Lipid extraction effects on stable isotope values ($\delta^{13}C$ and $\delta^{15}N$) of elasmobranch muscle tissue

Nigel E. Hussey a,⁎, Jill A. Olin a, Michael J. Kinney b, Bailey C. McMeans a, Aaron T. Fisk a

a Great Lakes Institute for Environmental Research, University of Windsor, 401 Sunset Avenue, ON, N9B 3P4, Canada
b Fishing and Fisheries Research Centre, School of Earth and Environmental Sciences, James Cook University, Townsville 4811, Queensland, Australia

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Given the known effect of lipid content on $\delta^{13}C$ values and the potential effect of urea on $\delta^{15}N$ values, examining the effects of lipid extraction, which can potentially extract both, is of particular importance for elasmobranch isotope ecology. Through analysing paired $\delta^{13}C$, total $\%C$, $\delta^{15}N$, total $\%N$ and $C:N$ values of non-lipid extracted (BULK) and lipid extracted (LE) muscle samples from twenty-one elasmobranch species, we assessed whether lipid extraction was required: (i) to remove lipids given reported low lipid content and, (ii) to determine if $\delta^{15}N$ values were affected and whether this relates to the retention of isotopically light urea by elasmobranchs. The mean ($\pm$ SD) $\delta^{13}C$ values of eight out of twenty-one species significantly increased following lipid extraction with two species, the Greenland (Somniosus microcephalus) and whale (Rhincodon typus) shark, showing a marked increase (5.0 ± 0.4‰ and 3.3‰, respectively). The mean ($\pm$ SD) and maximum increase in $\delta^{13}C$ values were 0.6 ± 1.2‰ and 5.9‰, respectively. For $\delta^{15}N$ data, thirteen species showed a significant increase following lipid extraction and a concomitant reduction in total percent nitrogen ($\%N$). The C:N ratio for these species also increased from unexpectedly low values of <3.0 to ~3.0, the value expected for pure protein. The mean and maximum observed increase in $\delta^{15}N$ values were 0.6 ± 0.6‰ and 2.3‰, respectively. There was no effect of increasing animal size on $\delta^{13}C$ and $\delta^{15}N$ difference (LE–BULK) for the two species examined. Field sampled animals (sampled immediately upon capture in the marine environment) showed a greater $\delta^{15}N$ difference than animals sampled in the laboratory (sampled several hours after capture in the marine environment) (1.0 ± 0.5‰ and 0.4 ± 0.4‰ respectively), while estuarine sampled animals (sampled immediately) showed the smallest difference (0.1 ± 0.6‰). The $\delta^{13}C$ data demonstrate that lipid extraction is required to remove lipids from elasmobranch muscle tissue given both intra- and inter-species variability. In addition, the increase in $\delta^{15}N$ values, decrease in $\%N$ and increase in C:N ratio indicate that lipid extraction is removing soluble urea. Given lower $\delta^{15}N$ diet-tissue discrimination factors for large marine predators, removal of urea is required to elucidate accurate trophic position estimates and relative food web position of elasmobranchs and for diet reconstruction. It is recommended that investigators undertake lipid extraction trials on elasmobranch muscle tissue to determine effects on $\delta^{13}C$ and $\delta^{15}N$ values on a species-by-species basis.

⁎ Corresponding author. Tel.: + 1 519 253 3000x4957.
E-mail address: nehussey@uwindsor.ca (N.E. Hussey).

1. Introduction

The retention of geochemical signatures in animal tissues and their systematic transfer through food webs has led to the development of important ecological tools to investigate aquatic and terrestrial ecosystems. Two of the most commonly applied tools are the stable isotopes of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$), isotopic ratios which have been reported across a broad range of taxa and ecosystems (Hobson, 1999; Peterson and Fry, 1987; Wolf et al., 2009). Typically, the stable carbon isotope ratio ($^{13}C/^{12}C$ relative to a standard) of an animal’s tissue provides a time integrated measure of the carbon sources to an animal’s diet, while the nitrogen isotope ratio ($^{15}N/^{14}N$ relative to a standard) indicates the relative trophic position of the organism within the food web (DeNiro and Epstein, 1981; Minagawa and Wada, 1984; Peterson and Fry, 1987).

With advancements in our knowledge of the transfer of $^{13}C$ and $^{15}N$ through food webs, more complex questions are now being addressed; for example, community trophic structure and niche width (Jackson et al., 2011; Layman et al., 2007a,b), specialist vs. generalist feeding behaviour (Matich et al., 2010; Newsome et al., 2009a) and intra-species variability in ontogenetic feeding patterns (Newsome et al., 2009a,b; Vander Zanden et al., 2010). Critical to the successful application of stable isotopes to more complex ecological questions, however, is confidence in species-specific sample storage and preparation techniques prior to analytical determination (Kim and Koch, 2011). Although much work has focused on the variable effects of (i) storage techniques/materials (Arrington and Winemiller, 2002; Kim and Koch, 2011; Lesage et al., 2010) and (ii) lipid extraction techniques on stable isotope values (for example: Logan and Lutcavage, 2008; Sotiropoulos...
et al., 2004; Sweeting et al., 2006), questions remain about the influence of lipid extraction on δ13C and δ15N values, particularly in elasmobranchs. As well, less attention has been given to the influence of lipid extraction on total percent nitrogen and carbon (SN and SC, respectively) which are important metrics in interpreting stable isotope data and are critical for lipid correction methods (Post et al., 2007; Reum 2011).

