels (*Modiolus modiolus*) and blue mussels (*Mytilus edulis*) for 20:5n-3 (-25 to -26 ‰), while the $\delta^{13}C$ value of 22:6n-3 was lower (-26 to -30 ‰) and varied with location. All other FA, with the exception of 18:4n-3, in sampled areas were more enriched relative to 20:5n-3 and 22:6n-3. Since both 20:5n-3 and 22:6n-3 are derived from phytoplankton, Murphy and Abrajano (1994) suggested that the depletion of ^{13}C in these two FA in mussels could be due to fractionation during synthesis by phytoplankton. Such fractionation could help explain the lower $\delta^{13}C$ values of 20:5n-3 and 22:6n-3 relative to the other FA in Atlantic pollock. However, in a species more closely related to pollock, Atlantic cod (*Gadus morhua*), both 20:5n-3 and 22:6n-3 had $\delta^{13}C$ values of -27 ‰, similar to most FA observed (~ -26 to ~ -28 ‰; Copeman et al., 2009). The differences between cod and pollock may be partly explained by sampling location; the cod were collected from a sheltered near-shore location in eastern Newfoundland, while the pollock were collected from coastal Nova Scotia. It is possible that very different phytoplankton and zooplankton communities were consumed by these fish. Also, the cod were almost undoubtedly consuming a different diet than the pollock, as the cod were much smaller (~6 cm vs. 34-51 cm); different diets may point to different

carbon sources and therefore isotopic signatures. If fractionation of non-essential FA occurs to a greater extent than PUFA, as the data here suggested, it would be expected that non-essential FA would become progressively enriched with trophic level. Therefore, variation with fish size may have less to do with source material and more to do with fractionation. In polar cod (*Boreogadus saida*) collected in the Beaufort Sea little variation in the $\delta^{13}C$ values was seen in the seven FA analyzed; both 20:5n-3 and 22:6n-3 had $\delta^{13}C$ values near -31 ‰ (Graham et al., 2014). The cod were much smaller (~60 mm) than the pollock analyzed here and were obviously from a different habitat and population. If fish collected in coastal Nova Scotia and Newfoundland showed sufficient differences in isotopic signatures, little similarity would also be expected in fish sampled in a seasonally ice-covered environment.

Despite the depletion of ^{13}C in 20:5n-3 and 22:6n-3 in the liver of initial pollock, an enrichment of approximately 6 ‰ was seen in fish fed the treatment diets relative to the initial samples. Although not showing such a large change in magnitude, $\delta^{13}C$ values of all other FA were also different in initial liver samples compared to liver from fish fed the experimental diets (Figure 3.2). The initial samples make it clear that the tissue did respond to the diet. For all FA except 18:2n-6, $\delta^{13}C$ of the initial liver was lower than the diet; however, at the end of the feeding experiment, all FA in the liver had higher $\delta^{13}C$ values than diet, clearly indicating that 20 weeks was likely a sufficient period of time for the fish to turn over FA and take on a new isotopic signature. The time period agrees with other studies with Atlantic salmon, Atlantic cod, rainbow trout (*Oncorhynchus mykiss*), and turbot (*Scophthalmus maximus*) (Castledine and Buckley, 1980; Regost et al., 2003b;

Kjær et al., 2009; Budge et al., 2012) where a new FA signature and, presumably FA $\delta^{13}C$ value, was incorporated in studies of 8-22 weeks duration.

In liver, the $\delta^{13}C$ values for 14:0, 16:0, 18:0, 20:5n-3, and 22:6n-3 did vary by treatment. While most of these differences were subtle, there was a trend to decreasing $\delta^{13}C$ with increasing concentration of FA in the diet. This decrease in $\delta^{13}C$ values contradicts the hypothesis that increasing FA concentration would lead to greater β-oxidation and therefore FA with higher $\delta^{13}C$, and suggests that some process was occurring that led to selective retention of FA containing the lighter isotope as dietary lipid level increased. By comparing blood chylomicrons to liver in pollock, AuCoin (2011) has shown that this difference is not caused by hydrolysis or re-esterification during assimilation of the FA in lipoproteins. For the saturated FA, *de novo* synthesis in the liver would be expected to result in higher $\delta^{13}C$ values relative to diet if carbon was from catabolism of lipids, since acetate precursors with the lowest amounts of ^{13}C would be preferentially incorporated; however, with a dietary surplus of FA, *de novo* synthesis would likely be suppressed. Therefore, liver of fish fed the diet with the highest lipid content, Diet 3, may show the least influence of *de novo* synthesis, and have lower $\delta^{13}C$ values that are more similar to diet. However, the same explanation cannot be applied to the essential FA 20:5n-3 and 22:6n-3.

