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METHODS

Direct determination of fatty acids in fish tissues: quantifying top predator trophic connections

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Abstract Fatty acids are a valuable tool in ecological studies because of the large number of unique structures synthesized. They provide versatile signatures that are being increasingly employed to delineate the transfer of dietary material through marine and terrestrial food webs. The standard procedure for determining fatty acids generally involves lipid extraction followed by methanolysis to produce methyl esters for analysis by gas chromatography. By directly transmethylating ~50 mg wet samples and adding an internal standard it was possible to greatly simplify the analytical methodology to enable rapid throughput of 20–40 fish tissue fatty acid analyses a day including instrumental analysis. This method was verified against the more traditional lipid methods using albacore tuna and great white shark muscle and liver samples, and it was shown to provide an estimate of sample dry mass, total lipid content, and a condition index. When large fatty acid data sets are generated in this way, multidimensional scaling, analysis of similarities, and similarity of percentages analysis can be used to define trophic connections among samples and to quantify them. These routines were used on albacore and skipjack tuna fatty acid data obtained by direct methylation coupled with literature values for krill. There were clear differences in fatty acid profiles among the species as well as spatial differences among albacore tuna sampled from different locations.

Keywords Signature fatty acids · Direct transmethylation · Multivariate statistics · Albacore tuna · Great white shark

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Introduction

In marine ecosystems, lipids provide the densest form of energy, which is transferred from algae to fish via the food web. Among the lipids, certain essential fatty acids—in particular the omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA)—are considered to be important determinants of ecosystem health and stability. Fatty acids are also a valuable tool in ecological studies because of their heterogeneous nature. Information provided by groups of fatty acids or fatty acid signatures may be used to delineate carbon cycling and the transfer of material through food webs.

Lee et al. (1971) were the first to show a direct transfer of dietary fatty acids into consumer storage lipids in the marine environment. Such a transfer from phytoplankton to copepods suggests it is energetically more efficient to incorporate dietary fatty acids without modification, allowing their use as biomarkers. Fatty acid biomarkers are now widely used in marine trophic studies (Parrish 2013), and they are also being increasingly used in terrestrial food web studies. In particular, they are being used to unravel complex trophic interactions in soil systems (Ruess et al. 2005; Pollierer et al. 2010; Haubert et al. 2011; Ferlian et al. 2012).

The standard procedure for determining fatty acids involves lipid extraction with organic solvents followed by methanolysis to produce fatty acid methyl esters (FAME) for analysis by gas chromatography (GC) and GC–mass spectrometry (GC/MS). A drawback to using signature FA in large-scale ecosystem studies is the relatively long processing time and associated higher costs. A possible way of increasing sample throughput was suggested by the work of Lewis et al. (2000), who used a direct transesterification method on freeze-dried microalgae (thraustochytrids). In this way they avoided the laborious lipid extraction step, and they found that direct transmethylation of microheterotrophs actually yielded more fatty acids than standard procedures.

In the work reported in the present paper, the direct transesterification method on dry samples was also verified against traditional methods using albacore tuna and great white shark muscle and liver samples. The possibility of using small wet samples was then investigated as well as the use of an internal standard to further simplify the methodology. The rapid approach presented here can enable larger, more cost-effective sampling in order to provide not only dietary information but also a measure of nutritional condition.

When large fatty acid data sets are generated using such a method, multivariate statistics such as principal components analysis (PCA) help to define the trophic connections among the samples but they do not quantify them. Other multivariate routines, such as analysis of similarities (ANOSIM) and similarity of percentages analysis (SIMPER), use a resemblance matrix to give numerical values for the similarity within a group and others for the similarity between groups. Here, we used multidimensional scaling (MDS), ANOSIM, and SIMPER on albacore tuna and skipjack tuna fatty acid profile data obtained by direct methylation to first define the trophic connections among these samples and then to quantify them. In this way, we can start to understand how to better quantify feeding relationships in widely spread species such as tuna, and to identify spatial differences in population structure of commercially important species.

Materials and methods

Fish samples

Albacore tuna (*Thunnus alalunga*) was collected by tuna longliners operating in the Coral Sea and in the waters off western New Zealand and eastern Tasmania in the Tasman Sea between January 2008 and December 2010. For each tuna specimen, a section of dorsal muscle tissue was obtained and kept on ice. In addition, liver tissue and different muscle tissues were sampled from three great white sharks (*Carcharodon carcharias*) collected through the shark control program in New South Wales. All tissue samples were kept at −20 °C until lipid extraction.

Lipid extraction of fish tissue

Samples of 1–2 g of frozen fish tissue were ground in a mortar with a pestle. Small subsamples (0.3–0.8 g) of albacore and shark tissue were extracted according to the Bligh and Dyer (1959) procedure for fish tissue. Before starting, all glassware was rinsed with Milli-Q® water (Millipore, Milford, MA, USA) and nanograde dichloromethane. A mixture of chloroform:methanol:Milli-Q® water (10:20:7.5 ml) was added to the tissue in a 100-ml separatory funnel. The funnel was shaken well with venting and left overnight. The next day, the solution was broken into two phases by adding 10 ml chloroform and 10 ml saline Milli-Q® water (9 g of sodium chloride/L) to give a final chloroform:methanol:water solvent ratio of 1:1:0.9. The lower layer was drained into a 50-ml round-bottom flask and the lipid was concentrated by rotary evaporation after adding three drops of methanol. The extract was transferred in chloroform into a pre-weighed glass 2-ml vial. The vial was placed in a warm heating block and the solvent was blown down under nitrogen gas. The round-bottom flask was rinsed twice with chloroform into the vial. The extract was dried in the vial to constant weight and 1,500 µl of chloroform added. The total solvent extract was stored in the freezer.