Lipid extraction (LE) of tissues is typically performed using a chloroform–methanol extraction following Bligh and Dyer (1959) although several techniques are available (Logan and Lutcavage, 2008). Lipids are depleted in 13C relative to carbohydrates and proteins (DeNiro and Epstein, 1977), requiring their extraction prior to stable isotope analysis to standardise data among individuals and across species within a food web. Chloroform–methanol is an effective solvent for removing lipids (Logan and Lutcavage, 2008; Sweeting et al., 2006;), but has also been found to alter δ15N values with an increase of ~1‰ widely reported [range: 0.25–0.78‰; Ingram et al., 2007; Pinnegar and Polunin, 1999; Post et al., 2007; Sotiropoulos et al., 2004;], yet see Murry et al., 2006 (~1.5‰). Investigators have mixed opinions in terms of these observed δ15N effects. Ingram et al. (2007) reported that LE had minimal effect on δ15N values and would not confound interpretation of food web data, but their meta-analysis combined data from a wide range of organisms inhabiting distinct environments. In contrast, Murry et al. (2006) suggested that increases in δ15N values following LE would complicate comparative studies of δ15N across food webs. Accepting that diet tissue discrimination factors (Δ15Nconsumer − Δ15Nprey and Δ13C = Δ15Cconsumer − Δ13Cprey) can be variable among species, but in general are predicted to decrease with increasing trophic step (or with increasing prey δ15N values) (Caut et al., 2009; Dennis et al., 2010; Hussey et al. 2010a), the effects of LE on δ15N values could have implications for studying upper trophic level species, for example large sharks and billfish. Moreover, although lipid correction formulae/s have been proposed for standardising δ13C values using C:N ratios without the need for chloroform–methanol extraction (Logan et al., 2008; Post et al., 2007; Sweeting et al., 2006), these equations may not be appropriate for all species (Fagan et al., 2011; Lesage et al., 2010). For elasmobranchs, this discrepancy is pertinent, considering the C:N ratio of bulk muscle tissue (tissue that has not been lipid extracted) is below the expected pure protein value of ~3 which form the basis of most lipid correction formulae/s (Hussey et al., 2010a; Kim and Koch, 2011).

Elasmobranchs are unique in that they maintain high levels of urea and trimethylamine oxide (TMAO) in their tissues for the purposes of osmoregulation (Olson, 1999). Fisk et al. (2002) suggested that the mismatch between δ13C and contaminant estimated trophic position of Greenland sharks (Somniosus microcephalus) was due to the presence of urea. Urea is a waste product of metabolism and consequently is depleted in 15N which would result in an artificially low δ15N value and a lower trophic position estimate than would be expected (Fisk et al., 2002). As solvents such as chloroform–methanol can extract urea (Christie, 1993), we would expect to see an increase in δ15N values and a concomitant decrease in δ13C in muscle tissue following lipid extraction (Hussey et al., 2010a). If this were the case, lipid extraction of elasmobranch tissue may be an effective mechanism for removing soluble urea content and standardising δ15N values among individuals and across species with variable urea tissue concentrations. Two studies to date have addressed the issue of urea effects on δ13C values in elasmobranchs with conflicting results. Logan and Lutcavage (2010) reported that urea content did not have an adverse effect on δ15N values of coastal sharks (Leucoraja spp.), and spiny dogfish (Squalus acantius) muscle tissue and blood following both lipid extraction and water rinsing. In contrast, Kim and Koch (2011) found that lipid extraction and combined lipid extraction and water rinsing significantly altered δ15N values of leopard shark muscle tissue (Triakis semifasciata) which the authors directly attributed to urea removal. Considering this disparity and the fact that muscle tissue urea content is variable between species (Gordievskaya 1973), rescalcs as individuals move between freshwater and marine environments (Pillans et al., 2005) and is rapidly broken down following death, further work is required to investigate potential urea extraction effects on δ15N values across a broad range of elasmobranch species following standard chloroform–methanol extraction techniques.

The aims of this paper were to assess the effects of standard chloroform–methanol lipid extraction on the stable isotope values (δ15N and δ13C) of white muscle tissue sampled from 21 species of elasmobranch (sharks and a skate). Specifically the study aimed to determine: (i) if lipid extraction of elasmobranch muscle tissue is required for interpreting δ13C values considering assumed low lipid content (Bone and Roberts, 1969; Hussey et al., 2010a) and (ii) species-specific effects of lipid extraction on δ15N, δ13C and C:N values given the potential confounding effect of urea retention in this physiologically unique group of marine vertebrates. Given the increasing use of stable isotopes in the study of elasmobranchs (Hussey et al., 2012), understanding potential species, animal size, sex and environment-driven effects of lipid and potential urea content on stable isotope values is important for improving estimates of trophic position, for the examination of diet, feeding ecology and movement, and in understanding overall food web dynamics.