Although it contradicts the proposed hypothesis, β-oxidation may still play a role but with an opposite effect to that predicted. It may be that β-oxidation of FA occurs at a constant rate necessary to maintain the metabolic functions of the fish, regardless of FA concentration in the diet. If true, then fish fed Diet 3 would have more FA available for deposition in their liver than fish fed Diet 1, meaning that FA of fish fed Diet 3 would

bear a greater resemblance to the $\delta^{13}C$ of diet, and, in effect, better mask the impact of any enrichment in $\delta^{13}C$ due to oxidation. This effect was observed only at total FA concentrations > 75.1 mg g^{-1}; therefore, it may not be detected when examining wild fish with diets lower in fat. In fact, several studies monitoring gene expression and enzyme activity in carp (*Ctenopharyngodon idella*) and bream (*Megalobrama amblycephala*) have shown a decrease in β-oxidation with increasing dietary fat content (Du et al., 2006; Lu et al., 2014). While more work is needed to determine the real influence of fat content on oxidation, the results of the current study offer empirical support for the idea of decreased β-oxidation with increasing fat content.

Of the five FA that showed a significant decrease in $\delta^{13}C$ with increasing dietary FA concentration, only 22:6n-3 in fish fed Diet 3 had a negative discrimination factor (Figure 3.3), indicating that 22:6n-3 was more depleted in ^{13}C in the tissue than in the feed. Aucoin (2011) calculated a similar negative discrimination factor in liver after feeding pollock a fish oil-based diet for 20 weeks, suggesting that the result for 22:6n-3 here is not false. Compared to all other major FA, 22:6n-3 had quite constant proportions in the liver (Figure 2.3), despite varying concentrations and proportions in the diet. This constant proportion of 22:6n-3 may point to a specific role for 22:6n-3 in fish physiology, rendering it less representative of the $\delta^{13}C$ of dietary FA. It is worth noting that 18:1n-7 was the only other FA that showed near constant proportions in the liver across the three treatments; while not significantly different between treatments, discrimination factors for that FA were also negative (Figure 3.3).

Since there are biochemical restrictions on the synthesis of PUFA, it was expected that there would be minimal modification of $\delta^{13}C$ in PUFA between diet and tissue,

similar to that seen with essential amino acids (AA) (McMahon et al., 2010; McMahon et al., 2015), where the discrimination factors of essential AA clustered closer to zero than non-essential AA in both fish and birds. The results of FA follow a similar pattern to AA; fish fed the diet with the lowest FA concentration, Diet 1, had discrimination factors for two of three PUFA (18:2n-6 and 22:6n-3) that were not significantly different than zero. Most of the non-essential FA had discrimination factors that were different than zero and > 0.6 ‰. The discrimination factor for 20:5n-3 was different than zero, but the biological significance of that is questionable as the error associated with that FA was larger than all others. Greater sample size would increase our confidence in this result.

When comparing fish fed Diet 3, with FA concentrations ~ 10 %, to fish fed a fish oil based diet of ~ 20 % lipid WWB (Aucoin, 2011), the results were more in agreement, showing near identical discrimination factors for 18:2n-6 and similar values for 20:5n-3. In both this current project as well as Aucoin (2011), with the high fat diets, 22:6n-3 had consistent negative discrimination factors (< -1 for both). The $\delta^{13}C$ values for PUFA in the diets used for pollock here were ~ 2-3 ‰ greater than those used by Aucoin (2011). While Caut et al. (2009) have shown that fractionation of bulk $\delta^{13}C$ values vary predictably with $\delta^{13}C$ of diet, the consistency of the results from feeding Diet 3 with those of Aucoin (2011) suggests that fractionation of $\delta^{13}C$ of individual FA is not subject to the same variation.

Overall, the results suggest that PUFA hold great promise as biological tracers of carbon source. When fed the lowest fat diet, pollock liver did not fractionate 18:2n-6 or 22:6n-3, and 20:5n-3 showed only slight fractionation. These FA may prove useful as tracers since 18:2n-6 can be used as a terrestrial marker, while 22:6n-3 and 20:5n-3

typically have marine or aquatic sources. Offering multiple markers for a single isotope presents a clear advantage over bulk analyses. Use of FA-specific isotopic data also introduces the potential of coupling isotopic fingerprinting of PUFA, similar to those of AA (Larsen et al., 2009), with FA signatures based on proportional composition. However, while the FA signature approach is well validated for proportional compositions (Iverson et al., 2004) and these experiments with $\delta^{13}C$ of PUFA are promising, further work is needed before the application of these combined techniques to wild populations can be considered.