Transmethylation

Procedures used for fatty acid methylation were based on those of Miller et al. (2006). Depending on the lipid weight in the vial, 20–200 µl of albacore or shark total solvent extract was taken from the 1,500 µl in the vial and placed in clean, labeled test tubes. This was blown dry before adding 3 ml of methylating solution (methanol:dichloromethane:concentrated hydrochloric acid 10:1:1 v/v/v) that had been made within a week. The tube was capped under Teflon, vortexed, and placed in a heating block at 80–85 °C for 2 h. After cooling, 1–1.5 ml of Milli-Q® water were added. This was extracted with 1.8 ml of hexane:dichloromethane (4:1 v/v) by shaking and then vortexing, after which the tube was centrifuged for 5 min. The upper, organic layer containing the fatty acid methyl esters (FAME) was transferred to a vial and blown down. The 1.8-ml extractions were repeated twice, blowing down in between. After adding 1,500 µl of internal standard solution (8.62 ng/µl of 19:0 FAME) to give a 12.93-µg addition, 0.2 µl were injected from this solution into a gas chromatograph (GC).

Direct transmethylation of dry tissue without lipid extraction

A small sub-sample (70 ± 50 mg wet weight) of fish tissue was weighed into pre-weighed glass test tubes which were freeze-dried overnight. These were reweighed to obtain dry weights before adding 3 ml of the same methylating solution that was used above for the lipid extracts. The same methylation procedure was followed until the addition of 400 µl of the internal standard solution to give a 3.448-µg addition of 19:0 FAME.

Direct transmethylation of wet tissue without lipid extraction

After the grinding step, 0.04–0.05 g of wet fish tissue were weighed into pre-weighed and tared glass test tubes fitted with screw caps. The tubes were reweighed accurately and then samples were directly transmethylated in methanol: dichloromethane:concentrated hydrochloric acid (10:1:1 v/v) for 2 h at 80–85 °C. After cooling and adding 1.5 ml of Milli-Q® water, a known concentration of internal injection standard (23:0 FAME) was added in 1.5 ml of hexane, followed by 0.3 ml of dichloromethane. The internal standard solution consisted of 11.59 ng/µl of 23:0 FAME, so there was a 17.39-µg addition. After thorough mixing, the tube was centrifuged at 2,000 rpm for 5 min to break phase. The upper, organic layer was removed and reduced under a nitrogen stream. About 1 ml of chloroform was added and 0.2 µl from this solution were injected into the GC.

Fatty acid and sterol analysis

Samples were analyzed in chloroform using an Agilent Technologies (Palo Alto, CA, USA) 7890B GC equipped with a non-polar Equity™-1 fused silica capillary column (15 m × 0.1 mm internal diameter and 0.1 µm film thickness), a flame ionization detector, a split/splitless injector, and an Agilent Technologies 7683B series autosampler. Samples (0.2 µl) were injected in splitless mode at an oven temperature of 120 °C with helium as the carrier gas. The oven temperature was raised to 270 °C at 10 °C per min, and then to 310 °C at 5 °C per min. Peaks were quantified using Agilent Technologies ChemStation software.

FAME were identified by GC/MS using a Finnigan Thermoquest GCQ GC/MS fitted with an on-column injector and Thermoquest Xcalibur software (Austin, TX, USA). The GC had a nonpolar HP-5 crosslinked methyl silicone–fused silica capillary column (50 m × 0.32 mm internal diameter) which was of similar polarity to that described above. Helium was used as the carrier gas.

Statistical analysis

Fatty acid relationships were investigated using Plymouth Routines in Multivariate Ecological Research (PRIMER), a software package used for analyzing ecological data. Analysis of similarities (ANOSIM), a multivariate nonparametric analog of univariate ANOVA tests, and similarity of percentages analysis (SIMPER) were used to identify the fatty acids that primarily provided the discrimination between observed sample clusters. The similarity percentages routine is a multivariate analysis that uses a resemblance matrix to give the average percentage similarity within a group and the average percentage similarity between groups. SIMPER permutations and one-way ANOSIM were performed on untransformed percentage composition data using a nonparametric Bray–Curtis similarity matrix.

Results

Lipid analyses

Separate subsamples were used to compare fatty acid proportions and total fatty acid to total cholesterol ratios with and without prior Bligh and Dyer extraction. The fatty acid to cholesterol ratio and the proportions of all 14 fatty acids and fatty aldehydes with means that were >0.75 % of the total were all highly correlated ($p < 0.001$). Figure 1 shows the regressions for the six major contributors among the fatty acids and fatty aldehydes in the samples (mean values up to 23 % of total), while Fig. 2 shows the regression for the six minor fatty acids, with means of between 0.8 and 3.6 % of the total. Another smaller set of subsamples was then used to compare the direct determination of fatty acid proportions and total fatty acid to total cholesterol ratios in wet and dry white shark tissues. Slopes and r^2 values were calculated for the fatty acid to cholesterol ratio and all fatty acids and fatty aldehydes present at mean proportions greater than 0.9 %. Most of the slopes were between 0.83 and 1.09 and most of the r^2 values were greater than 0.95 for the white shark samples.

The separate subsamples were also used to compare direct total fatty acid and total cholesterol determinations on wet and dry samples. Regressions for the two direct methods were highly significant ($r^2 > 0.97$, $p < 0.001$), with intercepts close to the origin and slopes near unity. Other subsamples were then used to quantitatively compare total fatty acid and total cholesterol determinations with and without prior Bligh and Dyer extraction (Fig. 3).