2. Materials and methods

2.1. Sampling location

The effects of lipid extraction on δ13C and δ15N values were tested on a total of 21 species of elasmobranch (20 sharks and 1 skate) from 8 families (Carcharhinidae, Lamnidae, Sphyrnidae, Somniosidae, Odontaspidae, Triakidae, Rhincodontidae and Rajidae, respectively) (Table 1). Sharks and skates were sampled from four distinct geographical regions; Florida (USA), Townsville (Australia), Cumberland Sound (Canadian Arctic) and Durban (South Africa) (Table 1). Specific details on sampling protocols for each region are as follows:

2.1.1. Florida (USA)

Juvenile bull sharks (Carcharhinus leucas) were sampled from known nursery habitats of the Caloosahatchee and Myakka Rivers of south western Florida using shallow water (< 10 m) bottom-set longlines (length: 400–800 m) set for periods of 0.5–2.0 h. Bonnethead (Sphyra tiburo), Atlantic sharpnose (Rhizoprionodon terraenovae) and blacktip sharks (Carcharhinus limbatus) were sampled from the Pine Island Sound, Charlotte Harbour Estuary of south western Florida, immediately adjacent to the Caloosahatchee River. Sharks were caught using a 360 m gill net (stretched mesh size: 11.8 cm), set at 1.5–2.0 m depth, for periods of 0.5–1.0 h. All sharks were euthanised in the field directly following capture. Each shark was measured [pre-caudal PCL: fork length (FL) and total length (TL) - cm], sexed and white muscle tissue was excised from the dorsal region, anterior to the first dorsal fin. Muscle samples were stored on ice and then stored frozen on return to the laboratory (~20 °C).

2.1.2. Townsville (Australia)

Eight shark species (Table 1) were sampled during fisheries independent surveys conducted in Cleveland Bay, Townsville. Sharks were caught using either: (i) shallow water (< 20 m) bottom-set longlines (length: ~800 m) set for ~1.0 h or, (ii) a 300 m gill net (stretched mesh size: 4.5 cm) set in shallow water (< 15 m) for 0.5–1.0 h. Each shark was measured (FL and TL - cm), sexed and a small biopsy of white muscle tissue was taken from the area anterior to the first dorsal fin. Sharks were then released alive. Muscle tissue samples were placed on ice until they were returned to the lab where they were stored frozen (~20 °C).

2.1.3. Cumberland Sound (Canadian Arctic)

Greenland sharks (S. microcephalus) and Arctic skates (Amblyraja hyperborea) were caught using deep water (~200 m) bottom-set longlines...
lines (length: ~120 m), with set times ranging from 5 to 24 h. Sharks and skates were euthanized upon capture, measured [TL and disc width (DW) − cm], sexed and sampled for stable isotope analysis. White muscle tissue was excised from the area immediately posterior to the first dorsal fin of Greenland sharks, and from the dorsal surface of the pectoral fin, adjacent to the vertebral column of Arctic skates. Samples were stored frozen (−20 °C) on return to the laboratory.

2.1.4. Durban (South Africa)

Eleven shark species were sampled from animals incidentally caught in beach protection nets along the KwaZulu-Natal coast of South Africa (Table 1). Beach nets consisted of two 106.8 m long black gill nets (stretched mesh size: 51 cm) joined together to form ‘double nets’ (length: 213.5 m), with the exception of Durban, Brighton Beach and Anstey’s Beach where gill nets were 308.4 m length. All beach protection nets were set in exposed water of 10 °C). Nets were serviced on a daily basis and shark mortalities in good condition were retrieved and sampled for stable isotope analysis.

### Table 1

<table>
<thead>
<tr>
<th>Species Common name</th>
<th>Species code</th>
<th>Collection location</th>
<th>Sample number</th>
<th>Sampling (field/lab)</th>
<th>Habitat (marine/estuarine/freshwater)</th>
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<tr>
<td>Sphyra tiburo</td>
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<td>5</td>
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<tr>
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<td>5</td>
<td>F</td>
<td>M-E</td>
</tr>
<tr>
<td>Sphyra zygaena</td>
<td>SMO</td>
<td>South Africa</td>
<td>8</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
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<td>South Africa</td>
<td>6</td>
<td>L</td>
<td>M</td>
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<td>F/F/L</td>
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<td>M</td>
</tr>
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<td>4/9</td>
<td>F/L</td>
<td>E(F)/M</td>
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<td>L</td>
<td>M</td>
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<tr>
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<td>9</td>
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<td>M</td>
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<tr>
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<td>Australia</td>
<td>8</td>
<td>F</td>
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</tr>
<tr>
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<td>9</td>
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<td>M-E</td>
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<td>JAV</td>
<td>Australia/South Africa</td>
<td>8/5</td>
<td>F/L</td>
<td>E/M</td>
</tr>
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<tr>
<td>Skates</td>
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<td></td>
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</tr>
<tr>
<td>Amblyraja hyperborea</td>
<td>SKA</td>
<td>Canadian Arctic</td>
<td>8</td>
<td>F</td>
<td>M</td>
</tr>
</tbody>
</table>

* Referred to as raggie shark in South Africa, sand tiger in North America and Europe and grey nurse shark in Australia.
* Blacktip sharks sampled from Australia may be either Carcharhinus limbatus or Carcharhinus tilstoni.

