Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

(This is a sample cover image for this issue. The actual cover is not yet available at this time.)

This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Journal of Experimental Marine Biology and Ecology 434–435 (2012) 7–15

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

# 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

ARTICLE INFO

*Article history:*
Received 3 October 2011
Received in revised form 6 July 2012
Accepted 17 July 2012
Available online xxxx

*Keywords:*
Intra- and inter-species variability
Lipids
Sharks
Skate
Stable isotopes
Urea

ABSTRACT

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 (±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 (±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.

Crown Copyright © 2012 Published by Elsevier B.V. All rights reserved.

## 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

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

0022-0981/$ – see front matter. Crown Copyright © 2012 Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jembe.2012.07.012

Author's personal copy



et al., 2004; Sweeting et al., 2006), questions remain about the influence of lipid extraction on $\delta^{13}C$ and $\delta^{15}N$ values, particularly in elasmobranchs. As well, less attention has been given to the influence of lipid extraction on total percent nitrogen and carbon (%N and %C, 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 $^{13}C$ 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 $\delta^{15}N$ 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 (~1.59‰)]. Investigators have mixed opinions in terms of these observed $\delta^{15}N$ effects. Ingram et al. (2007) reported that LE had minimal effect on $\delta^{15}N$ 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 $\delta^{15}N$ values following LE would complicate comparative studies of $\delta^{15}N$ across food webs. Accepting that diet tissue discrimination factors ($\Delta^{15}N = \delta^{15}N_{consumer} - \delta^{15}N_{prey}$ and $\Delta^{13}C = \delta^{13}C_{consumer} - \delta^{13}C_{prey}$) can be variable among species, but in general are predicted to decrease with increasing trophic step (or with increasing prey $\delta^{15}N$ values) (Caut et al., 2009; Dennis et al., 2010; Hussey et al. 2010a), the effects of LE on $\delta^{15}N$ 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 $\delta^{13}C$ 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 that 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 $\delta^{15}N$ 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 expected to be depleted in $^{15}N$ which would result in an artificially low $\delta^{15}N$ 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 $\delta^{15}N$ values and a concomitant decrease in %N 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 $\delta^{15}N$ values among individuals and across species with variable urea tissue concentrations. Two studies to date have addressed the issue of urea effects on $\delta^{15}N$ values in elasmobranches with conflicting results. Logan and Lutcavage (2010) reported that urea content did not have an adverse effect on $\delta^{15}N$ values of coastal skates (*Leucoraja* spp.) and spiny dogfish (*Squalus acanthias*) 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 $\delta^{15}N$ 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), rescales 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 $\delta^{15}N$ 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 ($\delta^{15}N$ and $\delta^{13}C$) 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 $\delta^{13}C$ values considering assumed low lipid content (Bone and Roberts, 1969; Hussey et al., 2010a) and (ii) species-specific effects of lipid extraction on $\delta^{15}N$, %N 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 $\delta^{13}C$ and $\delta^{15}N$ values were tested on a total of 21 species of elasmobranch (20 sharks and 1 skate) from 8 families (Carcharhinidae, Lamnidae, Sphyrnidae, Somniosidae, Odontaspididae, Triakidae, Rhincodontidae and Rajiidae, 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 from 0.5–2.0 h. Bonnethead (*Sphyrna 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 euthanized 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 hr 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 long

Author's personal copy



**Table 1**
Muscle samples used to examine the effect of standard chloroform/methanol lipid extraction on $\delta^{15}N$ and $\delta^{13}C$ values in elasmobranchs.