The regression of summed fatty acid concentrations obtained after extraction on those obtained by direct transmethylation passed through the origin with and without the inclusion of lipid rich liver samples. Both regressions

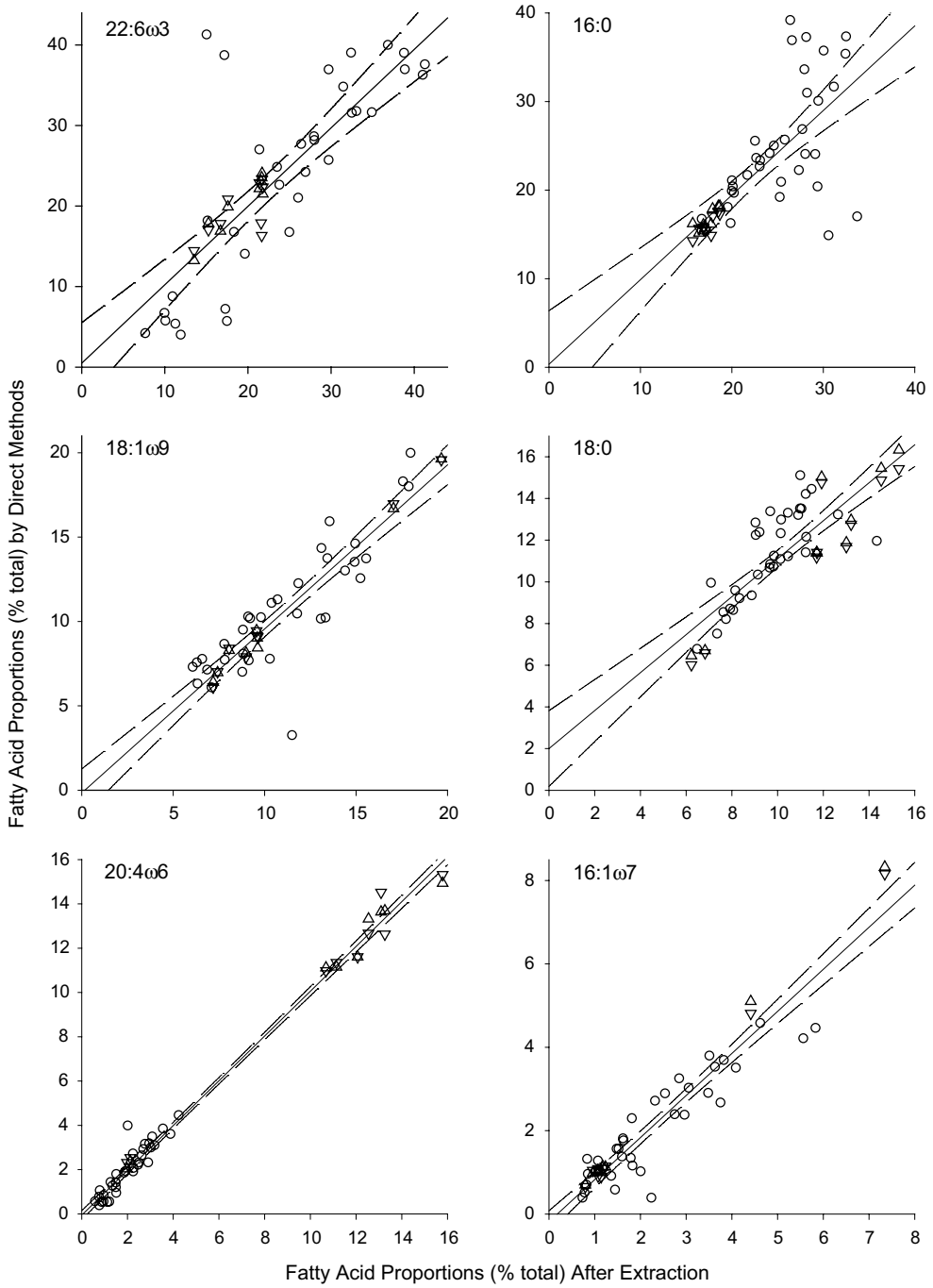

Fig. 1 Regressions for the six major contributors among the fatty acids and fatty aldehydes in albacore tuna (*open circles*) and great white shark tissues (*triangles*). Fatty acid proportions (percentage total fatty acids) obtained after methylation of total lipid extracts were compared with those obtained by the direct transmethylation methods. Albacore samples and half the white shark samples (*inverted open triangles*) were compared with transmethylation after freeze-drying; the remaining white shark samples were compared with transmethylation of wet samples (*open triangles*). *Dashed lines* are 95 % confidence intervals. r^2 values for the linear regressions range from 0.53 to 0.99 and slopes range from 0.91 to 1.01, $n = 52$

were highly significant ($r^2 = 0.75$, $p < 0.001$) but the slope obtained with liver samples included was significantly greater than 1 ($p < 0.001$), indicating that direct transmethylation underestimated the total fatty acids in the liver. The slope without the liver samples was 0.97 ± 0.08 (Fig. 3a).

The regression of total cholesterol concentrations obtained after extraction on those obtained after direct transmethylation passed through the origin with the inclusion of lipid rich liver samples (Fig. 3b). When these samples were excluded, the regression had to be forced through