White muscle tissue was sampled from the muscle block anterior to the first dorsal fin adjacent to the vertebral column and stored frozen (−20 °C). The tissue and solvent were then either (i) centrifuged for 3 min and then decanted, before a second addition of 5 ml of 2:1 chloroform–methanol was added followed by a further round of agitation and centrifuging before the final decant. The resulting pellet was left to dry overnight in a fume cupboard to allow for the evaporation of any remaining solvent (Australia). Between 400–600 μg of paired tissue samples, lipid extracted tissue (LE) and non-lipid extracted tissue (BULK) for each shark/skate were then weighed into tin capsules and stable carbon and nitrogen isotope ratios were provided from a continuous flow isotope ratio mass spectrometer (IRMS, Finngnan MAT Deltaplus, Thermo Finngnan, San Jose, CA, USA) equipped with an elemental analyser (Costech, Valenica, CA, USA). All shark and skate LE and BULK tissue samples were analysed using the above mass spectrometer.

Stable isotope abundances are expressed in delta (δ) values as the deviation from standards in parts per thousand (‰) using the following equation:

\[
\delta X = \left[ \frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right] \times 1000
\]

where X is 15N or 13C and R is the ratio 15N/14N or 13C/12C. The standard reference material was Pee Dee Belemnite carbonate for CO2 and atmospheric nitrogen for N2. The analytical precision for δ15N data was 0.24 and 0.14‰ and for δ13C data was 0.10 and 0.07‰ for NIST standard 8414 (bovine muscle) and an internal lab fish muscle standard (Tilapia), respectively, across multiple runs and more than 400 total standards analysed. Analytical accuracy was 0.14‰ for δ15N data and 0.05‰ for δ13C data based on a single run of NIST standard sucrose (n = 13) and ammonium sulphate (n = 13).

2.3. Statistical analysis

The difference in δ13C, total δC, δ15N, total δN and C:N ratio between LE and BULK tissue were calculated for all 21 species to show the relative magnitude and direction of change following LE (Difference = LE − BULK). Wilcoxon signed rank tests were used to assess whether the observed paired differences were significant for 20 species where
sufficient data were available (n≥3; Table 1). To examine the effect of sex on the difference between LE and BULK tissue δ¹⁵N and δ¹³C values on species, species basis, a non-parametric Mann–Whitney test was performed. For the scalloped hammerhead (Sphyrna lewini) and white (Carcharodon carcharias) sharks where a large size range of animals were sampled from the same geographic location, least squares linear regressions were used to assess whether the difference between LE and BULK tissue δ¹³C and δ¹⁵N values varied systematically with size. Given that (i) urea is rapidly broken down following death and (ii) urea concentrations in elasmobranch muscle tissue rescales between freshwater (low urea content) and marine (high urea content) environments, we examined the effect of time of sampling (minutes vs. hours) and environment (marine vs. estuarine) on the difference between LE and BULK tissue δ¹⁵N values. To do this, species LE and BULK δ¹⁵N data were binned into three main environment/sampling regime categories and a general linear model (GLM) performed. The environment/sampling regime categories were defined as: MARINEFIELD, MARINELABORATORY and ESTUARINEFIELD, where MARINE and ESTUARIE are the environment where the animal was sampled and FIELD and LABORATORY are the tissue sampling method for the sharks; either directly in the field immediately following euthanasia or prior to release of live animals (minutes) or at the laboratory following the retrieval of shark mortalities (hours), respectively. To assess if geographical sampling region had an effect on the difference between LE and BULK tissue LE we performed either Mann–Whitney (where samples were from 2 locations) or Kruskal–Wallis (for 3 locations) tests on δ¹⁵N and δ¹³C values for all species sampled across more than one of our study regions. This resulted in the testing of the difference in δ¹⁵N and δ¹³C data for scalloped hammerhead, sharpnose, blacktip, bull and java (Carcharhinus amblyominus) sharks. All data were normally distributed and equal in variance, consequently no data transformations were required prior to statistical testing; Wilcoxon signed rank, Mann–Whitney and Kruskal–Wallis tests were used given small sample sizes. A criterion of p<0.05 was used for all analyses.

3. Results

Lipid extraction had species-specific effects on δ¹³C, δ¹⁵N, δ¹⁵N, δ¹⁵N and C:N ratio of the 20 sharks and one skate examined (Fig. 1A–E). A total of 8 of the 20 elasmobranch species analysed showed a significant (p<0.05) increase in δ¹³C values following LE, with the largest positive effect measured in the Greenland shark (5.0±0.4‰) and the largest negative effect in the bonnethead shark (−0.1±0.8‰) (Fig. 1A; Suppl. A). The whale shark also showed a large increase in δ¹³C values (3.3‰) between LE and BULK tissue, accepting a sample size of 2. Java and sharpnose (Rhizoprionodon acutus) sharks had the largest δ¹³C SD associated with the difference between LE and BULK tissue (1.1‰ and 1.2‰, respectively; SD range: 0.1‰–1.2‰). There was a significant increase in total %C for all species except bonnethead, smooth hound (Mustelus mosis), Atlantic sharpnose and sand tiger sharks (Carcharias taurus) (Fig. 1B; Suppl. A). A plot of the difference in δ¹³C vs. the difference in total %C as a proxy for lipid content) between LE and BULK tissue found no clear relationship between the two variables except for the Greenland and whale shark (Fig. 2). For δ¹⁵N data, 13 of the 20 elasmobranch species tested showed a significant increase in δ¹⁵N following LE (Fig. 1C; Suppl. A). The largest positive mean effect was measured in the Arctic skate (1.4±0.2‰) and the largest negative mean effect in the sand tiger and smooth hound shark (−0.1±0.3‰ and −0.1±0.3‰, respectively). Overall the δ¹⁵N SD range of the difference between LE and BULK tissue (0.2‰–0.6‰) was less than that recorded for δ¹³C SD range. Total %N declined for 9 species but an increase was recorded for the whale and Greenland shark (Fig. 1D; Suppl. A). The C:N ratio increased for 17 species (Fig. 1E; Suppl. A).