| Species | Common name | Species code | Collection location | Sample number | Sampling (field/lab) | Habitat (marine/estuarine/freshwater) |
|---|---|---|---|---|---|---|
| Sharks | | | | | | |
| *Sphyrna tiburo* | Bonnethead | BNT | USA | 5 | F | E |
| *Carcharhinus sorrah* | Spotail | SPOT | Australia | 8 | F | M |
| *Mustelus mosis* | Smooth hound | SMH | South Africa | 5 | L | M |
| *Rhizoprionodon terraenovae* | Atlantic sharpnose | ATSHP | USA | 5 | F | M–E |
| *Sphyrna zygaena* | Smooth hammerhead | SMO | South Africa | 8 | L | M |
| *Carcharias taurus* | Raggie/sandtiger[a] | RAG | South Africa | 6 | L | M |
| *Carcharhinus limbatus*[b] | Blacktip | BLA | Australia/USA/South Africa | 6/4/7 | F/F/L | M–E/M |
| *Carcharhinus macloti* | Hardnose | HARD | Australia | 9 | F | M |
| *Carcharhinus brevipinna* | Spinner | SPN | South Africa | 8 | L | M |
| *Carcharhinus obscurus* | Dusky | DUS | South Africa | 9 | L | M |
| *Carcharhinus leucas* | Bull | BUL | USA/South Africa | 4/9 | F/L | E(F)/M |
| *Carcharodon carcharias* | Great white | GRE | South Africa | 18 | L | M |
| *Galeocerdo cuvier* | Tiger | TIG | South Africa | 9 | L | M |
| *Rhizoprionodon taylori* | Australian sharpnose | ASHP | Australia | 9 | F | M–E |
| *Sphyrna lewini* | Scalloped hammerhead | SCA | Australia/South Africa | 3/21 | F/L | M |
| *Carcharhinus fitzroyensis* | Creek whaler | CREK | Australia | 8 | F | M |
| *Rhizoprionodon acutus* | Sharpnose/milk | SHP | Australia | 9 | F | M–E |
| *Carcharhinus amboinensis* | Java/pigeye | JAV | Australia/South Africa | 8/5 | F/L | E/M |
| *Rhincodon typus* | Whale | WH | South Africa | 2 | L | M |
| *Somniosus microcephalus* | Greenland | GRN | Canadian Arctic | 10 | F | M |
| Skates | | | | | | |
| *Amblyraja hyperborea* | Arctic skate | SKA | Canadian Arctic | 8 | F | M |

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

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 10–15 m depth. For specific details on net installations and changes in net installations over time please refer to Dudley et al. (2005). Nets were serviced on a daily basis and shark mortalities in good condition were retrieved and transported to either the main KwaZulu-Natal Sharks Board (KZNSB) laboratory in Durban or a coastal substation. An additional species, whale sharks (*Rhincodon typus*) were sampled from beach stranded animals in KwaZulu-Natal. At the KZNSB laboratory, each shark was measured (PCL, FL and TL – cm), sexed and during dissection, 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).

*2.2. Stable isotope analysis*

Muscle tissue samples from all four geographical regions were processed in the same laboratory (Chemical Tracers Laboratory - Great Lakes Institute for Environmental Research, University of Windsor, Canada). Muscle tissue was freeze dried for 48 h (Thermo Savant ModulyoD-1, Thermo Scientific) and homogenised using either a SPEX CertiPrep 8000-D ball milling unit (SPEX CertiPrep; Metuchen, NJ, USA) or by hand using a mortar and pestle. Lipid extraction was undertaken by agitating the dried powdered muscle tissue in a 2:1 chloroform–methanol solution for 24 h following the Bligh and Dyer (1959) method. The tissue and solvent were then either (i) filtered and the resulting residue/filter paper dried at 60 °C for 48 h to evaporate the remaining solvent (USA, South Africa and Canadian Arctic) or (ii) 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, Finnigan MAT Delta$^{plus}$, Thermo Finnigan, 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:

$$\delta X = \left[\left(R_{sample}/R_{standard}\right) - 1\right] \times 1000 \quad (1)$$

where X is $^{15}N$ or $^{13}C$ and R is the ratio $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The standard reference material was Pee Dee Belemnite carbonate for $CO_2$ and atmospheric nitrogen for $N_2$. The analytical precision for $\delta^{15}N$ data was 0.24 and 0.14‰ and for $\delta^{13}C$ 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 $\delta^{15}N$ data and 0.05‰ for $\delta^{13}C$ data based on a single run of NIST standard sucrose ($n = 13$) and ammonium sulphate ($n = 13$).