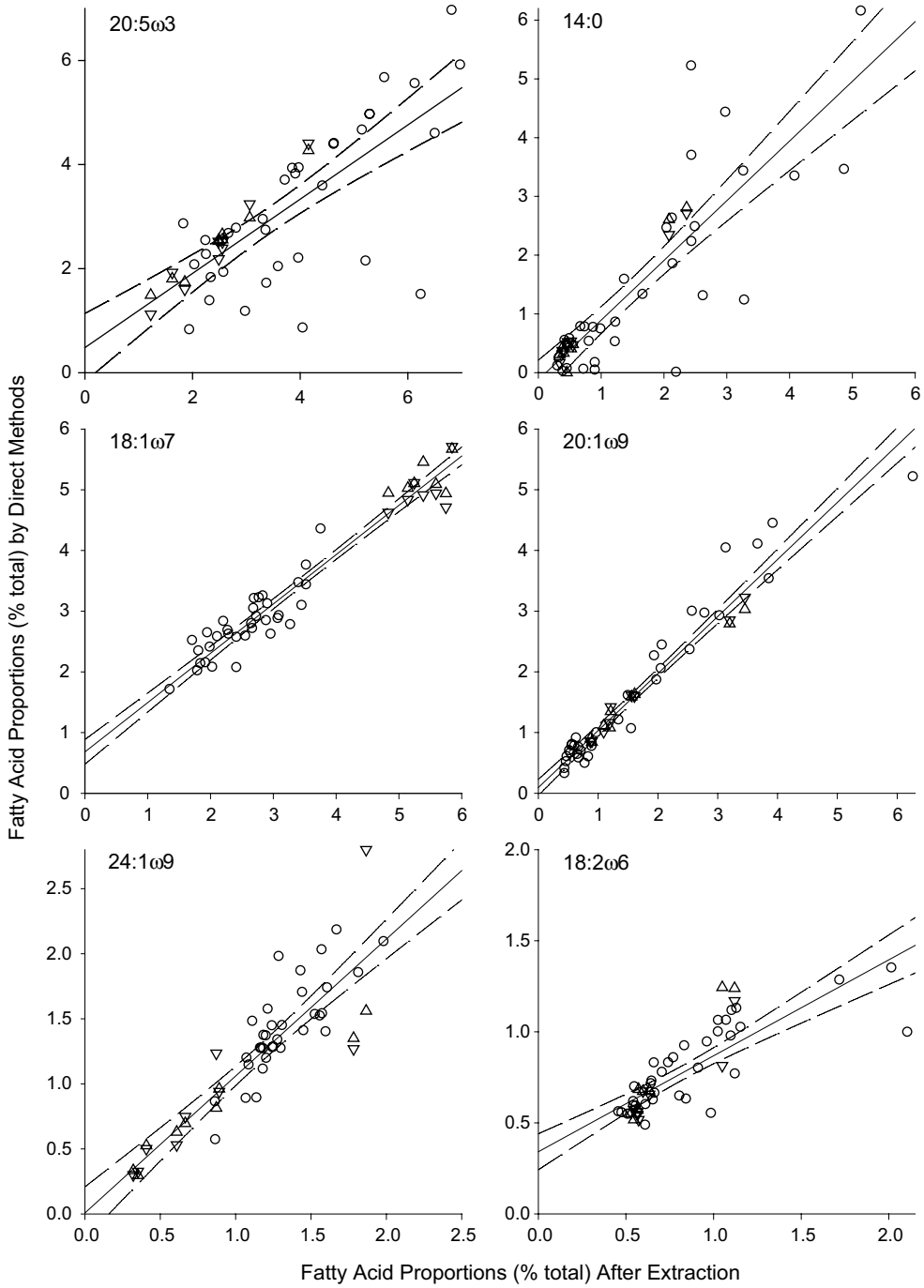

Fig. 2 Regressions for six minor contributors among the fatty acids and fatty aldehydes in albacore tuna (*open circles*) and great white shark tissues (*triangles*). Fatty acid proportions (percentage total fatty acids) obtained after methylation of total lipid extracts were compared with those obtained by the direct transmethylation methods. Albacore samples and half the white shark samples (*inverted open triangles*) were compared with transmethylation after freeze-drying; the remaining white shark samples were compared with transmethylation of wet samples (*open triangles*). *Dashed lines* are 95 % confidence intervals. r^2 values for the linear regressions range from 0.58 to 0.95 and slopes range from 0.53 to 1.05, $n = 52$

the origin, indicating less reliability in the quantification of cholesterol at low concentrations.

The regression of summed fatty acids on lipid mass determined gravimetrically from the same samples passed through the origin with and without the inclusion of lipid rich liver samples. The slopes of the regressions indicate that fatty acids accounted for 60–70 % of the lipid mass, the higher value being with liver samples