There was no statistical effect of sex on the difference between LE and BULK tissue δ¹³C and δ¹⁵N values accepting small sample sizes in certain cases (Suppl. B). In addition, there was no significant effect of size on the difference between LE and BULK tissue δ¹³C and δ¹⁵N values for the great white and scalloped hammerhead sharks sampled from South Africa (Fig. 3).

When considering the data for all species as a function of environment/sampling regime, MARINEFIELD sampled sharks showed the largest change in δ¹⁵N values between LE and BULK tissue, followed by MARINELABORATORY sharks and then ESTUARINEFIELD sampled sharks (Fig. 4; GLM: F2,157=49.62, p<0.0001; r²=34%). The one exception to this pattern was the field sampled smooth hound shark which exhibited a minimal δ¹⁵N shift following LE (Fig. 4). Juvenile bull sharks (mean FL: 76.7±14.3 cm; range: 64.5–97.4 cm) sampled in an estuarine system showed a minimal shift in δ¹⁵N values between LE and BULK tissue when compared to large bull sharks (mean FL: 180.5±14.3 cm; range: 165.0–206.2 cm) sampled from the marine environment even though the latter animals were sampled in the laboratory (Figs. 4 and 5). Similarly juvenile java sharks (mean FL: 81.3±17.1 cm; range: 62–104 cm) sampled near a freshwater input showed a negative shift in δ¹⁵N values between LE and BULK tissue compared to a positive shift in δ¹⁵N values of large sharks (Mean FL: 142.0±15.5 cm; range: 123.0–157.8 cm) sampled in the marine environment (Figs. 4 and 5). Sharpnose sharks and juvenile blacktip sharks, which move between both marine and estuarine environments, showed a change in δ¹⁵N between LE and BULK tissue which was intermediate to MARINEFIELD and ESTUARINEFIELD (Fig. 4).

For δ¹⁵N, there was no effect of geographic sampling location (USA, Australia and South Africa) on the difference between LE and BULK tissue with the exception of bull sharks (Fig. 5a; Suppl. C). Small bull sharks sampled from the USA showed less of an increase in δ¹⁵N following LE than large individual sampled from South Africa likely related to the size class of animal sampled and the environment rather than geographic location (see above: Suppl. C). Java sharks sampled in Australia also showed a trend of a smaller increase in δ¹⁵N than animals sampled from South Africa, but the data were more variable and non-significant (Fig. 5a; Suppl. C). For δ¹³C, there was no effect of geographic sampling location on species-specific shifts in δ¹³C values between LE and BULK data with the exception of scalloped hammerheads (Australia vs. South Africa) and blacktip sharks (Australia vs. South Africa vs. Florida; Fig. 5b; Suppl. C).

4. Discussion

Understanding the effects of lipid extraction on tissue-specific stable isotope values is of central importance for accurately interpreting δ¹³C and δ¹⁵N data in ecological studies. Given that lipids are generally depleted in δ¹³C (DeNiro and Epstein, 1977; McConnaughey and McRoy, 1979), it was not unexpected that while lipid extraction increased the δ¹³C values for several of the elasmobranchs analysed, in general the observed increase was minimal, indicative of low lipid content (Bone and Roberts, 1969; Devadoss, 1984; Hussey et al., 2010a). The magnitude of the lipid extraction effect on δ¹³C values was, however, species-specific.

Although the mean increase in δ¹³C between BULK and LE tissue for most species was minimal (mean ± SD: 0.6±1.2‰, both the Greenland and whale shark showed a marked increase (5.0±0.4‰ and 3.3‰ (n=2), respectively). In the case of the Greenland shark, high lipid content in the muscle tissue of polar fish is widely reported (Eastman and DeVries, 1982; Freidrich and Hagen, 1994) and is thought to assist buoyancy, given most polar fish do not have swim bladders. In addition, lipid reserves in muscle tissue are thought to provide calorific stores (Sidell et al., 2005). Considering that sharks lack swim bladders, the high lipid content found in the muscle tissue of Greenland sharks may assist in regulating buoyancy and providing energy stores for this polar species. Similarly, the large size of the

1 Species were divided according to the environment where they were sampled (see Table 1) and from known habitat preferences reported in the literature.

tropical whale shark may require lipid stores in muscle tissue to maintain buoyancy. The scalloped hammerhead shark showed the next largest change in \( \delta^{13}C \) values between BULK and LE tissue (mean \( \pm \) SD; 0.5 \( \pm \) 0.3), in agreement with the relative higher lipid content recorded in the whole body tissue of this species (2.1% dry weight; Devadoss, 1984). Logan and Lutcavage (2010) and Reum (2011) also reported that spiny dogfish (Squalus acanthias) \( \delta^{13}C \) values increased significantly following lipid extraction.