CHAPTER 4.0 CONCLUSION

Results of this feeding trial directly influence our ability to interpret and rely on the results of experiments conducted in the wild. CC developed during this project show that they do vary with fat content. Higher fat diets in general give CC closer to 1, so that fish liver more closely resembles FA profiles of the diet than fish fed lower fat diets. CC developed from feeding fish Diet 1, the lower lipid concentration, may be the most reliable, as this diet more closely resembles the diet of wild fish, and could be applied to study diets of wild gadoids. While the FA data did not show evidence of an increase in β-oxidation of FA with increasing fat content, it did show that the FA signature of storage tissues was not influenced solely by dietary FA profile. Fat content of the diet also had a direct effect on FA composition of storage tissues.

Little variation was seen in discrimination factors developed from the three treatment diets and the corresponding liver. Regardless of diet, most discrimination factors for saturated and MUFA in fish showed significant deviations from zero. In contrast, few discrimination factors for PUFA were significantly different than zero. Therefore, it can be assumed that if $\delta^{13}C$ of a PUFA was sampled from liver of a wild fish, $\delta^{13}C$ of FA in that sample would be related to the prey consumed by that animal. In other words the $\delta^{13}C$ of 20:5n-3, for instance, sampled from a pollock in the wild would be expected to be similar to that of the same FA in the prey it consumed. Like the FA profiles, the patterns observed cannot be explained by increased β-oxidation due to an increase in fat content. As other studies have shown (Du et al., 2006; Lu et al., 2014), increasing the dietary fat content can cause a decrease in β-oxidation. This description better explains the stable isotope data found in pollock.

There are a number of areas where improvements could be made to the experimental design and future work should take these into account. The most obvious improvement would be to collect liver and analyze it for enzyme activity. In doing so, the variation in oxidation with dietary fat content can be directly determined. The experimental fish received diets formulated to resemble those of wild diets, only differing by fat concentration; however, the diets did not fully meet expectations. For example, the low fat diet (4.9 %) was still not as low in fat as that of wild fish. It would have also been useful to feed diets with higher fat contents. For instance, use of 12 % fat, as was used in Aucoin (2011), would have facilitated comparisons with that work. Last, when estimating the proportions of prey species of wild fish diets using QFASA, an incomplete prey library was used. For the estimates to be more accurate and not sensitive to missing data, a complete prey library should have been utilized. Simulation studies, such as those described by Iverson et al. (2004), would have eliminated this problem of missing data and given a clear picture of the effect of varying CC on diet estimates.

Despite the limitations of the feeding trial, the CC that have been determined for Diet 1 can be applied, with care, to assess diets of wild gadoids, as this diet most resembles the FA concentration of diets of fish found in the wild. Also, the discrimination factors that have been measured for Atlantic pollock FA metabolism could eventually be used to outline food webs when more data are available on the isotope fractionation associated with the metabolism of zooplankton and higher animals. This is important background information in order to begin to use CSIA to estimate diets in wild fish and represents a starting point for other studies investigating fractionation in $\delta^{13}C$ of FA from diet to tissue.

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APPENDIX
FATTY ACID RESULTS

Appendix I: FA lists used in QFASA diet estimates.

Full FA	10 Largest FA	Dietary FA	Extended Dietary FA
Saturated	Saturated	Saturated	Saturated
14:0	14:0		14:0
i-15:0	16:0		16:0
15:0	18:0		17:0
16:0			18:0
i-17:0			
ai-17:0			
17:0			
18:0			
20:0			
Monounsaturated	Monounsaturated	Monounsaturated	Monounsaturated
14:1n-9	18:1n-11	20:1n-11	16:1n-7
16:1n-11	18:1n-9	20:1n-9	18:1n-9
16:1n-9	20:1n-9	20:1n-7	18:1n-7
16:1n-7	22:1n-11	22:1n-11	18:1n-5
16:1n-5		22:1n-9	20:1n-11
17:1(b)[a]		22:1n-7	20:1n-9
17:1			20:1n-7
18:1n-13			22:1n-11
18:1n-11			22:1n-9
18:1n-9			22:1n-7
18:1n-7			
18:1n-5			
20:1n-11			
20:1n-9			
20:1n-7			
22:1n-11			
22:1n-9			
22:1n-7			
24:1n-9			
Polyunsaturated	Polyunsaturated	Polyunsaturated	Polyunsaturated
16:2n-6	18:2n-6	16:2n-6	16:2n-6
16:2n-4	20:5n-3	16:2n-4	16:2n-4
16:3n-4	22:6n-3	16:3n-4	16:3n-4
16:4n-1		16:4n-1	16:4n-1
18:2d5,11		18:2n-6	18:2n-6
18:2n-6		18:2n-4	18:2n-4
18:2n-4		18:3n-6	18:3n-6
18:3n-6		18:3n-4	18:3n-4
18:3n-4		18:3n-3	18:3n-3