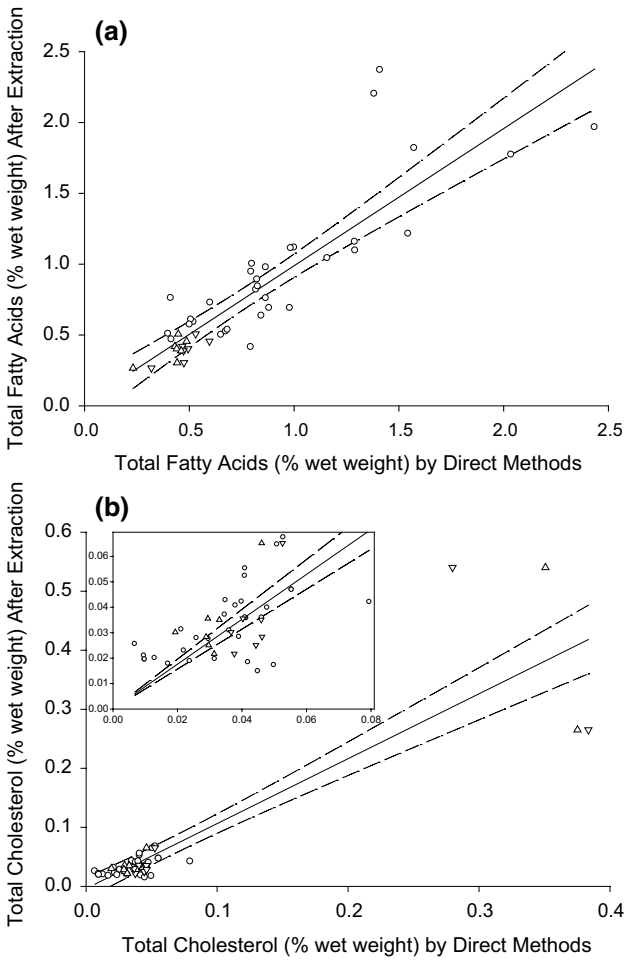

Fig. 3 Total fatty acid and total cholesterol regressions for albacore tuna (*open circles*) and great white shark tissues (*triangles*). Fatty acid and cholesterol concentrations (percentage wet weight) obtained after methylation of total lipid extracts were compared with those obtained by the direct transmethylation methods. Albacore samples and half the white shark samples (*inverted open triangles*) were compared with transmethylation after freeze-drying; the remaining white shark samples were compared with transmethylation of wet samples (*open triangles*). *Dashed lines* are 95 % confidence intervals. **a** Linear regression of total fatty acids in muscle tissues. $y = 0.02 + 0.97x$, $r^2 = 0.75$, $p < 0.001$, $n = 46$. **b** Linear regressions of total cholesterol in muscle and liver tissues. $y = -0.004 + 1.10x$, $r^2 = 0.77$, $p < 0.001$, $n = 50$. *Inset*: same data set minus shark liver samples $y = 0.88\,x$, $p < 0.001$, $n = 46$

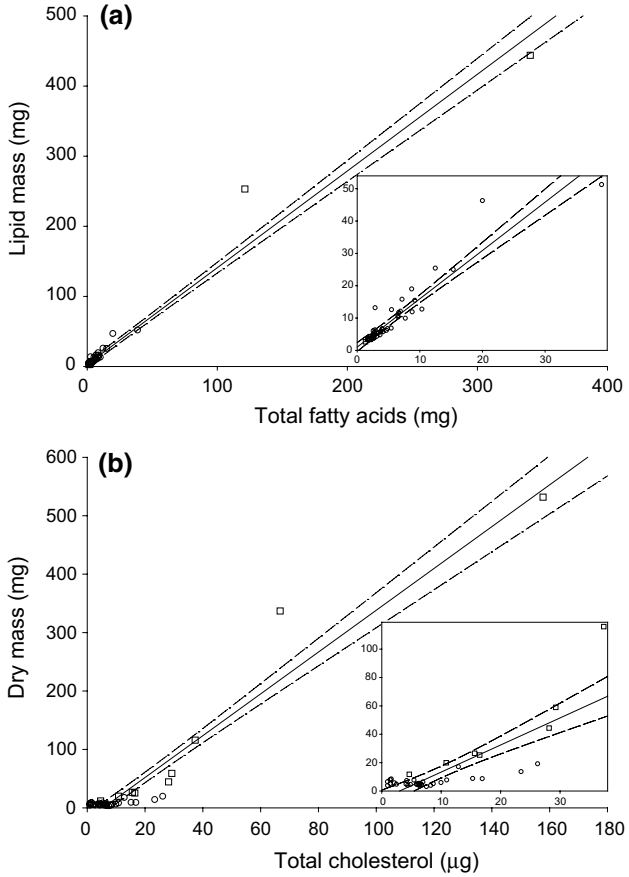

Fig. 4 Mass and lipid regressions for albacore tuna (*open circles*) and great white shark muscle and liver tissues (*open squares*). *Dashed lines* are 95 % confidence intervals. **a** Linear regression of lipid mass (mg) on total fatty acids (mg). Lipid mass was determined gravimetrically on Bligh and Dyer extracted samples and compared with summed fatty acids from the same samples. Lipid mass (mg) = 2.9 + (1.4 × total fatty acids), $r^2 = 0.97$, $p < 0.001$, $n = 47$. *Inset*: same data set minus shark liver samples [lipid mass (mg) = 1.0 + (1.5 × total fatty acids), $r^2 = 0.89$, $p < 0.001$, $n = 45$]. **b** Linear regression of dry mass (mg) on total cholesterol (µg). Cholesterol was determined as the free sterol by GC analysis after direct methylation of freeze-dried tissue samples. Dry mass (mg) = −19.6 + (3.6 × total cholesterol), $r^2 = 0.93$, $p < 0.001$, $n = 39$. *Inset*: same data set minus shark liver samples [dry mass (mg) = −5.7 + (1.9 × total cholesterol), $r^2 = 0.66$, $p < 0.001$, $n = 37$]

included. By reversing the regression, a calibration graph for total lipid is obtained consisting of a highly significant linear regression (Fig. 4a). Similarly, a calibration graph for dry mass based on cholesterol can be obtained if the sample is freeze-dried prior to direct transmethylation (Fig. 4b).

When lipid mass determined gravimetrically was regressed on summed fatty acids from the same samples (Fig. 4a), the coefficient for the constant was not significant, indicating a zero–zero intercept. The slope obtained with and without the shark liver data remained between 1.4 and 1.5 and the line passed through the origin, indicating that the relationship was robust. This was not the case for the regression of dry mass on total cholesterol, where the slope decreased from 3.6 to 1.9 (Fig. 4b). Also, separate significant linear regressions ($p < 0.001$) could be obtained for albacore tuna samples [dry mass (mg) = 3.0 + (0.45 × total cholesterol), $r^2 = 0.53$, $n = 30$] and shark samples [dry mass (mg) = −20.9 + (3.7 × total cholesterol), $r^2 = 0.94$, $n = 9$].

