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Effect of temperature and tissue type on fatty acid signatures of two species of North Pacific juvenile gadids: A laboratory feeding study

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ABSTRACT

The utility of the fatty acid biomarker (FAB) approach in fisheries ecology is limited by our understanding of how biotic and abiotic factors determine dietary markers in fish tissues. An 8-week laboratory experiment was conducted on two species of juvenile gadids (Pacific cod, *Gadus macrocephalus* and walleye Pollock, *Theragra chalcogramma*) reared at 3 °C or 9 °C and fed a diet enriched with either oils of marine origin or terrestrial plant origin. Non-linear models were fitted to investigate how tissue type and temperature mediated the proportion of FABs in fish. Across temperatures, fatty acid (FA) profiles were similar for both species of gadids. FAs also showed high temporal sensitivity across temperatures, and were evident in fish after only one week of feeding. Pacific cod held at 9 °C and fed a terrestrial plant oil (TPO) enriched diet had significantly higher C_{18} polyunsaturated FAs (PUFAs) in their liver than cod held at 3 °C after one week, but this temperature effect diminished as tissues reached equilibrium with their diet. C_{18} PUFAs were significantly higher in liver than in muscle. Differential proportions of C_{18} PUFAs among tissues provide temporal patterns that may help with disentangling the timing of off-shore–inshore nursery migrations in juvenile fish. Calibration coefficients were determined to explain the relationship between FAs in the diet and FAs in fish tissues. These coefficients will support future development of quantitative estimates of diet in juvenile low-fat fish.

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1. Introduction

For decades scientists have been searching for rules to explain observed patterns of energy flow in food webs (Elton, 1927), reflecting the importance of predator–prey relationships in explaining patterns of community organization (Paine, 1980). There are a number of constraints to accurately determine predator–prey relationships in juvenile fish via traditional gut analyses (Cortes, 1997; Vander Zanden et al., 1997). Briefly, these include underrepresentation of small and/or soft

prey items and missing diet data beyond the most recent meal. Therefore, trophic ecologists now use biomarker methods (e.g., lipid classes, fatty acids, bulk stable isotope and compound specific isotopes) to resolve food web linkages (Canuel et al., 1997; Jaschinski et al., 2008; Kharlamenko et al., 2001).

In the last three decades, fatty acid biomarkers (FABs) have been used to define food web relationships in both marine (Dalsgaard et al., 2003; Sargent, 1976) and freshwater ecosystems (Arts and Wainman, 1999). The FAB approach is based on the distinctive FA signatures of primary producers (Budge et al., 2006; Iverson, 2009) which are integrated conservatively into secondary consumers such as zooplankton (Sargent, 1989; Sargent et al., 1987; Stevens et al., 2004). In fish, FABs have been used qualitatively to identify both dietary and habitat shifts in larval and juvenile stages (St. John and Lund, 1996). However, most studies on juvenile fish have used a qualitative approach to FAB analysis, with few quantitative examples of diet estimation (Budge et al., 2012; Dalsgaard and St. John, 2004).

Quantitative fatty acid signature analysis (QFASA), the use of unique prey species FA signatures to assign quantitative estimates of predator diet, is now being applied to higher trophic levels such as marine mammals (Beck et al., 2007), sea birds (Iverson et al., 2007) and polar bears (Thiemann et al., 2008). A prerequisite to the QFASA approach is controlled laboratory studies in which predators are fed one prey diet until

the predator tissue resembles the prey as closely as possible (Budge et al., 2011, 2012; Wang et al., 2010). This results in a "calibration coefficient" for each FA which takes into account predator metabolism and is the ratio of the FA in the predator relative to the proportion in its diet. However, significant knowledge gaps in how fish modify prey fatty acids through elongation and desaturation still prevent the quantitative application of the FAB approach (Budge et al., 2011, 2012). In addition, little is known about how FA metabolism in fish is affected by a range of abiotic and biotic factors that determine growth (temperature, ration, species-specific physiology, prey type and ontogeny). Proportions of FAs measured in fish may be further mediated by growth independent effects such as tissue type and condition, resulting in interactions between growth and these factors.

In the Northeast Pacific semi-pelagic walleye pollock (*Theragra chalcogramma*) and demersal Pacific cod (*Gadus macrocephalus*) are the two most commercially important species in the Alaskan finfish fishery. Both species have pelagic larvae (4–30 mm) that settle at ~45 mm into complex biogenic habitat (eelgrass, kelp) in shallow, coastal areas (2–4 m; Laurel et al., 2007). These nursery areas can be within meters of shore and are prone to terrestrial run-off through annual late-spring freshets when juvenile gadids begin to settle. Short chain C_{18} PUFAs ($18:2n-6$ and $18:3n-3$) are not unique to terrestrial material and are also found in high levels in green algae and eelgrass blades (Brett et al., 2009; Copeman et al., 2009). However, previous studies on cold water marine systems using both FABs and compound specific isotopes of FAs have shown that elevated proportions (>4%) of these two FAs in marine systems are indicative of nearshore dietary carbon input, often of terrestrial origin (Budge et al., 2001; Copeman et al., 2009; Ramos et al., 2003). Controlled laboratory studies looking at the retention of dietary nearshore FA indicators may help indicate residency times, a key component of understanding habitat connectivity and nursery function for coastal gadids.

We conducted a laboratory study to examine the proportions of $18:3n-3$ and $18:2n-6$ in the muscle and liver of two species of juvenile gadids. The level of FAs in fish fed two different gel food diets was measured in relation to species and culture temperature during an 8-week feeding trial. Diets were identical, except that one was enriched with marine oil (MO, cod liver) while the other was enriched with terrestrial plant oil (TPO, flax seed). Over our 8-week study we specifically examined the degree to which tissue proportions of C_{18} PUFAs were: 1) species-specific, 2) mediated by culture temperature, 3) specific to tissue type (liver, muscle) and 4) vary temporally between tissue types (i.e. FAB in liver:FAB in muscle). A further objective of our study was to provide the first calibration coefficients for low fat juvenile marine fish. These coefficients for both liver and muscle tissues could be used to develop quantitative approaches to diet determination in cold-water marine fish.

2. Materials and methods

2.1. Fish husbandry and sampling

Juvenile walleye pollock were collected in early June 2008 with lights and lift nets suspended from a dock in Pt. Townsend Bay, Washington, USA (48°6′N 122°48′W). Fish were held for 24 h in ambient seawater prior to shipment to the National Marine Fisheries Service laboratory at Hatfield Marine Science Center (HMSC) in Newport, Oregon, USA. At HMSC, pollock (20–40 mm initial total length) were fed a mixture of krill (*Euphausia superb*) and gel food (Table 1) to satiation 3 times per week. Pollock were held in flow-through seawater tanks (3140 L) at 9 °C until the start of the experiment in October.

Pacific cod were collected in Kodiak, Alaska (USA) by beach seine in mid July 2008 and were allowed to depurate for 24 h prior to overnight shipment in containers with an overlying saturated O_2 layer. On arrival at HMSC, cod (20–50 mm initial total length) were held in 3104 L tanks

Table 1
Ingredients in maintenance gel-food (herring), MO enriched gel-food (Pacific cod fillets and cod liver oil) and TPO enriched gel-food (Pacific cod fillets and flax seed oil) diets.