Intra-species variability in \( \delta^{13}C \) values between BULK and LE tissues indicated potential disparate lipid content between individuals of some species. Intra-specific variation in lipid content of teleost fish is widely reported and has been related to age, sex and sampling depth and according to species-specific life-stages/strategies (Freidrich and Hagen, 1994). Furthermore, individual \( \delta^{13}C \) variation following LE of fish muscle for stable isotope analysis is common (Logan and Lutcavage, 2008; Murry et al., 2006). Considering the importance of standardising individual \( \delta^{13}C \) values of a species, LE is required for stable isotope studies investigating individual level variation within a population; for example studies looking at community measures of niche width (Jackson et al., 2011; Layman et al., 2007a,b) or specialists vs. generalists, where multiple tissues of individual animals are compared (Matich et al., 2010). Furthermore, the difference in \( \delta^{13}C \) between BULK and LE muscle tissue varied for two species by geographic sampling location. Davidson et al. (2011) reported that the total amount of lipids in the liver of blacktip sharks sampled from South Africa was higher than individuals sampled from the Atlantic Ocean and Gulf of Mexico. Our data contrast this finding for muscle tissue, indicating that blacktip sharks from South Africa had lower lipid content than those of Florida. Regardless, both of these data indicate that lipid content can vary for a single species sampled from different geographic locations.

With the exception of the Greenland and whale shark, total %C increased for all species following lipid extraction. In addition, there was no clear relationship between the difference in \( \delta^{13}C \) values vs. difference in total %C between LE and BULK tissue for all species, except the Greenland and whale shark. This was likely a result of the higher proportional content of nitrogenous waste (%N) removed from the sample relative to the low lipid content for most species (Hussey et al., 2010a; see below). These data would suggest that current multi-species lipid correction models that use the C:N ratio data as a measure of lipid content may not be appropriate for elasmobranch muscle tissue in agreement with
Following LE, filtering (South Africa, USA and Canadian Arctic) vs. centrifu
gue (Australia), may have affected the magnitude of change in $\delta^{15}N$ values. Aside from known LE effects of different solvents on $\delta^{13}C$ values (Logan and Lutcavage, 2008), we are not aware that centrifuging or fil-
tering can differentially alter $\delta^{13}C$ values. Nevertheless, the fact that the differ-
ence in $\delta^{13}C$ values following LE varied across and within elasmo-
branch species enforces the requirement to determine tissue lipid con-
tent and/or test the effect of lipid extraction on $\delta^{13}C$ values of muscle
tissue on a species by species basis.

The effect of the lipid extraction process on $\delta^{15}N$ values of elasmo-
branchs was more variable than $\delta^{13}C$, both in terms of intra- and
inter-species differences. In most instances, $\delta^{15}N$ values increased fol-
lowing lipid extraction with a mean ($\pm$ SD) increase of; (i) $0.6 \pm 0.6\%$
across all species, (ii) $1.0 \pm 0.5\%$ across field sampled marine species
(excluding the smoothhound), (iii) $0.1 \pm 0.6\%$ across field sampled estu-
arine species, and (iv) $0.4 \pm 0.4\%$ across laboratory sampled ma-
rine species. Our reported mean $\delta^{15}N$ increase for field sampled estu-
arine species and laboratory sampled sharks was similar to that for
teleost fish following standard lipid extraction (Ingram et al., 2007;
Pinnegar and Polunin, 1999; Post et al., 2007). The maximum in-
creases in $\delta^{15}N$ data recorded for the Greenland shark (2.3%),
the creek whaler and the spottail shark (2.1%), the blacktip shark
(1.9%) and the Arctic skate (1.8%) were generally higher than that
reported in the literature [but see maximum values reported by
Logan and Lutcavage, (2008); Logan et al. (2008) (2.0% and 2.9%, re-
spectively) and Mintenbeck et al. (2008) (1.7%)].

The large directional change in $\delta^{15}N$ values of field sampled ma-
rine elasmobranchs, in conjunction with the lesser effect of lipid ex-
traction on $\delta^{13}C$ values of laboratory sampled sharks and juvenile
bull sharks which inhabit freshwater/brackish systems, would sug-
gest that nitrogenous waste products including ammonia, ammonium
and urea are being removed during LE. The non-protein nitrogen frac-
tion of shark muscle tissue comprises free amino acids, urea and
trimethylamine oxide (TMAO). Following death, urea within muscle
tissue is rapidly hydrolysed into ammonia by the action of urease
while TMAO is reduced to trimethylamine (TMA) by TMAO reductase.
Both of these enzymes (urease and TMAO reductase) typify the action
of psychrotrophic bacteria which is characterised by a distinctive
noxious odour. During the laboratory dissection of sharks in South
Africa, there was a strong odour, indicative that urea/TMAO break-
down was underway. This odour penetrates clothing and laboratory
utensils, indicating that ammonia/TMA leach out of the muscle tissue.
The conversion of urea, which is a metabolic waste product and
therefore expected to be depleted in $^{15}N$ (Fisk et al., 2002), and
leaching of resultant ammonia/TMA products would result in the ob-
served lesser effect of lipid extraction on $\delta^{15}N$ values from the labo-
ratory sampled marine elasmobranchs relative to fresh animals. In
addition, juvenile bull sharks reside in freshwater/brackish systems
for the first two years of life (Heupel et al., 2010; Simpfendorfer et
al., 2005) and adjust the retained levels of urea and associated
TMAO to scale with the low salinity conditions (Goldstein et al.,
1968; Pillans et al., 2005). Equally, this would result in the observed
lesser effect of lipid extraction on $\delta^{15}N$ values between BULK and LE
tissue because there would be less urea and TMAO in their tissues.
For Arctic skate and Greenland sharks, which showed a large change
in $\delta^{15}N$ values following lipid extraction, anecdotal evidence indicates
high TMAO and urea concentrations in muscle tissue of these species
(MacNeil et al., 2012). Elevated urea concentrations in hibernating
ecothemeric amphibians in cold climates, has been suggested as a
mechanism to counteract freezing episodes by acting as a cryoprotective
agent (Costanzo and Lee, 2008). The requirement of urea for osmoregulation plus potential cryoprotective properties may explain
reported high urea concentrations in these polar species and associat-
ed changes in $\delta^{15}N$ values following lipid extraction.