*2.3. Statistical analysis*

The difference in $\delta^{13}C$, total %C, $\delta^{15}N$, total %N 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

Author's personal copy



sufficient data were available ($n \geq 3$; Table 1). To examine the effect of sex on the difference between LE and BULK tissue $\delta^{15}N$ and $\delta^{13}C$ values on a species by 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 $\delta^{13}C$ and $\delta^{15}N$ 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 $\delta^{15}N$ values. To do this, species LE and BULK $\delta^{15}N$ 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: $MARINE_{FIELD}$, $MARINE_{LABORATORY}$ and $ESTUARINE_{FIELD}$, where MARINE and ESTUARINE are the environment where the animal was sampled[1] 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 we performed either Mann–Whitney (where samples were from 2 locations) or Kruskal–Wallis (for 3 locations) tests on $\delta^{15}N$ and $\delta^{13}C$ values for all species sampled across more than one of our study regions. This resulted in the testing of the difference in $\delta^{15}N$ and $\delta^{13}C$ data for scalloped hammerhead, sharpnose, blacktip, bull and java (*Carcharhinus amboinensis*) 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 $\delta^{13}C$, %C, $\delta^{15}N$, %N 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 $\delta^{13}C$ 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; Supl. A). The whale shark also showed a large increase in $\delta^{13}C$ values (3.3‰) between LE and BULK tissue, accepting a sample size of 2. Java and sharpnose (*Rhizoprionodon acutus*) sharks had the largest $\delta^{13}C$ 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; Supl. A). A plot of the difference in $\delta^{13}C$ 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 $\delta^{15}N$ data, 13 of the 20 elasmobranch species tested showed a significant increase in $^{15}N$ following LE (Fig. 1C; Supl. 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 $\delta^{15}N$ SD range of the difference between LE and BULK tissue (0.2‰–0.6‰) was less than that recorded for $\delta^{13}C$ SD range. Total %N declined for 9 species but an increase was recorded for the whale and Greenland shark (Fig. 1D; Supl. A). The C:N ratio increased for 17 species (Fig. 1E; Supl. A).

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

There was no statistical effect of sex on the difference between LE and BULK tissue $\delta^{13}C$ and $\delta^{15}N$ values accepting small sample sizes in certain cases (Supl. B). In addition, there was no significant effect of size on the difference between LE and BULK tissue $\delta^{13}C$ and $\delta^{15}N$ 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, $MARINE_{FIELD}$ sampled sharks showed the largest change in $\delta^{15}N$ values between LE and BULK tissue, followed by $MARINE_{LABORATORY}$ sharks and then $ESTUARINE_{FIELD}$ sampled sharks (Fig. 4; GLM: $F_{2,197}=49.62$, $p<0.0001$; $r^2=34\%$). The one exception to this pattern was the field sampled smooth hound shark which exhibited a minimal $^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ values between LE and BULK tissue compared to a positive shift in $\delta^{15}N$ 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 $\delta^{15}N$ between LE and BULK tissue which was intermediate to $MARINE_{FIELD}$ and $ESTUARINE_{FIELD}$ (Fig. 4).

For $\delta^{15}N$, there was no effect of geographic sampling location (USA, Australia and South Africa) on the difference between BULK and LE values with the exception of bull sharks (Fig. 5a; Supl. C). Small bull sharks sampled from the USA showed less of an increase in $\delta^{15}N$ 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: Supl. C). Java sharks sampled in Australia also showed a trend of a smaller increase in $\delta^{15}N$ than animals sampled from South Africa, but the data were more variable and non significant (Fig. 5a; Supl. C). For $\delta^{13}C$, there was no effect of geographic sampling location on species-specific shifts in $\delta^{13}C$ 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; Supl. C).