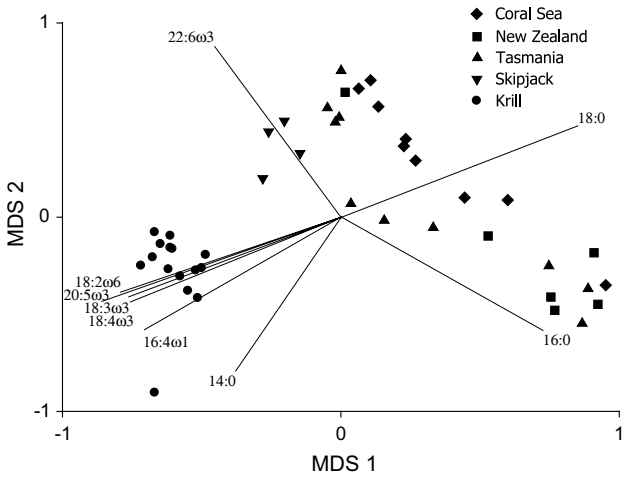

Fig. 5 MDS plot of untransformed fatty acid proportions in albacore tuna (Coral Sea, New Zealand, Tasmania), skipjack tuna, and krill samples. The resemblance matrix was created using Bray–Curtis similarity coefficients. Axes are Pearson correlations with the variables. Vectors with Pearson correlations >0.85 are shown for albacore tuna and skipjack tuna fatty acid data obtained by direct methylation coupled with literature values for krill (Virtue et al. 1995). 2D stress 0.05

Multivariate analyses

Figure 5 shows a two-dimensional configuration plot of a nonmetric multidimensional scaling (MDS) analysis of a resemblance matrix of fatty acid data. The lower triangular matrix was created using Bray–Curtis similarity coefficients. The albacore tuna data determined by direct transmethylation were supplemented with skipjack tuna (*Katsuwonus pelamis*) data determined the same way and with historical data for krill (Virtue et al. 1995). Only fatty acids which were identified in each sample set were included in the multivariate analyses of the 44 samples, giving a total of 23 fatty acids. The samples in the MDS plot were identified by taxon and, in the case of the albacore tuna, by sampling location as well. The skipjack tuna and krill were both sampled off Tasmania.

Samples for albacore tuna caught in the Tasman Sea off Tasmania and New Zealand showed the greatest dispersion, while the krill and skipjack tuna samples were much more closely clustered among themselves (Fig. 5). These spatial patterns can be quantified by SIMPER, with Tasman Sea albacore tuna samples having similarities of <77 % and with skipjack tuna and krill having similarities of >89 % (Table 1). Using the PERMANOVA+ add-on to PRIMER v6, it is possible to test if there are significant differences in dispersion among the groups. There is no difference in pairwise comparisons among the albacore groups ($p = 0.13$–0.65). Table 1 is generated from the same add-on but identical data can be obtained from a SIMPER analysis. SIMPER also shows that 16:0 and 22:6ω3 (DHA) were always among the top four contributors to the similarity in each of the five groups contributing 9–37 % each. This reflects the fact they were quantitatively the dominant fatty acids, with proportions which were on average an order of magnitude greater (23–25 % of total fatty acids) than any of the other fatty acids (mean = 0.05–9.4 %). In skipjack tuna and krill only, 20:5ω3 (EPA) was the third largest contributor to the similarity at 10–20 % contribution. When the two tuna taxa are compared with the diet component, krill, those samples taken off Tasmania were located closest together in the MDS plot, and this again is reflected in skipjack tuna and krill having the highest similarity at 74 %, followed by Tasmanian albacore tuna and krill at 65 %.

The closeness of skipjack tuna and krill and that of Tasmanian albacore tuna and krill (Fig. 5) was confirmed by one-way ANOSIM (Table 1). Again, skipjack tuna and krill and Tasmanian albacore tuna and krill are more similar ($R < 0.85$) than the remaining albacore tuna samples, which are the most dissimilar to krill ($R > 0.95$).

Table 1 SIMPER and ANOSIM R values for fatty acid proportions in albacore tuna (Coral Sea, New Zealand, Tasmania), skipjack tuna, and krill samples

Average similarity within/between groups (%)						*R*
	Krill	New Zealand	Coral Sea	Tasmania	Skipjack	Krill
Krill	89.9					
New Zealand	59.2	76.9				0.959
Coral Sea	63.1	73.2	82.4			0.956
Tasmania	64.8	74.1	77.4	76.0		0.826
Skipjack	73.7	65.7	78.1	76.6	92.8	0.845

These routines were used on albacore tuna and skipjack tuna fatty acid data obtained by direct methylation coupled with literature values for krill (Virtue et al. 1995)

R statistics are for pairwise tests with krill; all values are significant, $p = 0.1$ %

Discussion

Lipid analyses

Proportions of the major fatty acids from the direct transmethylation methods compared well with each other and with data obtained after extraction (Fig. 1), with intercepts close to the origin and slopes near unity. Interestingly, one of the two dominant fatty acids in the samples, 16:0, had the lowest r^2 value of the 12 most abundant fatty acids in the samples. Although the intercept and slope remain not significantly different to 0 and 1, respectively ($p > 0.7$), it does underline the possibility of contamination with this ubiquitously abundant fatty acid. The fatty acid with the highest r^2 value, 20:4ω6, had an interesting bimodal distribution of data points (Fig. 1), with albacore muscle and shark liver samples having much lower proportions of 20:4ω6 (0.3–4.5 %) than shark muscle samples (10–16 %). Higher levels of long-chain ω6 fatty acids have been used to help infer that white sharks feed more generally rather than specialize on marine mammals (Pethybridge et al. 2014).

For minor fatty acids present at mean levels below 4 % (Fig. 2), the relationship between data obtained from the direct transmethylation methods and those obtained after extraction remained strong ($p < 0.001$; power = 1.00 with $\alpha = 0.05$); however, the values for r^2 and slope deteriorated, especially for 18:2ω6 (mean of 0.8 %), which is usually a minor fatty acid in marine samples. There may have been problems with the integration of very small GC peaks from small samples. Nonetheless, most of the minor fatty acids had r^2 values of >0.72 and slopes >0.81.