Ingredient	Maintenance diet[a] (g/kg)	MO diet (g/kg)	TPO diet (g/kg)
Herring	139.5	0.0	0.0
Pacific cod	0.0	138.0	138.0
Cod liver oil	0.0	11.1	0.0
Flaxseed oil	0.0	0.0	11.1
Freeze dried krill	7.0	6.9	6.9
Squid	69.8	69.0	69.0
Otohime EP4[b]	212.2	209.9	209.9
TwinLab Amino Fuel[c]	16.2	16.0	16.0
Powdered gelatin[d]	137.1	135.6	135.6
Water	417.6	413.0	413.0
Vitamins[e]	0.6	0.6	0.6

Contained: pro vitamin A, vitamin A, vitamin C, vitamin D, vitamin E, thiamin, riboflavin, niacin, vitamin B-6, folic acid, vitamin B12, biotin, pantothenic acid, calcium, iron, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, choline, inositol and floraglo lutein.
[a] Fed for >6 weeks prior to onset of feeding experiment.
[b] Otohime EP4, extruded pellet manufactured by: Marubeni Nisshin Feed Co., Ltd., Tokyo, Japan and imported by: Reed Mariculture Inc., Campbell, CA 95008, USA. Feed contains: krill meal, fish meal, squid meal, wheat flour, potato starch, corn starch, fish oil, calcium phosphate, betaine, soy lecithin, licorice plant and wheat germ.
[c] Amino Fuel, Ideasphear Inc., American Fork, UT, USA.
[d] Knox unflavored Gelatine, Krafts Foods Global Inc., Northfield, IL, USA.
[e] TwinLab Daily One Caps with Iron, Ideasphere Inc., American Fork, UT, USA.

at 9 °C on the same feeding schedule as pollock until the beginning of the feeding trial in early October.

Prior to the experiment, 25 randomly selected walleye pollock and 25 Pacific cod were measured to assess their initial size at the beginning of the experiment: total length of 61.6 ± 4.3 mm and 61.8 ± 3.7 mm, respectively. Five days prior to the experiment, tanks were randomly stocked with either 12 juvenile pollock per tank or 10 juvenile cod. Dietary trials were run in twenty-four cylindrical upwelling 100 L tanks with water flow-through at 1.5 L·min^{-1}. Twelve of the tanks were gradually lowered to 3 °C over a 3-day period (2°/day) while the other tanks remained at 9 °C. The 24 experimental tanks consisted of 3 tanks for each species (cod or pollock) at each temperature (9 °C or 3 °C) replicated for two different dietary treatments (MO or TPO enriched gel food).

The feeding trial started October 7, 2008 and fish were hand-fed enriched gel-food diets to satiation 5× weekly. Diets were formulated to contain the same level of lipid per wet weight and the same lipid class composition (Tables 1 & 2). Gelatinized diets, previously shown to provide adequate nutrition for Pacific marine juveniles gadids (Hurst et al., 2010), were modified by addition of either marine oil (MO, cod liver oil) or terrestrial plant oil (TPO, flax seed oil). Diets contained a combination of squid, krill, Pacific cod fillets, commercial food, amino acid supplements, and vitamins (Table 1). Ingredients were blended together and then bound by addition of a warm gelatin. Diets were frozen immediately after addition of the gelatin. Small (~1 cm wide) strands of gel food, suitable for juvenile fish gape size, were produced by grating frozen blocks of gel food using a cheese grater.

Three pollock and three cod were sampled for lipid analyses at the beginning of the experiment (time-0) prior to the introduction of diet treatments. Fish were first euthanized and then blotted dry with paper towels and total length (mm) and wet weight (g) were recorded. During tissue sampling, liver and white muscle were sampled separately from each animal. White muscle (0.25–0.35 g wet weight) was collected from each fish along the dorsal margin, by first dissecting the skin and then sampling halfway along the anterior–posterior plane. Whole liver samples (0.02 to 0.20 g) were also collected by dissection on ice. Lipid sampling was conducted on three fish per species at time zero and on one fish per tank at the end of weeks 1, 2, 4 and 8 giving a total of 3 samples of liver and 3 samples of muscle per treatment at four time periods.

Table 2
FA composition of MO enriched and TPO enriched experimental gel-food diets fed to juvenile gadids for 8 weeks (n = 6, average ± SEM).

	Cod liver oil diet	Flaxseed oil diet
Total lipid per wet wt (mg·g^{-1})	40.3 ± 1.4	37.0 ± 5.9
Triacylglycerols	52.6 ± 2.8	55.9 ± 1.2
Free FAs	9.8 ± 0.8	8.5 ± 0.8
Sterols	2.3 ± 0.5	1.7 ± 0.0
Acetone mobile polar lipids	4.5 ± 1.3	3.5 ± 0.9
Phospholipids	29.2 ± 1.7	29.1 ± 1.8
FAs		
14:0	5.9 ± 0.1	4.4 ± 0.1
16:0	17.1 ± 0.5	15.2 ± 0.6
18:0	3.3 ± 0.1	3.5 ± 0.1
Sum of SFA	**27.6 ± 0.5**	**24.2 ± 0.1**
16:1n−7	6.6 ± 0.2	4.7 ± 0.1
18:1n−7	3.6 ± 0.1	3.4 ± 0.1
18:1n−9	11.2 ± 0.3	11.6 ± 0.4
20:1n−9	2.4 ± 0.2	2.2 ± 0.3
20:1n−11	1.8 ± 0.2	1.1 ± 0.5
22:1n−11 + 13	3.1 ± 0.3	2.2 ± 0.5
Sum of MUFA	**31.6 ± 1.1**	**27.7 ± 1.1**
16:2n−4	1.2 ± 0.1	0.9 ± 0.1
18:2n−6	**2.6 ± 0.0**	**5.4 ± 0.1**
18:3n−3	**0.8 ± 0.2**	**13.9 ± 0.7**
18:4n−3	2.2 ± 0.0	1.7 ± 0.0
20:4n−6	0.9 ± 0.0	0.7 ± 0.0
20:5n−3	13.1 ± 0.2	10.1 ± 0.4
22:5n−3	1.7 ± 0.0	1.2 ± 0.0
22:6n−3	13.0 ± 0.4	11.0 ± 0.6
Sum of PUFA	**39.9 ± 0.9**	**47.4 ± 1.0**
Sum of 18:2n−6 + 18:3n−3	**3.5 ± 0.2**	**19.3 ± 0.8**

Lipid samples were placed in chloroform under nitrogen immediately and were stored at −80 °C for less than 4 months prior to extraction.

Total length (TL, mm) and wet weight (wwt, g) were measured on all remaining fish (n = 136) at the end of the 8-week feeding trial. These data, along with the 25 fish per species measured at time-0 and all length weight data from fish used in lipid analyses, were combined to calculate growth rates during the 8-week experimental trial. Average growth rates were calculated as the change in total length per day (mm per day) = ((mm week 8 − mm week 0) / days), or as specific growth rate (SGR = ((ln(wwt week 8) − ln(wwt week 0)) / day) * 100).

2.2. Lipid analysis

Lipids were extracted in chloroform/methanol according to Parrish (1987) using a modified Folch procedure (Folch et al., 1957). Lipid classes were determined using thin layer chromatography with flame ionization detection (TLC/FID) with a MARK VI Iatroscan (Iatron Laboratories, Tokyo, Japan) as described by Parrish (1987). Extracts were spotted on silica gel coated Chromarods and a three stage development system was used to separate lipid classes. The first separation consisted of 25- and 20-min developments in 98.95:1:0.05 hexane:diethyl ether:formic acid. The second separation consisted of a 40-min development in 79:20:1 hexane:diethyl ether:formic acid. The last separation consisted of 15-min developments in 100% acetone followed by 10-min developments in 5:4:1 chloroform:methanol:water. Data peaks were integrated using Peak Simple software (ver. 3.67, SRI Inc.) and the signal detected in millivolts was quantified using lipid standards (Sigma, St. Louis, MO, USA). Lipid classes are expressed per wet weight (mg g^{-1} wet weight) and as proportions (% total lipid).