Full FA	10 Largest FA	Dietary FA	Extended Dietary FA
18:3n-3		18:4n-3	18:4n-3
18:4n-3		18:4n-1	18:4n-1
18:4n-1		20:2n-6	20:2n-6
20:2n-6		20:3n-6	20:3n-6
20:3n-6		20:4n-6	20:4n-6
20:4n-6		20:3n-3	20:3n-3
20:3n-3		20:4n-3	20:4n-3
20:4n-3		20:5n-3	20:5n-3
20:5n-3		21:5n-3	21:5n-3
21:5n-3		22:4n-6	22:4n-6
22:4n-6		22:5n-6	22:5n-6
22:5n-6		22:6n-3	22:5n-3
22:5n-3			22:6n-3
22:6n-3			

[a] FA with 17 carbon atoms and 1 double bond of unknown position.

Appendix II: Concentrations (mg g^{-1} wet weight; mean +/- sd) of the 51 FA identified in the experimental diets.

FA	Diet 1		Diet 2		Diet 3	
	Mean	SD	Mean	SD	Mean	SD
Total	73.04	2.71	112.82	3.95	136.16	4.72
Saturated						
14:0	3.42	0.19	6.12	0.92	7.81	0.39
i-15:0	0.14	0.01	0.23	0.03	0.28	0.01
15:0	0.28	0.02	0.46	0.05	0.54	0.02
16:0	12.55	0.82	18.1	1.61	20.99	0.84
i-17:0	0.14	0.01	0.22	0.02	0.25	0.01
ai-17:0	0.07	0.00	0.10	0.01	0.11	0.00
17:0	0.27	0.02	0.41	0.03	0.48	0.02
18:0	2.43	0.16	3.51	0.34	4.12	0.17
20:0	0.19	0.02	0.35	0.02	0.41	0.04
Monounsaturated						
14:1n-9	0.08	0.01	0.15	0.01	0.16	0.01
16:1n-11	0.29	0.01	0.44	0.05	0.54	0.02
16:1n-9	0.15	0.01	0.23	0.03	0.28	0.01
16:1n-7	3.95	0.23	7.26	1.09	9.24	0.46
16:1n-5	0.15	0.01	0.24	0.02	0.28	0.01
17:1(b)	0.19	0.02	0.29	0.03	0.34	0.02
17:1	0.17	0.01	0.26	0.03	0.32	0.01
18:1n-13	0.06	0.02	0.09	0.03	0.08	0.01
18:1n-11	0.11	0.02	0.14	0.10	0.23	0.06
18:1n-9	7.54	0.42	10.16	0.94	11.84	0.50
18:1n-7	2.18	0.12	3.27	0.36	3.93	0.15
18:1n-5	0.10	0.01	0.19	0.03	0.25	0.01
20:1n-11	0.26	0.01	0.55	0.13	0.78	0.04
20:1n-9	1.58	0.09	4.45	1.05	6.39	0.04
20:1n-7	0.24	0.01	0.46	0.10	0.58	0.05
22:1n-11	1.97	0.11	6.75	1.76	10.08	0.56
22:1n-9	0.38	0.02	0.66	0.12	0.89	0.05
22:1n-7	0.09	0.01	0.17	0.03	0.22	0.01
24:1n-9	0.36	0.02	0.55	0.08	0.70	0.03
Polyunsaturated						
16:2n-6	0.10	0.01	0.17	0.02	0.21	0.01
16:2n-4	0.55	0.03	0.99	0.15	1.26	0.07
16:3n-4	0.68	0.04	1.18	0.16	1.46	0.08
16:4n-1	1.21	0.08	2.13	0.29	2.62	0.14
18:2Δ5,11	0.11	0.01	0.18	0.02	0.21	0.01
18:2n-6	6.64	0.39	5.86	0.40	5.12	0.08
18:2n-4	0.23	0.02	0.38	0.05	0.47	0.02
18:3n-6	0.13	0.10	0.22	0.03	0.27	0.01