The direct transmethylation methods compared well quantitatively with data obtained after extraction except at high fatty acid concentrations or at low cholesterol concentrations (Fig. 3). Total fatty acid concentrations were underestimated in liver samples, suggesting that much smaller samples should have been taken for direct determination. However, processing such small samples might call into question how representative the data would be of the entire liver. Under these circumstances, several replicate subsamples would have to be processed and the data averaged in order to obtain representative values for shark liver. The problem at the opposite end of the concentration scale for cholesterol probably relates to the fact that it is the last peak to exit the GC column when there is the most column bleed and more importantly when peaks are at their broadest. This combination means that such peaks are the most prone to signal-to-noise ratio problems. This could be partially resolved by adding a second derivatization step specifically for sterols, such as silylation (Miller et al. 2008), and/or the potential use of a higher final oven temperature.

The key to achieving good qualitative and quantitative comparisons with standard procedures may well be to minimize the amount of water in relation to methylating solution. Esters of carboxylic acids undergo acidic hydrolysis in a reversible reaction:

$$\mathrm{RCOOR'} + \mathrm{H_2O} \rightleftarrows \mathrm{RCOOH} + \mathrm{R'OH},$$

so methyl esters ($\mathrm{RCOOCH_3}$) of fatty acids (FAME) are more likely to hydrolyze with increasing water concentration.

By transmethylating small wet samples it is possible to greatly increase sample throughput, especially if the internal standard is added directly after methylation; however, information is lost compared to the longer processing protocols. When the lipid is extracted from the tissue prior to derivatization, it is possible to obtain a lipid mass for the sample as well as other information such as lipid class composition using companion analyses including HPLC or TLC–FID. If the sample is freeze-dried prior to direct derivatization it is possible to obtain a dry mass for the sample. Figure 4 shows that the same information may be calculated from the GC output obtained from wet derivatized samples. Lipid mass was highly correlated ($r = 0.98$, $p < 0.001$) with the sum of fatty acids in the samples, and dry mass was highly correlated ($r = 0.96$, $p < 0.001$) with the sum of free cholesterol and that released during direct methylation of samples. The slopes and intercepts of the samples with and without the inclusion of shark liver samples were very similar for the lipid mass data, but not for the dry mass data. Thus, for greater predictive power, a calibration curve should be constructed with tissues of only one type. In addition, close inspection of dry mass data points for muscle tissue shows that there is some segregation of data according to taxon. So, for further predictive power, the calibration should be done for tissues of one type for each taxon.

In addition to the speed of processing, the decision whether to use wet or dry samples can affect precision. In ecological studies, fatty acid data are usually processed as a percentage of total fatty acids and not as a concentration of individual fatty acids or total fatty acids per unit volume or mass. The average coefficient of variation (CV) of the percent composition of fatty acids in triplicate samples of wet shark tissue that was directly derivatized was 8.3 %. This included 19 fatty acids with mean proportions of 0.5–22.2 % of total fatty acids. On the other hand, the determination of total fatty acid concentrations per wet weight in triplicate Bligh and Dyer extractions of tuna gave, on average, a CV of 19.9 %. This compares to an average CV of 23.4 % for triplicate direct determinations of tuna and shark samples. Both are quite high, reflecting the difficulty involved in obtaining consistent wet weight determinations of ground tissue subsamples, which is presumably

exacerbated by the much smaller size used in the direct determination. By comparison, the CV for total fatty acid concentrations per dry weight for shark muscle tissues that were freeze-dried prior to direct derivatization was as low as 6.1 %. For frozen wet samples, it should be possible to reduce the problem of having different amounts of water squeezed out of the tissue samples by using an ice cold mortar and pestle in a cold room.

The simultaneous determination of fatty acids and cholesterol (Fig. 3) is afforded in our study through the use of a nonpolar column, and it leads to the possibility of determining a condition index in the same analysis. Fraser (1989) proposed an index for physiological condition and survival potential in marine larvae based on triacylglycerol content. He took increasing or decreasing ratios to sterol to be indicative of "good" or "bad" larval condition, respectively, based on a highly positive correlation between sterol content and dry weight, as we see here (Fig. 4b). Triacylglycerol, a storage lipid, is composed almost entirely of fatty acids, and therefore the total fatty acid to cholesterol ratio might also be considered a condition index. Ratios obtained from Bligh and Dyer (1959) extracted albacore tuna samples suggest that tuna from New Zealand waters are in better condition than those from the Coral Sea (Parrish et al. 2014).

The rapid methylation method could be further sped up by dissolving the internal standard in a mixture of hexane: dichloromethane (4:1 v/v) and using an accurate solvent bottle-top dispenser, rather than adding the internal standard in hexane using a syringe as done here. This would increase sample throughput to ~25 in 6 or 7 h. Another time-saving step would be to not obtain an accurate wet weight of the sample and to just use the fatty acid and cholesterol data. This would double sample throughput to ~40 ready for an overnight run on the GC. It was hoped that an estimate of the wet weight might be obtainable from the total cholesterol data as it was for the dry weight (Fig. 4b); however, a maximum of only 38 % of the variation could be explained by the regression equations obtained. This is despite having a very high value for n (up to 261 mainly tuna muscle samples) and investigating with and without liver data and with and without shark data. All regressions of wet mass (mg) on total cholesterol (μg) were, nonetheless, highly significant ($p < 0.001$).