Total lipid was analyzed for FA composition. FA methyl esters (FAME) were prepared by transesterification with 14% BF$_3$ in methanol at 85 °C for 90 min (Morrison and Smith, 1964). The average Iatroscan determined derivatization efficiency for marine samples was ~85%. FAMEs were analyzed on an HP 6890 GC FID equipped with a 7683 autosampler and a ZB wax + GC column (Phenomenex, U.S.A.). The column was 30 m in length, with an internal diameter of 0.32 mm

and a 0.25 μm film. The column temperature began at 65 °C where it was held for 0.5 min. Temperature was increased to 195 °C (40 °C min^{-1}), held for 15 min then increased again (2 °C min^{-1}) to 220 °C. The final temperature was held for 3.25 min. The carrier gas was hydrogen, flowing at 2 mL·min^{-1}. The injector started at 150 °C and increased (200 °C·min^{-1}) to 250 °C. The detector temperature was 260 °C. Peaks were identified using retention times based on standards purchased from Supelco (37 components of FAME, BAME, PUFA 1, PUFA 3). Chromatograms were integrated using Galaxie Chromatography Data System (Ver. 1.9.3.2, Varian). Individual FAs are expressed as a percentage of total FAs.

2.3. Statistical analysis

The effect of week, tissue type, species, temperature, and diet on the proportion of 18:3n−3 and 18:2n−6 in juvenile gadid tissues was examined by GLM (Using SYSTAT 12 for windows). Interaction terms were significant, so separate 3-way ANOVAs were performed for different tissue types and different diets, to investigate the effect of species, temperature, and week on FA proportions. All proportional FA data were arcsine square root transformed, while week data was log transformed in order to meet the assumptions of normality. Significance was set at α = 0.05. Differences in average growth rate between treatments were analyzed using a one-way ANOVA with Tukey's multiple comparison tests, α = 0.05.

The retention of 18:3n−3 and 18:2n−6 was fitted to a 3-parameter exponential rise to a maximum model ($Y_x = Y_0 + a[1 − \exp(−bX)]$) (Cober et al., 2006; Sigma Plot version 10.0)). In the model, Y_0 refers to the proportion of FA (%) in the tissue at time-0, **a** is the maximum proportion found in the tissue (%), **b** relates to the initial slope of the FA retention curve (%·day^{-1}), and **X** refers to the days of feeding.

The ratio between the level of 18:2n−6 or 18:3n−3 in the liver and that in the muscle over the 8-week feeding trial was fitted to a dome shaped curve explained by the Gaussian 3-parameter peak model: $Y_1 = a * \exp(−.5 * ((X_1 − X_0) / b)^2)$. Y_1 refers to the ratio of FAB in the liver relative to the muscle at time 1, **a** is the maximum proportion in the liver:muscle, X_1 is the days of feeding, X_0 is the days until a slope was zero or at the max peak, and **b** is the width of the peak in days.

Calibration coefficients (CC) were calculated separately for liver and muscle tissues. CCs were calculated by dividing the percentage of an individual FA in the gadid tissue by the average percentage of the same FA in the diet (Budge et al., 2012; Iverson, 2009).

3. Results

3.1. Growth rates

Individual growth rates were not collected throughout the experiment in order to minimize handling stress to fish and because of limited numbers of juvenile gadids per tank and lethal lipid sampling. However, growth rates of cod and pollock remaining at the end of the 8-week growth period differed significantly with temperature (one-way ANOVA, $F_{7,151}$ = 9.92, p < 0.001, Fig. 1). There were no size differences between species reared at the same temperature, or between fish fed different diets within a temperature treatment.

3.2. Summary lipid data

Cod and pollock muscle tissue contained 5 to 7 mg·g^{-1} FAs per wet weight throughout the experiment with phospholipids (PL) as the major lipid class (Table 3). PL ranged from 74 to 76% at time-0 to 84 to 86% at the end of the experiment in both species. The major saturated and monounsaturated FAs in cod and pollock were 16:0 and 18:1n−9 and 18:1n−7. Total PUFAs were ~40% at time-0 in cod and pollock, but increased in fish fed both MO enriched and TPO enriched diets to a high of ~53%. However, in fish fed the MO enriched diet this increase
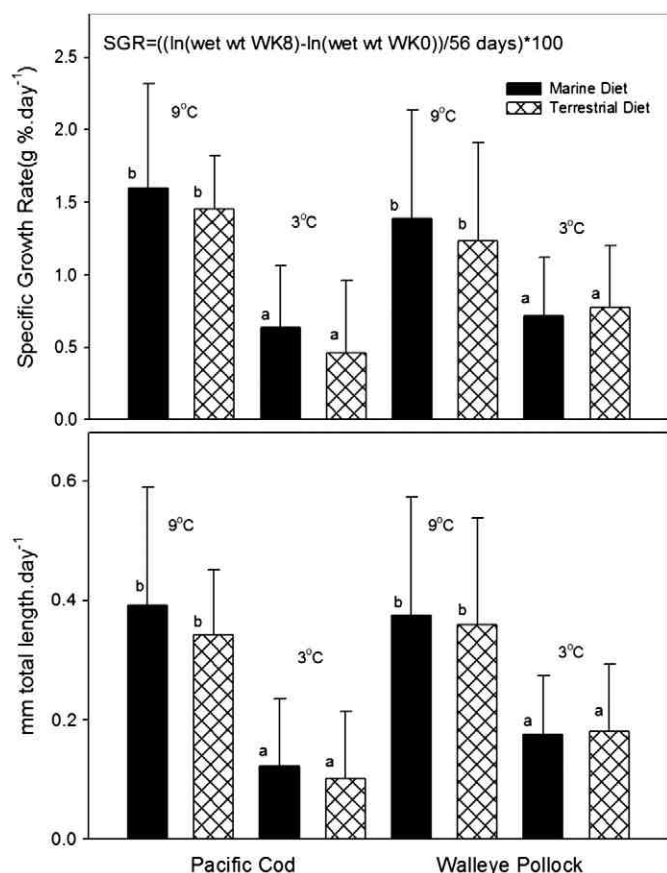
Fig. 1. Growth rates of juvenile cod (n = 10 to 14) and pollock (n = 24 to 26) reared at two temperatures and fed either a MO or TPO enriched diet for 8 weeks.

was caused by a rise in 20:5n−3 and 22:6n−3 (marine based PUFA), whereas fish in the TPO enriched diet treatment showed large increases in proportions of 18:2n−6 and 18:3n−3.

Cod and pollock livers had 115 mg·g⁻¹ and 386 mg·g⁻¹ lipid per wet weight (Table 3). At the end of the feeding trial, liver lipid ranged from 292 mg·g⁻¹ in cod fed the MO enriched diet to 542 mg·g⁻¹ in pollock fed the TPO enriched diet. Triacylglycerols (TAG), the major lipid class in gadid livers, ranged from 50% in cod at time-0 to 83% in pollock at time-0. Livers contained proportionally less PUFA than the muscle with a high of 40% and 54%, respectively (Table 3).