Further potential evidence for the removal of nitrogenous waste
(urea and TMAO) from elasmobranch muscle tissue pertains to the

Fig. 3. Difference in $\delta^{15}N$ and $\delta^{13}C$ between lipid extracted (LE) and non-lipid extracted
(BULK) muscle tissue with increasing size of animal. Note: grey dots = $\delta^{15}N$ and black
dots = $\delta^{13}C$.}

Reum (2011). More specifically, elasmobranch muscle tissue with lipid
content and urea may be complex to lipid correct, due to urea removal
and the associated effect on total $^N$N and consequently the C:N ratio
(see below). It is important to note that the method of solvent removal

Fig. 4. Mean ($\pm$SE) difference in $\delta^{15}N$ between lipid extracted (LE) and non-lipid
extracted (BULK) muscle tissue for all species as a function of environment/sampling re-
gime. Note: black = marine habitat with field sampling, white = estuarine habitat with
field sampling and light grey = marine habitat with laboratory sampling. An additional
category (dark grey) for sharks inhabiting both marine/estuarine environments and sam-
pled in the field is shown. Bull sharks and Java sharks are divided into small and large
sharks as there are known size-specific habitat preferences (Heupel et al., 2010; Knip et
al., 2011; Kinney unpublished data).

|$\delta^{15}C$| $F_{1,16} = 0.42, p = 0.524 / r^2 = 0.03$

|$\delta^{15}N$| $F_{1,16} = 0.00, p = 0.965 / r^2 = 0.00$

|$\delta^{15}C$| $F_{1,19} = 0.92, p = 0.182 / r^2 = 0.09$

|$\delta^{15}N$| $F_{1,19} = 0.92, p = 0.182 / r^2 = 0.09$

|$\delta^{15}C$| $F_{1,19} = 0.00, p = 0.524 / r^2 = 0.03$

|$\delta^{15}N$| $F_{1,19} = 0.00, p = 0.524 / r^2 = 0.03$

|$\delta^{15}C$| $F_{1,19} = 0.00, p = 0.524 / r^2 = 0.03$

|$\delta^{15}N$| $F_{1,19} = 0.00, p = 0.524 / r^2 = 0.03$
relative changes in the total carbon and nitrogen ratio (C:N) between BULK and LE tissue. The C:N ratio of pure protein in muscle tissue is ~3.0%. (DeNiro, 1987; Kilijunen et al., 2006), at which point lipid extraction is purportedly not required (Post et al., 2007). As a result, lipid extraction of elasmobranch tissue in certain cases has not been performed (Matich et al., 2010; Vaudo et al., 2010). The mean C:N ratios for elasmobranch BULK muscle tissue in this study were typically<3 (mean±SD: 2.8±0.2, n=186). The trend of an increase in the C:N ratio to ≥3 (mean±SD: 3.2±0.1) following lipid extraction for all species examined except the Greenland and whale shark, driven by the removal of 3N (i.e. nitrogenous waste), provides further confidence that isotopically light waste products are being removed and that extraction is required to elicit accurate and standardised δ15N data. This trend in the C:N ratio was in agreement with data previously presented for leopard shark (Triakis semifasciata) and spiny dogfish muscle tissue following lipid extraction (Kim and Koch, 2011; Logan and Lutcavage, 2010).

In contrast to our findings, Logan and Lutcavage (2010) reported that although urea removal resulted in a slight increase in δ15N values, the observed increase was not statistically significant. The authors concluded that urea did not adversely affect δ15N values of the coastal skate and the spiny dogfish. Similar to Logan and Lutcavage (2010), the marine field sampled smoothhound shark in this study showed no difference in δ15N values following lipid extraction, which may indicate a species-specific effect. Logan and Lutcavage (2010) stated that the minor increase in δ15N values following urea extraction via water rinses could possibly be attributed to the following: (i) urea is not isotopically ‘light’, (ii) the δ15N value of TMAO and osmolyte B-amino acids balances out the ‘lighter’ δ15N value of urea, or (iii) that urea concentrations are low in muscle tissue resulting in minimal effect on overall δ15N values. Generally, urea concentrations in elasmobranch muscle tissue are low (Ballantyne, 1997; Withers et al., 1994) and therefore a very large increase in δ15N values following urea removal would not be expected unless urea had an extremely low δ15N value.