## 4. Discussion

Understanding the effects of lipid extraction on tissue-specific stable isotope values is of central importance for accurately interpreting $\delta^{13}C$ and $\delta^{15}N$ data in ecological studies. Given that lipids are generally depleted in $^{13}C$ (DeNiro and Epstein, 1977; McConnaughey and McRoy, 1979), it was not unexpected that while lipid extraction increased the $\delta^{13}C$ 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 $\delta^{13}C$ values was, however, species-specific.

Although the mean increase in $\delta^{13}C$ 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

Author's personal copy



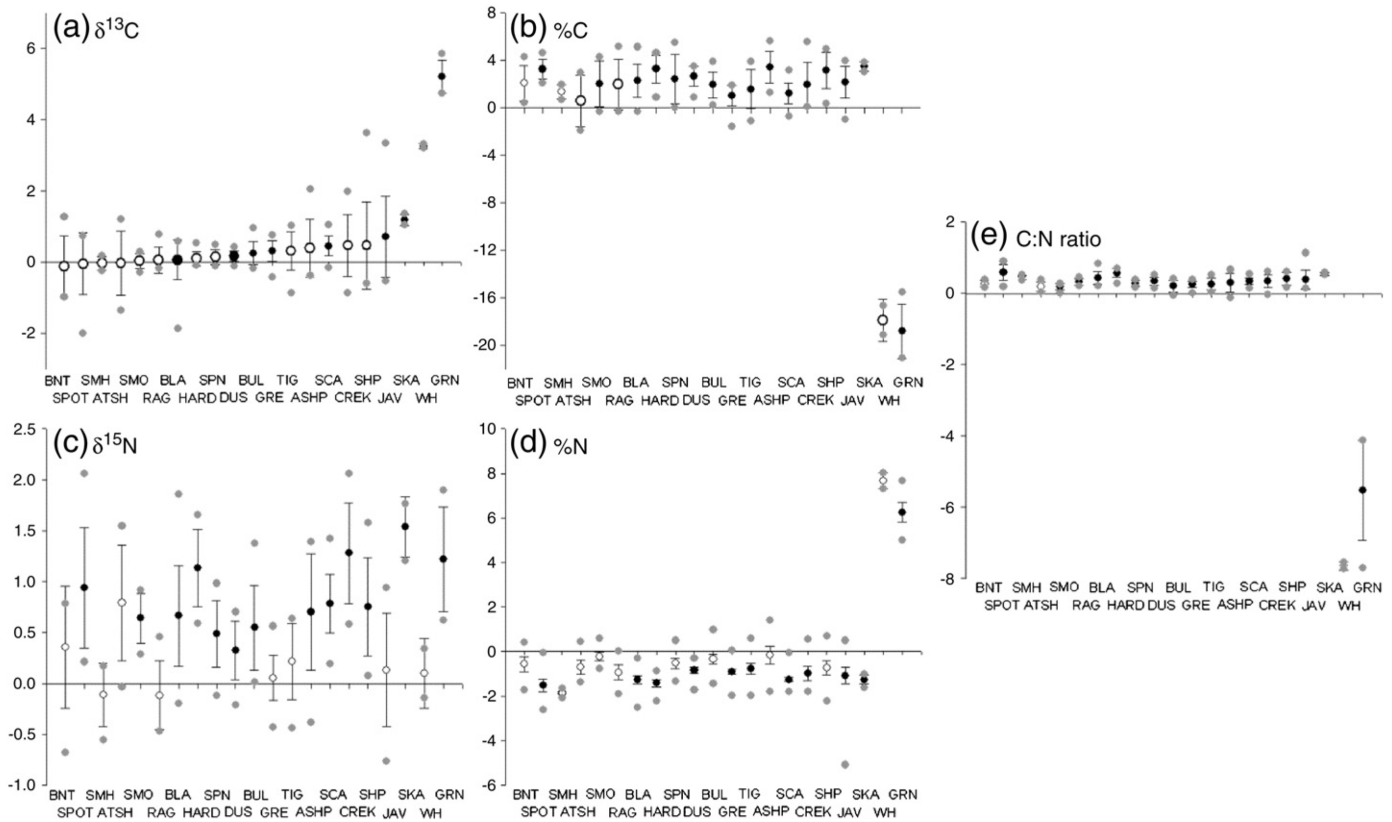


**Fig. 1.** Difference in (a) $\delta^{13}$C, (b) total %C, (c) $\delta^{15}$N, (d) total %N and (e) C:N ratios between lipid extracted (LE) and non-lipid extracted (BULK) elasmobranch muscle tissue for each species analysed. Solid grey circles are minimum and maximum values for