3.3. Temporal changes in the proportions of 18:3n−3 and 18:2n−6

GLM analysis of the effect of week, tissue type, species, temperature, and diet on the proportion of 18:3n−3 and 18:2n−6 in juvenile gadid tissues showed significant interaction terms. Further, there were several significant 3-way interactions (i.e. Tissue ∗ Week ∗ Diet, $F_{4,179} = 10.47$, $p < 0.001$) with tissue type in the multi-factorial GLM. Therefore, analysis of species, temperature and week of sampling effects on FA proportions were separately analyzed in smaller 3-way ANOVAs for each tissue type and each diet. Because there was no significant effect of species or temperature on the proportion of 18:3n−3 or 18:2n−6 in the muscles of marine gadids, species were pooled for the determination of temporal patterns in muscle (Figs. 2a, 3a).

There was a significant effect of both week and species on the proportion of 18:3n−3 and 18:2n−6 in the livers of gadids (i.e. for TPO enriched fish the levels of 18:3n−3 in liver, effect of week $F_{4,40} = 15.113$, $p < 0.001$ and effect of species $F_{1,40} = 8.249$, $p = 0.007$), and therefore, species were examined separately for the determination of temporal patterns in liver FAs (Figs. 2b & c, 3b & c).

The proportion of 18:3n−3 in the liver of gadids fed the MO diet was not affected by either species ($F_{1,40} = 2.69$, $p = 0.11$) or temperature ($F_{1,40} = 2.65$, $p = 0.11$); however, there was a small but significant increase in the proportion of 18:3n−3 from week 0 to week 8 in gadid livers ($F_{4,40} = 6.63$, $p = 0.001$), reflecting the sensitivity of liver to slight changes in diet (Fig. 2).

In juvenile gadids fed the TPO enriched diet, there was a significant interaction between the effect of species and temperature on the proportion of 18:3n−3 and 18:2n−6 in the liver. Therefore, separate 2-way ANOVAs were run to determine the effect of temperature and week on FA proportions in cod and pollock livers. In both species, week was significant ($p < 0.001$); however, temperature had a significant effect on the proportion of 18:3n−3 in cod livers ($F_{1,16}=7.32$, $p = 0.016$) but not in pollock ($F_{1,16} = 0.545$, $p = 0.471$, Fig. 2b & c). This temperature effect was only evident during the first week of feeding but was not significant after week 1.

3.4. Temporal changes in FA proportions

The model describing the relationship between the proportions of 18:3n−3 and 18:2n−6 in gadid tissue through 8 weeks of feeding (Figs. 2, 3 and Table 4) shows a rapid initial rate of increase followed by a decreased rate and eventual saturation maximum (Cober et al., 2006). Separate temporal FA accumulation graphs were constructed based on statistical differences detected by the GLM. Table 4 summarizes the model parameters and shows that
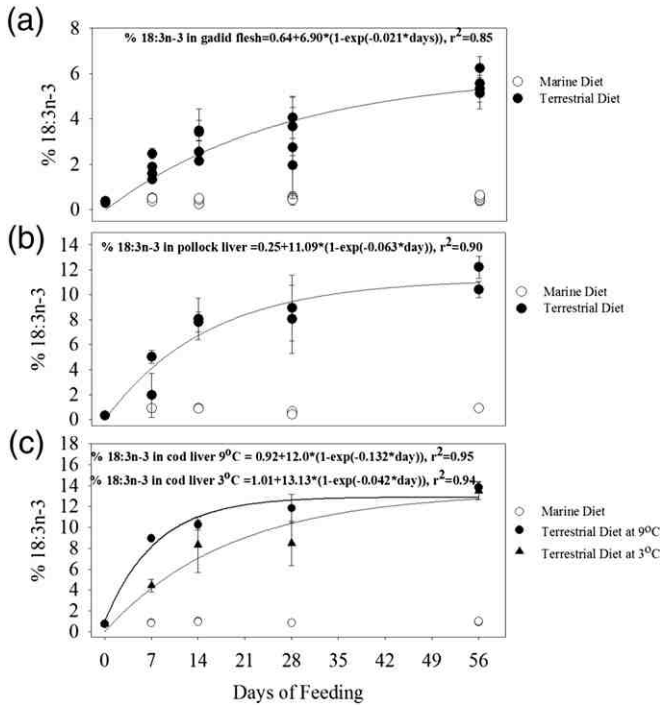
Table 3

Total lipid, FA concentrations and summary lipid proportions (>5% in either tissue type) for liver and muscle of juvenile gadids fed either a MO or TPO enriched diet for 8 weeks (temperatures are pooled due to lack of significant differences). Data are mean ± SEM, n = 3 fish at time-0, and n = 6 fish at week 8.

	Cod muscle Time-0	Cod MO muscle Week 8	Cod TPO muscle Week 8	Pollock muscle Time-0	Pollock MO muscle Week 8	Pollock TPO muscle Week 8	Cod liver Time-0	Cod MO liver Week 8	Cod TPO liver Week 8	Pollock liver Time-0	Pollock MO liver Week 8	Pollock TPO liver Week 8
Total lipids per wwt (mg·g⁻¹)	8.4 (0.6)	25.4 (16.5)	10.4 (1.2)	11.3 (0.9)	9.6 (1.7)	8.7 (1.6)	115 (15.0)	292 (57.7)	364.6 (78.8)	386 (108.7)	364 (46.1)	542 (60.2)
% TAG	1.4 (0.2)	1.1 (1.0)	1.6 (2.0)	2.2 (1.8)	1.2 (0.8)	1.2 (1.2)	49.9 (12.1)	69.6 (5.2)	69.9 (6.1)	82.9 (1.6)	77.1 (0.7)	80.3 (1.6)
% PL	73.9 (6.0)	85.3 (5.0)	85.6 (4.3)	75.9 (3.0)	83.7 (1.9)	84.8 (7.0)	21.4 (7.7)	13.2 (3.5)	17.7 (4.5)	5.7 (1.1)	7.9 (1.3)	8.0 (0.9)
Total FAs mg·g⁻¹ wwt	5.2 (0.5)	5.9 (1.4)	6.8 (0.8)	6.6 (1.4)	6.3 (1.3)	5.6 (1.1)	80.0 (13.0)	260.0 (52.5)	329.3 (75.5)	264.2 (77.6)	336.8 (42.3)	499.5 (57.4)
% total FAs[a]												
16:0	26.7 (7.3)	14.4 (0.6)	13.8 (0.1)	21.3 (3.6)	15.2 (0.1)	14.3 (0.3)	14.5 (0.3)	11.9 (0.7)	10.7 (0.5)	15.7 (0.8)	9.3 (1.9)	11.2 (0.5)
18:1n−9	11.9 (0.2)	13.0 (0.1)	13.0 (0.4)	12.6 (0.6)	12.4 (0.3)	13.2 (0.2)	17.1 (0.6)	20.1 (1.0)	21.8 (0.8)	20.3 (0.8)	21.3 (0.8)	22.0 (1.1)
18:1n−7	5.3 (0.5)	4.3 (0.2)	3.9 (0.2)	4.2 (0.1)	4.4 (0.1)	4.0 (0.1)	5.9 (0.1)	4.8 (0.3)	4.4 (0.3)	5.0 (0.1)	5.2 (0.4)	5.0 (0.2)
18:2n−6	0.7 (0.2)	1.5 (0.1)	3.0 (0.2)	0.9 (0.1)	1.5 (0.0)	2.5 (0.1)	1.7 (0.1)	2.5 (0.1)	5.4 (0.2)	1.6 (0.0)	2.2 (0.1)	4.6 (0.2)
18:3n−3	0.4 (0.0)	0.4 (0.1)	5.8 (0.4)	0.3 (0.0)	0.6 (0.1)	5.3 (0.3)	0.7 (0.0)	1.0 (0.0)	13.6 (0.5)	0.7 (0.0)	0.9 (0.0)	11.3 (0.6)
20:5n−3	10.6 (3.3)	16.6 (0.4)	14.8 (0.3)	12.5 (1.8)	16.1 (0.3)	14.3 (0.3)	12.5 (0.5)	12.9 (0.4)	8.6 (0.3)	10.2 (0.3)	12.6 (0.3)	9.1 (0.4)
22:6n−3	20.5 (6.0)	24.3 (0.8)	23.2 (0.8)	22.6 (2.9)	25.9 (0.6)	25.2 (1.1)	16.6 (0.4)	12.2 (1.0)	8.1 (0.4)	9.6 (0.2)	11.2 (1.1)	8.2 (1.6)

[a] Detailed lipid class and fatty acid data found in Appendix A (Supplementary material).