Multivariate analyses

A rapid direct tissue transmethylation method will facilitate the generation of data for fatty acid profiling—a technique that is being widely and increasingly employed to define trophic connections in the marine environment because of the large number of unique structures synthesized. They can be used as signatures to delineate the transfer of material through food webs, especially when analyzed with multivariate statistics. For example, Loseto et al. (2009) and Pepin et al. (2011) used principal components analysis (PCA) of FA to reveal size-related dietary differences in beluga whales and copepods, while Parrish et al. (2012) showed that there was little modification of dietary polyunsaturated FA (PUFA) in a copepod feeding study.

PCA is a powerful data reduction procedure that can handle a large number of variables simultaneously to provide a simple graphic representation of an entire data set; however, other multivariate statistical analyses can furnish a more quantitative analysis of patterns. For example, PRIMER provides measures of similarity within and between sample groups as well as graphic representations of data sets based on non-metric MDS analysis. By using MDS, some of the limitations of PCA are avoided (Clarke and Warwick 2001). MDS, ANOSIM, SIMPER, and permutational multivariate analysis of variance (PERMANOVA) are also all available in an open source *R* package, vegan (Oksanen et al. 2013). *R* is a free software environment for statistical computing and graphics.

Here, we used MDS to examine spatial differences among albacore tuna sampled from different locations in the southwestern Pacific (Parrish et al. 2014) and to define trophic connections among albacore and skipjack tuna and krill (Fig. 5). Most of the world's tuna harvest comes from the Pacific and it is dominated by skipjack tuna, and krill is a potentially dominant prey item.

The degree to which the two dimensional configuration plot distorts the sample relationship is called the "stress." Values below 0.05 give an "excellent representation," while those between 0.1 and 0.2 give "a potentially useful 2-dimensional picture" (Clarke and Warwick 2001). The stress value in Fig. 5 is low, while that obtained from a large 2-dimensional MDS analysis of fatty acids in primary producers (Kelly and Scheibling 2012) was 0.21, which the authors termed as "relatively high." Figure 5 shows the importance of 18:2ω6, 18:3ω3, 18:4ω3, EPA, 16:4ω1, and 14:0 in krill. EPA and 16:4ω1 are characteristic of diatoms (Kelly and Scheibling 2012), reflecting their prominence in the diets of these krill.

To quantify the relationships visualized in MDS plots, ANOSIM can be used to provide a measure of similarity between groups, and SIMPER can be used to examine the contribution of each FA to average resemblances between sample groups (Table 1). The ANOSIM analysis gives an *R* statistic which is usually between 0 and 1, as well as a significance level. *R* values ranged from <0.05 to >0.90 and *p* values from 0.001 to >0.05 in ANOSIM analyses of fatty acids from food web samples collected off eastern Australia (Hall et al. 2006; Pethybridge et al. 2011). Focusing on *p* values, Hall et al. (2006) demonstrated differences in diet within a species in an experimental estuarine food chain.

On the other hand, Pethybridge et al. (2011) focused on *R* values to characterize interspecific differences in resource-use patterns, identifying degrees of dietary specialization and niche partitioning among co-occurring species of deep-water sharks. They characterized *R* values >0.75 as indicating "well separated groups," while those <0.25 were "barely separated groups." By this measure, all tuna groups are well separated from krill (Table 1), but Tasmania albacore and skipjack tuna are less well separated, indicating a greater contribution to the diet.

Hall et al. (2006) used SIMPER to help determine which FA, and therefore dietary items, were responsible for the differences in diet within a species in the experimental estuarine food chain. They also compared the average within-group and between-group similarities, usually terming ≥90 % as "high." By this measure, the within-group similarity of krill and skipjack tuna in Table 1 is high. Kelly and Scheibling (2012) also used SIMPER to determine which FA contributed at least 10 % to within-group similarity for only one group of primary producers. In the case of diatoms, 14:0 fulfilled this criterion, and it was also within the top five contributors to the high similarity in krill (Table 1), further underlining the importance of diatoms in the diets of these krill.

The vectors in the MDS plot show the importance of the essential fatty acids DHA and EPA in determining similarities among samples. In the tuna MDS plot, DHA gave the highest Pearson correlation at 0.99, and EPA gave the third highest among the 23 fatty acids at 0.96. Varying the long-chain ω3 fatty acid content, in turn, also has implications for seafood quality. A meta-analysis of recent randomized controlled fish oil supplementation trials confirmed that provision of EPA and DHA reduces blood pressure (Miller et al. 2014). There are also consequences for inflammation, which is thought to connect chronic diseases such as cardiovascular disease, obesity, and allergic diseases such as asthma (Myers and Allen 2012), and for cognition and mental health (Lin et al. 2010; Smith et al. 2011; Loef and Walach 2013). The fatty acid composition of membrane phospholipids may be central to many of the mechanisms of the health effects of essential fatty acids (Parrish 2009; Lin et al. 2010), so that changes in supply of EPA and DHA in the natural environment, as well as in aquaculture, have implications for both fish and the human consumer.

Conclusions

We report the application of a rapid, direct transmethylation method that can facilitate the acquisition of large quantities of fatty acid data. We show that results derived from this method for fish muscle and liver tissue are comparable to those generated from longer and more costly traditional methods where lipid extraction is performed. When large fatty acid data sets are generated through a rapid transmethylation method, multivariate statistics such as multidimensional scaling, analysis of similarities and similarity of percentages analysis can be used to first define the trophic connections among the samples and then to quantify them. Such routines were used on southwestern Pacific albacore tuna and skipjack tuna fatty acid data obtained by direct methylation coupled with literature values for krill. PUFA primarily provided the discrimination between the observed sample clusters and the similarity within and between groups was quantified. Skipjack tuna and krill had the highest similarity at 74 %. DHA and EPA were important in determining similarities and differences in the commercially caught tuna with further implications for seafood quality. Used in conjunction with knowledge of FA turnover rates, this methodology can also provide input into our understanding of the residency times of a range of migratory species.

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