FA	Diet 1		Diet 2		Diet 3	
	Mean	SD	Mean	SD	Mean	SD
18:3n-4	0.09	0.00	0.15	0.02	0.19	0.01
18:3n-3	0.08	0.05	1.03	0.05	1.11	0.04
18:4n-3	1.24	0.08	2.17	0.30	2.70	0.15
18:4n-1	0.14	0.01	0.25	0.03	0.31	0.02
20:2n-6	0.28	0.08	0.31	0.12	0.32	0.12
20:3n-6	0.18	0.07	0.17	0.02	0.21	0.02
20:4n-6	0.82	0.05	1.17	0.11	1.38	0.06
20:3n-3	0.06	0.01	0.09	0.01	0.10	0.00
20:4n-3	0.40	0.02	0.64	0.09	0.81	0.04
20:5n-3	9.65	0.58	15.58	1.92	18.98	0.98
21:5n-3	0.41	0.03	0.67	0.08	0.81	0.04
22:4n-6	0.14	0.01	0.20	0.01	0.19	0.01
22:5n-6	0.23	0.02	0.33	0.03	0.38	0.02
22:5n-3	1.42	0.09	2.18	0.24	2.60	0.12
22:6n-3	8.20	0.50	10.99	0.85	12.37	0.65

Appendix III: Proportions (mass % of total FA; mean +/- sd) of 51 FA for initial liver samples, Diets 1, 2 and 3, and liver from fish fed the corresponding experimental diets.

FA	Initial Liver		Diet 1		Diet 1 Liver	
	Mean	SD	Mean	SD	Mean	SD
Saturated						
14:0	2.47	0.53	4.68	0.08	2.26	0.10
i-15:0	0.22	0.07	0.19	0.01	0.10	0.01
15:0	0.40	0.04	0.38	0.01	0.24	0.03
16:0	13.88	1.45	17.19	0.46	16.36	0.67
i-17:0	0.35	0.05	0.19	0.01	0.20	0.01
ai-17:0	0.22	0.04	0.09	0.00	0.13	0.01
17:0	0.41	0.07	0.37	0.01	0.30	0.02
18:0	5.56	0.83	3.33	0.09	7.31	0.54
20:0	0.09	0.02	0.26	0.02	0.15	0.01
Monounsaturated						
14:1n-9	0.21	0.11	0.11	0.00	0.07	0.01
16:1n-11	0.42	0.13	0.39	0.01	0.33	0.02
16:1n-9	0.45	0.07	0.21	0.00	0.33	0.02
16:1n-7	4.16	1.04	5.41	0.06	4.21	0.15
16:1n-5	1.04	0.03	0.20	0.00	0.12	0.01
17:1(b)	0.42	0.19	0.25	0.02	0.26	0.03
17:1	0.37	0.06	0.23	0.00	0.36	0.01
18:1n-13	0.25	0.16	0.09	0.02	0.06	0.02
18:1n-11	1.19	0.57	0.14	0.01	0.26	0.06
18:1n-9	16.08	3.01	10.32	0.09	23.66	1.49
18:1n-7	4.99	0.57	2.98	0.01	4.66	0.13
18:1n-5	0.39	0.06	0.14	0.01	0.21	0.01
20:1n-11	0.72	0.40	0.36	0.02	0.45	0.03
20:1n-9	5.58	2.06	2.16	0.03	1.93	0.12
20:1n-7	0.55	0.19	0.32	0.01	0.28	0.02
22:1n-11	3.39	1.96	2.69	0.06	1.44	0.10
22:1n-9	0.56	0.22	0.52	0.01	0.24	0.01
22:1n-7	0.10	0.09	0.12	0.00	0.07	0.01
24:1n-9	0.44	0.17	0.50	0.01	0.32	0.02
Polyunsaturated						
16:2n-6	0.07	0.02	0.13	0.00	0.09	0.01
16:2n-4	0.26	0.10	0.75	0.01	0.41	0.02
16:3n-4	0.18	0.08	0.93	0.01	0.55	0.02
16:4n-1	0.23	0.11	1.66	0.03	0.70	0.04
18:2d5,11	0.06	0.02	0.16	0.00	0.23	0.03
18:2n-6	1.77	0.63	9.10	0.25	5.97	0.23
18:2n-4	0.10	0.05	0.31	0.00	0.40	0.02
18:3n-6	0.13	0.03	0.18	0.00	0.15	0.01
18:3n-4	0.04	0.03	0.12	0.00	0.18	0.01
18:3n-3	1.03	0.41	1.14	0.02	0.84	0.04

FA	Initial Liver		Diet 1		Diet 1 Liver	
	Mean	SD	Mean	SD	Mean	SD
18:4n-3	1.29	0.18	1.70	0.03	1.21	0.05
18:4n-1	0.10	0.03	0.19	0.01	0.25	0.01
20:2n-6	0.57	0.20	0.37	0.11	0.34	0.02
20:3n-6	0.10	0.05	0.24	0.07	0.12	0.02
20:4n-6	1.41	0.89	1.12	0.01	0.83	0.06
20:3n-3	0.32	0.11	0.08	0.01	0.09	0.02
20:4n-3	0.59	0.06	0.54	0.01	0.50	0.02
20:5n-3	11.38	2.43	13.21	0.17	10.05	0.59
21:5n-3	0.27	0.04	0.57	0.01	0.45	0.03
22:4n-6	0.25	0.15	0.19	0.01	0.18	0.01
22:5n-6	0.19	0.05	0.31	0.01	0.20	0.02
22:5n-3	1.88	0.48	1.95	0.02	1.64	0.12
22:6n-3	13.74	4.51	11.23	0.14	8.38	0.84