Fig. 2. Proportion of 18:3n−3 in fish reared on either a MO or TPO enriched diet for 8 weeks: (a) muscle with species and temperature combined, (symbols represent mean ± SEM, n = 3 per symbol), (b) liver of pollock with temperatures combined (symbols represent mean ± SEM, n = 3 per symbol), and (c) liver of cod with temperatures separate (symbols represent mean ± SEM, n = 3 per symbol). Graphs are pooled/separated for species and temperature based on ANOVA results.
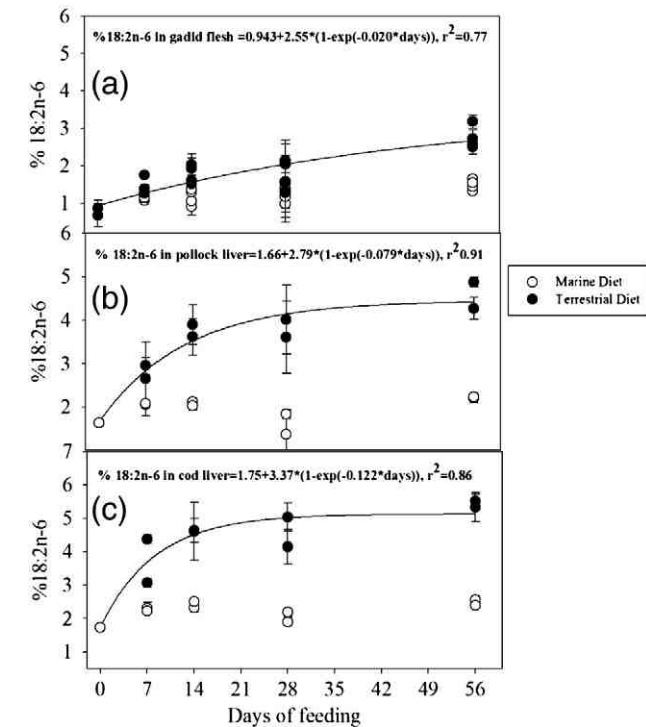

Fig. 3. Proportion of 18:2n−6 in fish reared on either a MO or TPO enriched diet for 8 weeks: (a) muscle with species and temperature combined, (symbols represent mean ± SEM, n = 3 per symbol), (b) liver of pollock with temperatures combined (symbols represent mean ± SEM, n = 3 per symbol), and (c) liver of cod with temperatures combined (symbols represent mean ± SEM, n = 3 per symbol). *Graphs are pooled/separated for species and temperature based on ANOVA results.
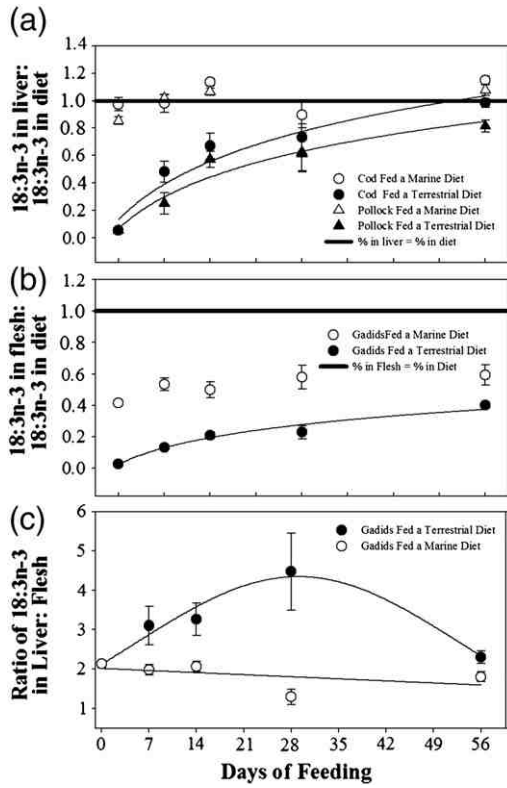
Table 4

Parameters for the rise to a maximum shaped retention of 18:3n−3, 18:2n−6 and the sum of 18:2n−6 plus 18:3n−3 in the liver and muscle of juvenile gadids. The equation $Y_x = Y_0 + a[1 − \exp(−bX)]$, exponential rise to a maximum, was used with the following parameter: Y_0 refers to the proportion of FA (%) in the tissue at time-0, a is the maximum proportion found in the tissue (%), b relates to the initial slope of the FA retention curve (%·day^{-1}), X refers to the days of feeding, while $1/b$ refers to the duration of the maximum slope (days) (data are means ± standard errors of the mean).

Treatment	Y_0 (%)	a (Max % FA)	b (% FA·day^{-1})	$1/b$ (days)	R^2
18:3n−3					
Gadid muscle	0.6 ± 0.3	6.9 ± 2.2	0.02 ± 0.01	47.6	0.9
Pollock liver	0.3 ± 0.9	11.1 ± 1.3	0.06 ± 0.02	15.9	0.9
Cod liver 9 °C	0.9 ± 1.2	12.0 ± 1.4	0.13 ± 0.04	7.69	1.0
Cod liver 3 °C	1.1 ± 1.5	13.1 ± 3.1	0.04 ± 0.02	23.8	0.9
18:2n−6					
Gadid muscle	0.9 ± 0.2	2.6 ± 1.1	0.02 ± 0.02	50.0	0.8
Pollock liver	1.7 ± 0.2	2.8 ± 0.3	0.08 ± 0.02	12.7	0.9
Cod liver	1.8 ± 0.4	3.4 ± 0.4	0.12 ± 0.04	8.2	0.9
Sum of 18:2n−6 + 18:3n−3					
Gadid muscle	1.58 ± 0.46	9.40 ± 3.18	0.02 ± 0.01	50.0	0.8
Pollock liver	2.27 ± 1.09	13.60 ± 1.61	0.06 ± 0.02	16.1	0.9
Cod liver	2.72 ± 1.64	15.27 ± 2.14	0.09 ± 0.03	11.4	0.9

the rate of initial retention was elevated in liver (0.1 to 0.04%·day^{-1}) compared to muscle (0.02%·day^{-1}). Retention rates were similar for 18:3n−3 (0.02%·day^{-1}) and 18:2n−6 (0.02%·day^{-1}) in muscle tissue, despite very different proportions of these FAs present in the TPO enriched diet (Table 2). Pollock had lower proportions of both 18:3n−3 and 18:2n−6 than cod in their tissues at time-0 (Y_0) and maxima (**a**) were also slightly lower at the end of the 8-week feeding period (Table 5). Trends for 18:3n−3 plus 18:2n−6 were similar to the individual component FAs, with higher initial slopes in liver (0.06 to 0.09%·day^{-1}) than in muscle (0.02%·day^{-1}) tissue. Cod liver (15.3%) trended towards a higher maxima equilibrium than pollock liver (13.6%, Fig. 2b & c).