each species. Solid black circles and open black circles are mean values (±SD) with significant and non significant paired t-tests, respectively (see Supplementary materials A). For species codes and sample sizes see Table 1. Note y-axis scales are different for each parameter.

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±SD; 0.5±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

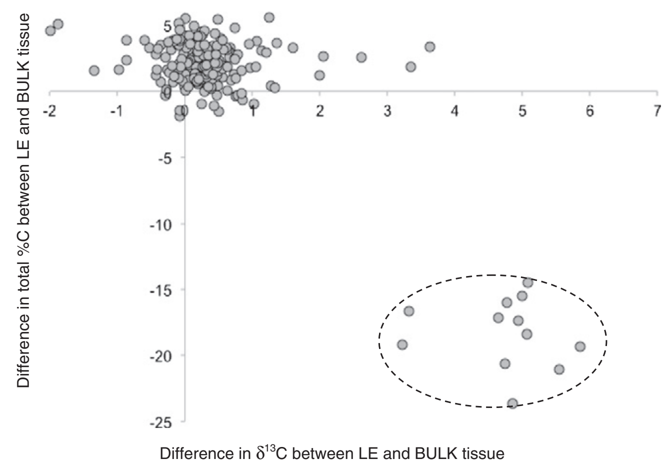


**Fig. 2.** The relationship between the difference in lipid extracted and non-lipid extracted (LE–BULK) $\delta^{13}$C and total %C values for 21 elasmobranch species. Circled data are those for the Greenland and whale shark.

Author's personal copy



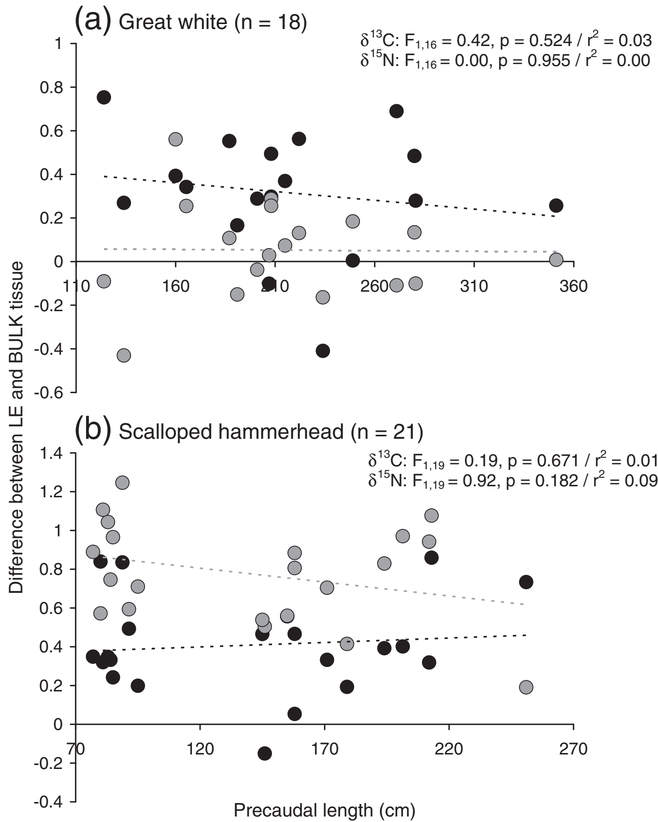


**Fig. 3.** Difference in $\delta^{15}N$ and $\delta^{13}C$ between lipid extracted (LE) and non-lipid extracted (BULK) muscle tissue with increasing size of animal. Note: grey dots = $\delta^{15}N$ and black dots = $\delta^{13}C$.