Following both lipid extraction using petroleum ether and combined lipid extraction and water rinses of muscle tissue of large sharks, Kim and Koch (2011) reported results in agreement with our data. As predicted, δ15N values increased significantly following both extraction methods indicating removal of 14N. The authors also reported the presence of urea in the water rinses and noted there was relatively little change in amino acid composition following treatments. Given that the increase in δ15N values was greater following combined lipid extraction and water rinsing, Kim and Koch (2011) recommended that both extraction techniques are required for effective urea removal. Dale et al. (2011) examined the stable isotopic composition of individual amino acids in the brown stingray (Dasyatis lata) and found that trophic enrichment factors were much lower than those reported for non-ureosmotic species. These results are similar to Hussey et al. (2010a) for stable isotope diet-tissue discrimination factors of large sharks, based on a semi-controlled feeding study. The results of these studies and the current data indicate that urea retention in elasmobranchs affects individual elasmobranch δ15N values. This effect likely stems from both soluble urea within the muscle tissue (which can be extracted) (Hussey et al., 2010a, 2012; Kim and Koch, 2011) and because muscle tissue may be depleted in 15N as a result of lower rates of deamination of glutamic acid resulting from its use in urea formation (Dale et al., 2011).

In contrast to elasmobranchs, most teleost fish are ammonotelic and do not retain urea for osmoregulation, but instead excrete nitrogen waste across their gills as ammonia (Wood, 1993). The reported increase in δ15N values in teleost fish following lipid extraction is thought to reflect the removal of; (i) ammonia/ammonium 15N depleted waste (Murry et al., 2006), and/or (ii) free amino acids associated with polar lipids (Pinnegar and Polunin, 1999). Although several lines of evidence indicate an effect of soluble urea in elasmobranch muscle tissue, we can not rule out, however, that the increase in δ15N values or a fraction of the increase following LE was a result of the removal of proteins associated with polar structure lipids (Pinnegar and Polunin, 1999; Sweeting et al., 2006) although Kim and Koch (2011) found no evidence to support this.

The variable intra- and inter-species effects of lipid extraction on δ15N values could reflect rescaled urea/TMAO levels in response to variable salinity levels experienced by these wide ranging predators related to depth, temperature, proximity to coastal areas, feeding regime and species-specific physiology. Further experimental work is required to quantify the δ15N, δ13C, Total 3C and Total 3N values of soluble urea, TMAO, ammonia, ammonium and free amino acids and their relative effect on stable isotope values in elasmobranch and teleost fish tissues. In addition, most tissue samples are stored on ice for periods of time prior to stable isotope analysis. Within the food processing industry, storage of elasmobranch muscle tissue on ice is a widely adopted technique for removing soluble urea to prepare flesh for human consumption (Mathew and Shamasundar, 2002). It is therefore possible that urea is removed during the freeze storage period and that variable periods of freeze storage could account for some of the observed variation between individuals and among species.

Assuming a δ15N diet tissue discrimination factor of 2.3 ± 0.2‰ for muscle tissue of large sharks (Hussey et al., 2010a,b), a mean δ15N

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Fig. 5. Species-specific boxplots representing differences in median (A) δ15N and (B) δ13C values between lipid extracted (LE) and non-lipid extracted (BULK) muscle tissue by geographic region: dark grey = USA, grey = Australia and white = South Africa.
increase in muscle tissue of 1.1‰ following removal of potential nitrogenous waste, would correspond to approximately a 0.5 trophic level shift. This has serious implications for interpreting the trophic position of elasmobranchs and their relative position in a given food web using stable isotopes. In agreement with Murry et al. (2006), if all data were either non-lipid extracted or lipid extracted, there may only be minor alteration to overall species trophic structure if only elasmobranchs are considered. It is important to note, however, that the observed large δ15N effect on elasmobranch muscle tissue as a result of urea retention when compared to teleost fish would alter the scaling of the trophic position estimates within a given food web if lipid correction were not undertaken. If studies utilise external data to set isotopic baselines or for comparative purposes and/or lipid extract only certain components of the food web (i.e. lipid rich species), interpreting δ15N trends of elasmobranchs will be complex and unreliable. Stable isotope analysis of individual amino acids of elasmobranch muscle tissue by Dale et al. (2011) also raises important questions over potential urea effects on δ15N values which cannot be accounted for through lipid extraction or water rinsing methods.

In conclusion, for most elasmobranch species examined there were minimal shifts in δ13C values following lipid extraction, but intra-species variation and statistically significant shifts for certain species indicate that preliminary lipid extraction trials should be undertaken prior to stable isotope analysis of all samples. Certainly for species in this study such as the Greenland and whale shark and previous data reported for the spiny dogfish, lipid extraction is necessary to standardise data among individuals and for cross species comparisons. When considering that δ15N values increased following lipid extraction, total percent nitrogen decreased and C:N ratios re-balanced to those expected for pure protein, the data strongly suggests that soluble urea in elasmobranch tissue can significantly alter δ15N values. Similar to δ13C, the δ15N effect was variable among species, likely as a result of different urea concentrations dependent on the environment the animal inhabits, sampling approach (field or laboratory) and physiology. Given lower diet-tissue discrimination factors for large sharks relative to teleost fish and the effect of urea content on δ15N values, lipid extraction will minimise urea effects consequently improving the interpretation of elasmobranch stable isotope data. Given the reported efficiency of water rinses on removing urea from elasmobranch muscle tissue (Kim and Koch, 2011), but the potential for water to remove low molecular weight proteins (Mathew et al., 2002), future work should directly quantify urea content of elasmobranch muscle tissue and measure urea concentrations in extracts following both lipid extraction and water rinsing techniques in addition to comparative stable isotope analysis.

Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jembe.2012.07.012.

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