3.5. Temporal changes in C_{18} PUFAs in the liver and muscle relative to diet

Fig. 4 shows ratios of 18:3n−3 in the liver:diet (Fig. 4a) and muscle:diet (Fig. 4b) for juveniles fed either a marine or a terrestrial-enriched diet. Despite very different levels of 18:3n−3 in the diet, the tissue:diet ratios were similar at the end of the experiment for fish fed both diets. In Fig. 4a, fish approached a 1:1 ratio of 18:3n−3 in the liver:18:3n−3 diet. Fish fed the cod-liver oil enriched marine diet remained near this 1:1 ratio throughout the 8-week feeding trial, indicating dietary stability. In contrast, the ratio in those fish switched to the terrestrial diet at week 0 increased from ~0.1:1 at week-0 to 0.5:1 at week-2, and to 1:1 (liver:diet) at week-8.

The proportion of 18:3n−3 in muscle tissue relative to the diet reached a maximum of approximately 0.5:1, which is lower than observed in the liver (Fig. 4a, b). Gadids fed a TPO enriched diet showed a low ratio (~0.025:1) at week-0; however, this ratio increased to a

Table 5

Parameters for the peaked shaped ratio of the proportion of 18:3n−3 and 18:2n−6 in the liver relative to the muscle for juvenile gadids. Gaussian 3 parameter peak model: $Y = a * \exp(−.5 * ((X_1 − X_0)/b)^2)$: Y refers to the ratio of 18:3n−3 in the liver relative to the muscle, a is the maximum proportion in the liver:muscle, X_1 is the days of feeding, X_0 is the days until a slope was zero or max peak, and b is the width of the peak in days (data are means ± standard errors of the mean).

FA ratio in liver to muscle	a (Max proportion, unitless)	X_0 (days)	b (days)	R^2
18:3n−3	4.3 ± 0.3	29.0 ± 1.6	24.2 ± 2.0	0.9
18:2n−6	3.1 ± 0.2	28.1 ± 2.4	28.2 ± 3.6	0.8

Fig. 4. Proportion of 18:3n−3 in the (a) liver (pooled temperatures) and (b) muscle (pooled temperatures and species) of juvenile gadids relative to the proportion in the diet and (c) ratio of 18:3n−3 in the liver relative to the proportion in the muscle for juvenile gadids switched to a TPO enriched diet. (a) Symbols are n = 6, mean ± SEM, (b) symbols are n = 12, mean ± SEM, and (c) symbols are n = 12 mean ± SEM.

high of ~0.5:1 after eight weeks of feeding, approaching the ratio in fish fed a marine diet. The same pattern was observed for 18:2n−6 and the sum of 18:2n−6 and 18:3n−3 (data not shown).

3.6. Temporal changes in the proportion of C_{18} PUFAs in the liver relative to muscle

Proportions of 18:3n−3 and 18:2n−6 in the liver relative to those in the muscle tissue of MO fed fish did not change significantly over the 8-week feeding trial (Fig. 4c). Therefore, the approximate 2:1 ratio in the liver relative to the muscle represents an equilibrium level in fish with no large recent dietary change. However, in fish fed the TPO enriched diet, the relationship between the proportions in liver relative to muscle tissue was described by a dome-shaped function (Gaussian 3 parameter peak model), indicating a rise in the ratio to a maximum at 1 month followed by a decline to original equilibrium levels at 2 months. The relationship between liver to muscle was similarly dome-shaped for 18:2n−6, even though this FA represented only 5.4% of the total FAs in the diet compared to 13.9% for 18:3n−3 (Fig. 4c, data only shown for 18:3n−3).

3.7. Calibration coefficients

CCs for most FAs were close to one; however, there was a difference in the patterns between muscle (Fig. 5) and liver (Fig. 6). Remarkably, the patterns observed in both tissue types show very little differences between the two species. Further, despite vast differences in the proportions of 18:2n−6 and 18:3n−3 in their diets, juvenile fish did not show variable CC for these specific FAs based on dietary levels.

Flesh tissue showed a higher retention of longer chain C_{22} PUFAs with CC reaching a high of ~4 for 22:6n−3 in both species fed both types of diets (Fig. 5). Lower CC for C_{18} PUFAs (~0.6) was found equally
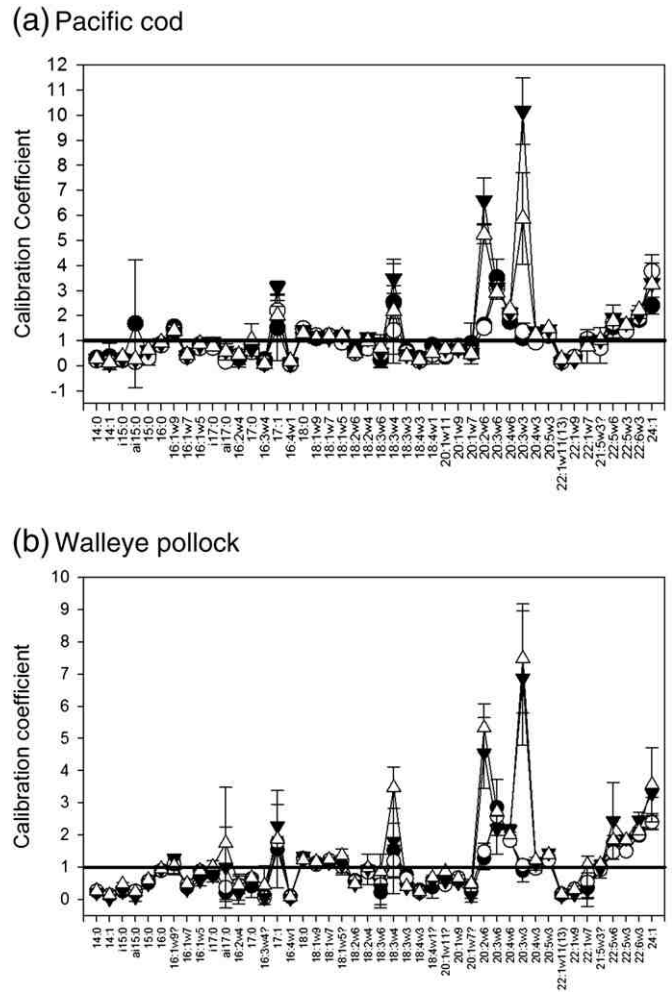

Fig. 5. Calibration coefficients (mean ± standard deviation (SD)) for each fatty acid calculated for the muscle of (a) Pacific cod and (b) walleye pollock after feeding a constant diet for 8 weeks. Black circles are marine diet at 9 ºC (n = 3), clear circles are MO enriched diet at 3 °C (n = 3), black triangles are TPO enriched diet at 9 °C (n = 3) and clear triangles are TPO enriched diet at 3 °C (n = 3).

in fish fed the MO and TPO enriched diets. Spikes of 20:2n−6 (5:1 to 6:1) and 20:3n−3 (9:1 to 7:1) were shown in cod and pollock fed the TPO enriched diets only, giving evidence for elongation and desaturation of excess C_{18} PUFAs.

Liver tissue did not show retention of C_{22} PUFAs relative to diet and generally most FAs had CCs close to one, except for spikes in C_{18} and C_{20} PUFAs found in both cod and pollock fed the TPO enriched diet (Fig. 6a, b). Elevated CCs at 20:2n−6 (~5:1) and 20:3n−3 (~6:1) were the most extreme cases.