FA	Diet 2		Diet 2 Liver		Diet 3		Diet 3 Liver	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Saturated								
14:0	5.33	0.04	3.09	0.17	5.73	0.04	3.35	0.13
i-15:0	0.20	0.00	0.13	0.01	0.20	0.00	0.14	0.01
15:0	0.41	0.00	0.30	0.01	0.40	0.01	0.31	0.01
16:0	16.20	0.15	15.67	0.52	15.42	0.15	14.81	0.36
i-17:0	0.19	0.01	0.20	0.01	0.18	0.00	0.20	0.01
ai-17:0	0.09	0.01	0.13	0.01	0.08	0.00	0.10	0.02
17:0	0.37	0.00	0.32	0.02	0.35	0.01	0.33	0.02
18:0	3.13	0.01	6.12	0.40	3.02	0.02	5.68	0.35
20:0	0.31	0.02	0.17	0.03	0.30	0.03	0.13	0.01
Monounsaturated								
14:1n-9	0.14	0.00	0.11	0.02	0.15	0.00	0.13	0.01
16:1n-11	0.39	0.01	0.37	0.02	0.39	0.00	0.38	0.01
16:1n-9	0.20	0.00	0.26	0.03	0.20	0.00	0.24	0.01
16:1n-7	6.34	0.04	5.21	0.17	6.78	0.04	5.73	0.12
16:1n-5	0.21	0.00	0.14	0.01	0.20	0.00	0.14	0.00
17:1(b)	0.26	0.01	0.26	0.02	0.25	0.02	0.25	0.02
17:1	0.23	0.00	0.33	0.03	0.23	0.00	0.32	0.01
18:1n-13	0.08	0.03	0.05	0.01	0.06	0.01	0.06	0.02
18:1n-11	0.09	0.07	0.50	0.12	0.17	0.05	0.60	0.14
18:1n-9	9.09	0.03	16.85	1.04	8.70	0.04	14.84	0.83
18:1n-7	2.90	0.01	4.35	0.10	2.89	0.02	4.31	0.12
18:1n-5	0.17	0.00	0.22	0.02	0.18	0.00	0.23	0.01
20:1n-11	0.46	0.01	0.77	0.08	0.57	0.01	0.91	0.07
20:1n-9	3.74	0.03	3.56	0.25	4.69	0.05	4.37	0.13
20:1n-7	0.40	0.02	0.35	0.02	0.42	0.03	0.36	0.02
22:1n-11	5.61	0.05	3.40	0.21	7.40	0.08	4.38	0.17
22:1n-9	0.57	0.01	0.40	0.02	0.65	0.01	0.48	0.02

FA	Diet 2		Diet 2 Liver		Diet 3		Diet 3 Liver	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
22:1n-7	0.15	0.01	0.08	0.01	0.16	0.00	0.09	0.01
24:1n-9	0.49	0.01	0.33	0.04	0.51	0.00	0.32	0.02
Polyunsaturated								
16:2n-6	0.15	0.00	0.12	0.01	0.15	0.00	0.13	0.01
16:2n-4	0.87	0.01	0.59	0.03	0.92	0.01	0.68	0.02
16:3n-4	1.03	0.01	0.74	0.04	1.07	0.01	0.83	0.04
16:4n-1	1.86	0.01	0.96	0.08	1.92	0.02	1.12	0.08
18:2d5,11	0.16	0.01	0.16	0.02	0.15	0.00	0.15	0.01
18:2n-6	5.60	0.10	4.68	0.17	3.76	0.13	3.34	0.06
18:2n-4	0.34	0.00	0.44	0.03	0.34	0.00	0.46	0.02
18:3n-6	0.19	0.00	0.17	0.01	0.20	0.00	0.17	0.01
18:3n-4	0.13	0.01	0.18	0.01	0.14	0.00	0.18	0.01
18:3n-3	0.94	0.01	0.82	0.02	0.82	0.01	0.77	0.02
18:4n-3	1.91	0.01	1.54	0.10	1.98	0.02	1.74	0.07
18:4n-1	0.22	0.01	0.27	0.01	0.23	0.00	0.28	0.02
20:2n-6	0.30	0.13	0.29	0.02	0.24	0.08	0.25	0.01
20:3n-6	0.15	0.02	0.13	0.01	0.15	0.01	0.12	0.01
20:4n-6	1.05	0.01	0.87	0.06	1.01	0.01	0.88	0.04
20:3n-3	0.08	0.00	0.09	0.01	0.08	0.00	0.10	0.01
20:4n-3	0.57	0.00	0.60	0.02	0.59	0.00	0.64	0.02
20:5n-3	13.76	0.09	12.06	0.47	13.93	0.09	13.17	0.43
21:5n-3	0.59	0.01	0.56	0.02	0.60	0.00	0.60	0.02
22:4n-6	0.19	0.00	0.16	0.02	0.14	0.01	0.14	0.01
22:5n-6	0.29	0.00	0.22	0.01	0.28	0.00	0.23	0.01
22:5n-3	1.94	0.01	1.94	0.24	1.91	0.01	1.98	0.14
22:6n-3	9.92	0.07	8.74	0.52	9.08	0.07	8.84	0.64