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 and consequently the C:N ratio (see below). It is important to note that the method of solvent removal following LE, filtering (South Africa, USA and Canadian Arctic) vs. centrifuge (Australia), may have affected the magnitude of change in $\delta^{13}C$ 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 filtering can differentially alter $\delta^{13}C$ values. Nevertheless, the fact that the difference in $\delta^{13}C$ values following LE varied across and within elasmobranch species enforces the requirement to determine tissue lipid content and/or test the effect of lipid extraction on $\delta^{13}C$ values of muscle tissue on a species by species basis.

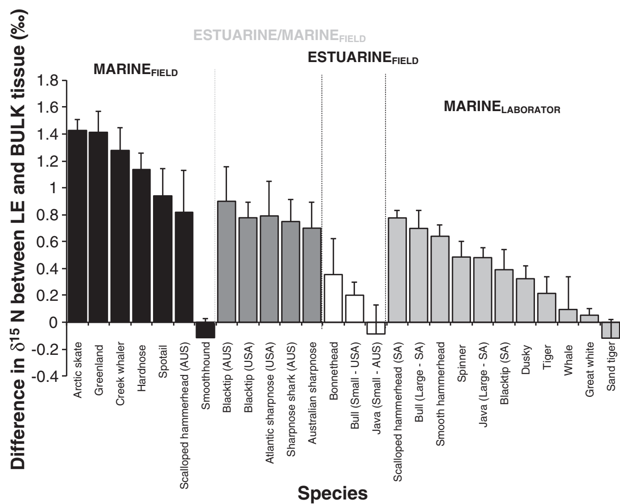


**Fig. 4.** Mean (±SE) difference in $\delta^{15}N$ between lipid extracted (LE) and non-lipid extracted (BULK) muscle tissue for all species as a function of environment/sampling regime. Note: black — marine habitat with field sampling, white — estuarine habitat with field sampling and light grey — marine habitat with laboratory sampling. An additional category (dark grey) for sharks inhabiting both marine/estuarine environments and sampled 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).

The effect of the lipid extraction process on $\delta^{15}N$ values of elasmobranchs was more variable than $\delta^{13}C$, both in terms of intra- and inter-species differences. In most instances, $\delta^{15}N$ values increased following lipid extraction with a mean (±SD) increase of; (i) 0.6±0.6‰ across all species, (ii) 1.0±0.5‰ across field sampled marine species (excluding the smoothhound), (iii) 0.1±0.6‰ across field sampled estuarine species, and (iv) 0.4±0.4‰ across laboratory sampled marine species. Our reported mean $\delta^{15}N$ increase for field sampled estuarine 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 increases 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‰, respectively) and Mintenbeck et al. (2008) (1.7‰)].

The large directional change in $\delta^{15}N$ values of field sampled marine elasmobranchs, in conjunction with the lesser effect of lipid extraction on $\delta^{15}N$ values of laboratory sampled sharks and juvenile bull sharks which inhabit freshwater/brackish systems, would suggest that nitrogenous waste products including ammonia, ammonium and urea are being removed during LE. The non-protein nitrogen fraction of shark muscle tissue comprises free amino acids, urea and trimethylamine oxide (TMAO). Following death, urea within muscle tissue is rapidly hydrolised 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 breakdown 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 observed lesser effect of lipid extraction on $\delta^{15}N$ values from the laboratory sampled marine elasmobranches 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 ectothermic 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 associated 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