4. Discussion

The application of FABs to food web relationships is still mostly qualitative despite three decades of marine lipid research (Dalsgaard et al., 2003), so FA analyses remain a 'blunt tool' for describing food web relationships (Sargent, 1976). However, with data on FA compositions of prey and rates at which such FAs are deposited in the tissue of predators, FAs can become quantitative determinants of diet (Iverson, 2009). While the FA composition of prey is best measured in situ, incorporation of FAs in predator tissues generally requires an experimental approach with sequential sampling in order to trace selective retention of specific FAs, detect de novo FA synthesis, and measure rates of tissue specific lipid/FA deposition (Copeman and Laurel, 2010; Copeman et al., 2002).
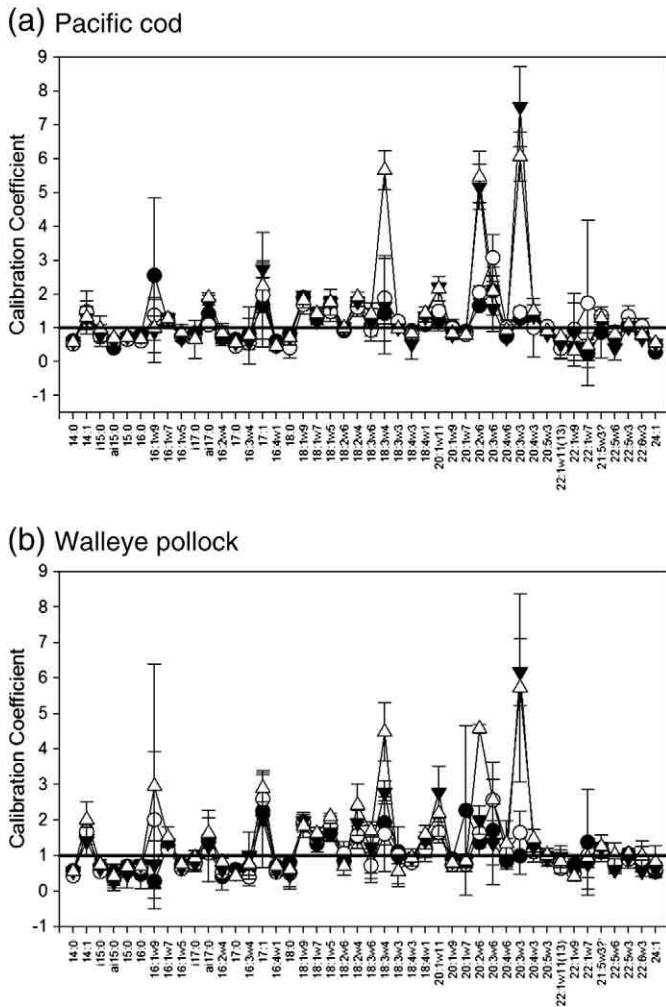
(a) Pacific cod

(b) Walleye pollock

Fig. 6. Calibration coefficients (mean ± standard deviation (SD)) for each fatty acid calculated for the liver of (a) Pacific cod and (b) walleye Pollock after feeding a constant diet for 8 weeks. Black circles are marine diet at 9 °C (n = 3), clear circles are MO diet at 3 °C (n = 3), black triangles are TPO enriched diet at 9 °C (n = 3) and clear triangles are TPO enriched diet at 3 °C (n = 3).

4.1. Temporal changes in FA composition

In this experiment, TPO based short chain PUFAs were rapidly evident in juvenile gadid tissues, with maximal change in the first four weeks of feeding (Figs. 2 & 3). This rapid and significant adjustment of FA composition in muscle and liver is consistent with other studies of age-0 fish in the laboratory (Copeman et al., 2002; Dalsgaard and St. John, 2004) and field (Copeman et al., 2009; Koussoroplis et al., 2010). Such rapid retention has also been observed in whole adult gadids after only 3 weeks of feeding (Kirsch et al., 1998). Thus, juvenile and adult fish muscle is sensitive to changes in dietary FA composition and nearshore C_{18} PUFAs are likely to be rapidly evident in fish tissues in the field. Further, juvenile fish are expected to show increased proportions of FAB more rapidly than seen for adult fish.

Dalsgaard and St. John reported a rate of $\delta^{13}C$ labeled $18:3n-3$ uptake in juvenile sand eel (*Ammodytes tobianus*) that corresponded to an exponential model based on growth and FA metabolism (Dalsgaard and St. John, 2004). This model predicted a FA uptake rate of $0.035 \cdot day^{-1}$ for $18:3n-3$ and $0.0086 \cdot day^{-1}$ for $16:0$, in whole bodies of sand eel. We estimated changes in FAs over time using a 3-parameter growth to a maximum model for muscle and liver separately. Results for $18:3n-3$ are within the range of Dalsgaard and St. John (2004), at

$0.021\% \cdot day^{-1}$ in the muscle and 0.6 to $0.13\% \cdot day^{-1}$ in the liver. Similar values were reported for $18:2n-6$ and the sum of the total FAs (Table 4), suggesting that similar rates of uptake may be valid for a number of juvenile fish species.

Copeman et al. (2009) reported levels of terrestrial-sourced FAs in both pelagic and settled juvenile Atlantic cod (*Gadus morhua*) from cold-water eelgrass beds in Bonavista Bay Newfoundland, Canada. Levels of $18:2n-6$ plus $18:3n-3$ in pelagic juveniles in September (3.9%) increased in settled juveniles to a maximum in November (6.2%), indicating a 2.3% proportional retention. A simple calculation of the proportional change in the sum of terrestrial FAs over the number of days in the nearshore (~56), yields a retention rate of $0.041\% \cdot day^{-1}$. This calculation for the transition from pelagic-offshore (marine) to demersal-inshore (terrestrial) enriched food webs does not account for concurrent changes in FA composition of zooplankton in the nearshore, yet it is consistent with other laboratory studies (e.g. Dalsgaard and St. John, 2004) and the rates for two Pacific gadids that we report here. Interestingly, the calculation for whole-bodied, field-captured Atlantic cod is intermediate ($0.041\% \cdot day^{-1}$) between the muscle ($0.020\% \cdot day^{-1}$) and liver ($0.075\% \cdot day^{-1}$) derived rates of the Pacific cod and walleye pollock (Table 4). This intermediate rate likely reflects the elevated C_{18} PUFAs in TAG from liver and the reduced C_{18} PUFAs in PL from muscle, as whole juvenile Atlantic cod contained 35% TAG and 70% PL (Copeman et al., 2008).

Although we did not perform a diet switch from TPO to MO enriched feeds, it should be possible to determine movement of fish from nearshore habitats into offshore regions by measuring a decrease in C_{18} PUFAs. Within the aquaculture field, there has been some quantification of these "wash-out" and dilution rates; when fish are switched from vegetable oil to fish oil enriched feeds just prior to harvest. Jobling (2003) found that Atlantic salmon, *Salmo salar* L., switched to MO enriched diets following the parr-smolt transformation showed FA percentages in their fillets that closely conformed to what would be expected from a dilution model.

4.2. Role of species and temperature

Temperature determines fish growth by increasing food consumption and driving the metabolic processes that can change growth efficiency (Brett, 1979). Previously it has been shown that there is a significant impact of temperature on behavior and growth rate, both in juvenile walleye pollock (Kooka et al., 2007) and Pacific cod (Hurst, 2007; Hurst et al., 2010). We did not track individual growth rates, however, temperature-mediated growth rates based on larger numbers of fish at time-0 and at 8 weeks are in agreement with previous reports in both species, showing on average 0.4 mm length·day⁻¹ at 9 °C and 0.2 mm day⁻¹ at 3 °C (Fig. 1, Hurst et al., 2010; Kooka et al., 2007).