Appendix IV: Preliminary and final measurements of weight and length of initial and treatment fish as well as percent lipids.

	Preliminary				Final				Percent Lipids (%)	
	Weight (g)		Length (cm)		Weight (g)		Length (cm)			
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Initial	373.10	94.01	33.97	2.70					25.92	10.02
Diet 1	375.89	47.10	33.31	1.41	1068.2	161.01	41.6	2.01	44.71	7.81
Diet 2	429.04	14.04	34.74	0.33	1342.7	267.03	45.4	3.5	53.12	8.57
Diet 3	405.44	139.32	33.96	2.21	1272.6	236.57	45.3	3.26	52.15	6.69

Appendix V: Individual QFASA diet estimates when CC_{D1}, CC_{D2}, and CC_{D3} are applied using the extended FA list. Proportion of each species in the diet by mass.

Fish	I-20	I-21	I-22	I-23	I-24	I-25	I-26	I-27	I-28	I-29	I-30
Diet 1											
Cod	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.00	0.00	0.00
Haddock	0.00	0.40	0.00	0.00	0.00	0.70	0.00	0.58	0.46	0.42	0.00
Herring	0.44	0.01	0.07	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mackerel	0.00	0.07	0.17	0.00	0.00	0.07	0.00	0.00	0.00	0.04	0.01
Northern Shrimp	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Northern Sandlance	0.17	0.23	0.23	0.15	0.05	0.19	0.13	0.03	0.19	0.14	0.12
Red Crab	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Red Hake	0.00	0.00	0.00	0.00	0.07	0.00	0.00	0.00	0.05	0.00	0.00
Rock Crab	0.39	0.15	0.43	0.8	0.89	0.00	0.87	0.00	0.30	0.40	0.87
Silver Hake	0.00	0.13	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.00	0.00
White Hake	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diet 2											
Cod	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.79	0.10	0.00	0.00
Haddock	0.00	0.00	0.00	0.00	0.00	0.17	0.00	0.13	0.00	0.21	0.00
Herring	0.41	0.00	0.05	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mackerel	0.00	0.19	0.25	0.00	0.02	0.25	0.00	0.00	0.00	0.19	0.07
Northern Shrimp	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
Northern Sandlance	0.13	0.17	0.20	0.13	0.03	0.15	0.11	0.02	0.18	0.10	0.09
Red Crab	0.07	0.07	0.39	0.11	0.00	0.00	0.11	0.00	0.00	0.00	0.01
Red Hake	0.00	0.04	0.00	0.00	0.09	0.03	0.00	0.07	0.35	0.00	0.00
Rock Crab	0.39	0.19	0.11	0.72	0.86	0.00	0.78	0.00	0.32	0.50	0.83
Silver Hake	0.00	0.33	0.00	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00
White Hake	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Diet 3											
Cod	0.00	0.00	0.00	0.00	0.00	0.22	0.00	0.84	0.00	0.00	0.00
Haddock	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00
Herring	0.46	0.00	0.08	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Mackerel	0.00	0.36	0.26	0.00	0.05	0.39	0.00	0.05	0.02	0.30	0.08
Northern Shrimp	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.12	0.00	0.00
Northern Sandlance	0.10	0.18	0.17	0.12	0.02	0.14	0.11	0.01	0.19	0.09	0.08
Red Crab	0.17	0.20	0.49	0.19	0.04	0.05	0.21	0.00	0.00	0.05	0.09
Red Hake	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.10	0.44	0.00	0.00
Rock Crab	0.27	0.00	0.00	0.63	0.83	0.00	0.68	0.00	0.23	0.54	0.75
Silver Hake	0.00	0.21	0.00	0.00	0.00	0.20	0.00	0.00	0.00	0.00	0.00
White Hake	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

Appendix VI: Calibration coefficients (mean +/- sd) of 51 FA of post-prandial liver in fish fed the experimental diets. Asterisks denote FA with p-value <0.05. 17:1(b) is a FA with 17 carbon and 1 double bond in an unknown location.