Despite these differences in growth, there were no significant differences in the proportion of C_{18} PUFAs in the muscle of gadids reared at either 3 °C or 9 °C even after 8 weeks of feeding. These patterns suggest a decoupling of growth rate and FAB composition in juvenile gadids. There is little known about the effect of temperature on the deposition of FAs in storage tissues, although Elsdon (2010) explicitly examined the effect of feeding duration and temperature on the FA composition of juvenile black bream (*Acanthopagrus butcheri*). Both diet and feeding duration were found to significantly affect the FA composition of juvenile fish, while temperature (16 or 23 °C) had no significant impact on FA composition.

Species differences in the accumulation of FAB in the muscle were not evident. Generally, Pacific cod juveniles are considered to be more tightly connected to coastal nursery areas than walleye pollock (Brodeur and Wilson, 1996; Hurst et al., 2009). In a recent study of nearshore habitat use in juvenile gadids, Laurel et al. (2007) showed higher abundance of age-0 juvenile Pacific cod and saffron cod (*Eleginus gracilis*) than walleye pollock during settlement (July and/or August) in the Kodiak, Alaska nearshore. However, all gadids preferentially

used nearshore macrophytes over bare substratum during and shortly after settlement (Laurel et al., 2009). Despite differences in behavior in the field, our lab based approach shows that FA metabolism and retention are generally similar for these low-fat marine juveniles.

4.3. Differences in liver and muscle FA composition

In both the North Atlantic and Pacific, many large-scale studies on FA signatures in fish have added to large prey databases that provide a platform on which to base trophic investigations of larger predatory fish, sea birds and marine mammals (Budge et al., 2002; Iverson et al., 2002). Given that fish are generally eaten whole, these analyses mostly encompass whole body lipids. However, temporal trends can be inferred from separate analysis of storage lipids (liver) and membrane lipids (muscle) thereby providing a better understanding of the feeding ecology and possible historical residency of the species (Copeman et al., 2002; Koussoroplis et al., 2010). Not surprisingly, we found that proportions of C_{18} PUFAs in the liver of juvenile gadids were saturated more quickly than muscle tissue.

Proportions of C_{18} PUFAs in the liver of fish fed a TPO enriched diet approached a maximum ratio of 1:1 (liver:diet), whereas their muscle levels approached a lower ratio of 0.5:1. Different ratios in the liver and muscle relative to the diet likely reflected the higher proportion of TAG in liver (~70 to 80% at week 8) compared to PL in muscle tissue (~85% at week 8). PLs are characterized by a common backbone with two FAs esterified at positions 1 and 2. Normally a SFA or MUFA is esterified to the $sn-1$ position while a PUFA is preferentially esterified to the $sn-2$ position (Bell and Tocher, 1989). Bell and Dick's (1991) study of PLs in cod fed on a natural marine diet reported major molecular species such as 16:0/22:6 and 16:0/20:5 in both the liver and muscle, but not 18:2 or 18:3. The significant increase in the proportion of C_{18} PUFAs here indicates that there is considerable flexibility in the $sn-2$ position of juvenile gadid PLs. Conversely, in early stages of marine fish the FA specificity in storage TAGs is less than in PLs (Copeman et al., 2002). These functional differences in the lipid classes that typify the liver and muscle of fish likely explain part of the differences in equilibrium levels of $18:3n-3$ in the muscle (0.5:1) and liver (1:1) tissues.

Differences in C_{18} PUFA proportions in liver and muscle of juvenile gadids resulted in the dome-shaped relationship between the proportions of C_{18} PUFAs in liver relative to muscle tissue (Fig. 4c). Maximal differences in the liver:muscle ratio were observed after 1-month of feeding on the TPO enriched diet. These differences seemed to have reached equilibrium levels after two months of feeding, as they were at a level similar to that found in the MO fed fish (2:1 liver:muscle). This relationship holds promise for estimating residency time in the nearshore, especially when coupled with size-at-age data collected in field studies. Based on the dome-shape relationship, the ratios of C_{18} PUFAs in the liver:muscle are similar after two and six weeks on a new diet (Fig. 4c). However, when this information is coupled with basic size-at-age population structure, it may be possible to assign a 6-week residency to a ~100 mm fish and a 2-week residency to a ~50 mm fish, despite a similar liver to muscle ratio of C_{18} PUFAs. A general increase in the proportion of C_{18} PUFAs that occurs with residency in the nearshore (2 weeks vs 6 weeks) could help validate the liver:muscle ratio, as could $\delta^{13}C$ FA analysis.

Koussoroplis et al. (2010) analyzed neutral and polar lipids for FA $\delta^{13}C$ in juvenile leaping gray mullet (*Liza saliens*) during settlement and demonstrated that the neutral lipids took on the dietary FA signal and isotopic values of the new lagoon habitat faster (at a smaller size) than the polar lipids from the same animals. Further, within a relatively short settlement growth period (20 mm to 50 mm, August to September) the isotopic values of the FAs in the neutral and polar lipids equilibrated. Supporting our results, Koussoroplis et al (2010) showed a similar time-scale to that observed here for two juvenile gadids: maximal differences in liver (neutral) to muscle (polar) were observed after one month followed by equilibration at 2 months.

4.4. CC in liver and muscle

These first CCs for individual FAs in low-fat marine juvenile fish were generally independent of dietary levels. For example, $18:3n-3$ was found at extremely high levels in the TPO enriched diet (13.9%) and much lower levels in the MO diet (0.8%). However, liver and muscle CCs for fish fed both diets were ~1.1 in liver and ~0.6 in muscle (Figs. 5 & 6). Generally, CCs were very different to those reported for sea birds (Wang et al., 2010) and mammals (Iverson et al., 2004; Thiemann et al., 2008) and more akin to those reported for salmon (Budge et al., 2012). CCs for salmon belly flap and muscle were generally similar, but we observed a more discrete difference between liver and flesh CC. This is likely due to the lean nature of gadid muscle and high levels of TAG associated with liver storage. Although CCs were generally independent of diet, some variability was associated with elevated C_{20} PUFAs in fish fed elevated C_{18} PUFAs. Increased proportions of these fatty acids are likely due to chain elongation and desaturation of C_{18} PUFAs in the liver.

4.5. Conclusions

Neither species nor temperature affected temporal patterns of FAB proportions in muscle of Pacific gadids over a 2-month study period. This indicates knowledge of abiotic factors such as temperature-history, which are difficult to reconstruct, are not actually necessary for the application of this technique to juvenile gadids.

Tissue specificity led to different proportions of FABs in the muscle and liver of gadids. This differential retention produced liver and muscle tissue ratios that show promise in determining nearshore residency times. Presence of significant dietary input of nearshore carbon could expand the definition of critical habitat for juvenile Pacific cod and saffron cod to include nearshore biogenic structure (eelgrass and kelp) as well as surrounding terrestrial buffer zones that supply carbon to the food web during summer and fall settlement.

Along with Budge et al., (2012), we provide some of the first CCs that could be used in the development of the QFASA approach to diet determination in fish. Given the requirements for quantitative FA dietary analysis (knowledge of the variability in the FAs of all prey items and conversion factors in the consumer); it may be impractical to take this same approach to studies of juvenile fish feeding ecology. The large number of zooplankton prey items, their small size, the difficulty in collecting and sorting individual species, as well as the time scale on which they are likely to vary their lipid composition make this approach difficult. However, simplification of prey items into feeding guilds (Piche et al., 2010) rather than species may allow practical application of the QFASA approach to juvenile marine fish feeding ecology.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2013.07.008.

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