FA	Diet 1		Diet 2		Diet 3	
	Mean	SD	Mean	SD	Mean	SD
Saturated						
14:0	0.48	0.00	0.58	0.01	0.58	0.00
*i-15:0	0.52	0.00	0.65	0.02	0.73	0.01
*15:0	0.63	0.00	0.73	0.02	0.77	0.00
16:0	0.95	0.00	0.97	0.01	0.96	0.01
i-17:0	1.08	0.01	1.05	0.01	1.09	0.00
ai-17:0	1.40	0.03	1.36	0.01	1.25	0.03
17:0	0.82	0.01	0.85	0.01	0.94	0.01
18:0	2.19	0.01	1.95	0.04	1.90	0.01
20:0	0.58	0.01	0.54	0.02	0.43	0.00
Monounsaturated						
*14:1n-9	0.66	0.00	0.77	0.02	0.83	0.01
*16:1n-11	0.84	0.01	0.95	0.00	0.96	0.00
*16:1n-9	1.55	0.02	1.27	0.02	1.15	0.01
*16:1n-7	0.76	0.01	0.83	0.01	0.84	0.00
*16:1n-5	0.58	0.00	0.67	0.01	0.71	0.00
17:1(b)	1.02	0.02	1.00	0.02	0.99	0.01
17:1	1.58	0.00	1.41	0.03	1.37	0.00
*18:1n-13	0.77	0.03	0.61	0.01	1.06	0.03
*18:1n-11	1.74	0.07	5.03	0.17	3.43	0.13
*18:1n-9	2.30	0.01	1.86	0.05	1.72	0.00
18:1n-7	1.56	0.00	1.50	0.01	1.50	0.01
*18:1n-5	1.56	0.00	1.30	0.01	1.27	0.00
*20:1n-11	1.25	0.02	1.64	0.03	1.56	0.01
20:1n-9	0.89	0.00	0.94	0.01	0.93	0.00
20:1n-7	0.87	0.01	0.86	0.00	0.85	0.01
22:1n-11	0.53	0.00	0.60	0.00	0.60	0.00
*22:1n-9	0.47	0.00	0.69	0.00	0.74	0.01
22:1n-7	0.56	0.01	0.56	0.00	0.57	0.01
24:1n-9	0.65	0.00	0.67	0.01	0.62	0.01
Polyunsaturated						
*16:2n-6	0.67	0.01	0.77	0.01	0.86	0.01
*16:2n-4	0.55	0.00	0.69	0.01	0.73	0.00
*16:3n-4	0.59	0.00	0.72	0.01	0.78	0.01
*16:4n-1	0.42	0.01	0.52	0.01	0.59	0.01
*18:2d5,11	1.50	0.02	1.03	0.02	0.97	0.03
*18:2n-6	0.66	0.00	0.84	0.01	0.89	0.01
*18:2n-4	1.29	0.00	1.32	0.01	1.37	0.02
18:3n-6	0.85	0.01	0.85	0.01	0.87	0.01

FA	Diet 1		Diet 2		Diet 3	
	Mean	SD	Mean	SD	Mean	SD
*18:3n-4	1.48	0.00	1.38	0.01	1.33	0.02
*18:3n-3	0.73	0.00	0.88	0.01	0.94	0.00
*18:4n-3	0.71	0.00	0.82	0.01	0.88	0.01
18:4n-1	1.29	0.01	1.26	0.02	1.26	0.02
*20:2n-6	0.90	0.02	0.95	0.01	1.05	0.01
*20:3n-6	0.49	0.01	0.86	0.01	0.80	0.01
*20:4n-6	0.74	0.00	0.83	0.00	0.87	0.00
*20:3n-3	1.01	0.03	1.18	0.01	1.29	0.04
*20:4n-3	0.91	0.00	1.05	0.01	1.08	0.00
*20:5n-3	0.76	0.00	0.88	0.01	0.95	0.00
*21:5n-3	0.80	0.00	0.94	0.01	1.01	0.01
*22:4n-6	0.92	0.01	0.84	0.02	1.02	0.00
*22:5n-6	0.66	0.00	0.76	0.00	0.81	0.00
*22:5n-3	0.84	0.00	0.97	0.01	1.02	0.01
*22:6n-3	0.75	0.00	0.87	0.01	0.96	0.00