Author's personal copy



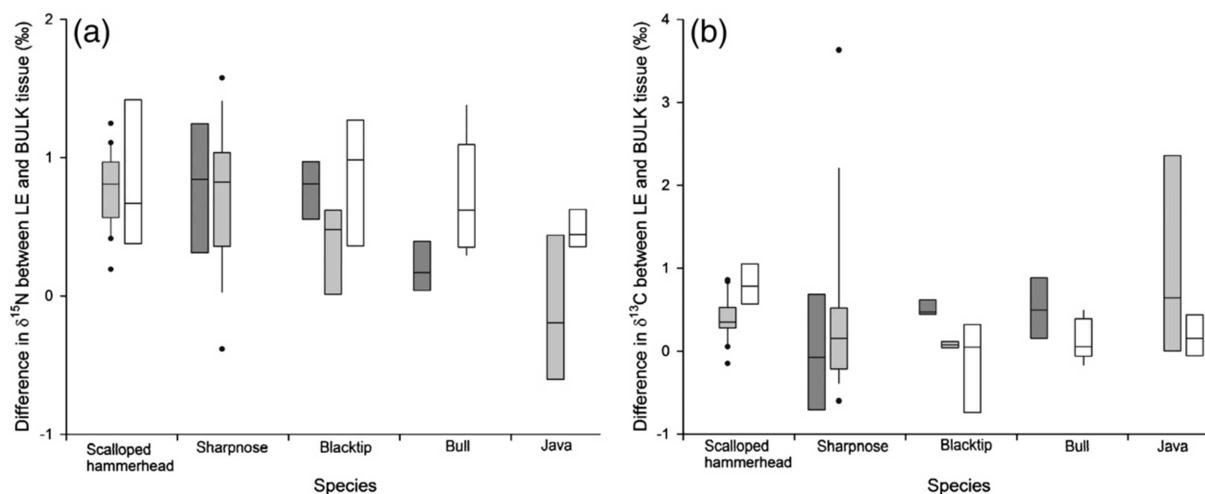


**Fig. 5.** Species-specific boxplots representing differences in median (A) $\delta^{15}$N and (B) $\delta^{13}$C values between lipid extracted (LE) and non-lipid extracted (BULK) muscle tissue by geographic region: dark grey — USA, grey — Australia and white — South Africa.

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 $\geq 3$ (mean ± SD; 3.2 ± 0.1) following lipid extraction for all species examined except the Greenland and whale shark, driven by the removal of %N (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 $\delta^{15}$N and %N data. This trend in the C:N ratio was in agreement with data previously presented for leopard shark (*Triakis semifascita*) 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 $\delta^{15}$N values, the observed increase was not statistically significant. The authors concluded that urea did not adversely affect $\delta^{15}$N 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 $\delta^{15}$N values following lipid extraction, which may indicate a species-specific effect. Logan and Lutcavage (2010) stated that the minor increase in $\delta^{15}$N values following urea extraction via water rinses could possibly be attributed to the following; (i) that urea is not isotopically 'light', (ii) the $\delta^{15}$N value of TMAO and osmolyte B-amino acids balances out the 'lighter' $\delta^{15}$N value of urea, or (iii) that urea concentrations are low in muscle tissue resulting in minimal effect on overall $\delta^{15}$N values. Generally, urea concentrations in elasmobranch muscle tissue are low (Ballantyne, 1997; Withers et al., 1994) and therefore a very large increase in $\delta^{15}$N values following urea removal would not be expected unless urea had an extremely low $\delta^{15}$N value.

Following both lipid extraction using petroleum ether and combined lipid extraction and water rinses of muscle tissue of leopard sharks, Kim and Koch (2011) reported results in agreement with our data. As predicted, $\delta^{15}$N values increased significantly following both extraction methods indicating removal of $^{14}$N. 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 $\delta^{15}$N 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 $\delta^{15}$N 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 $^{15}$N 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 $\delta^{15}$N values in teleost fish following lipid extraction is thought to reflect the removal of; (i) ammonia/ammonium $^{15}$N 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 $\delta^{15}$N 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 $\delta^{15}$N 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 $\delta^{15}$N, $\delta^{13}$C, Total %C and Total %N 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 $\delta^{15}$N diet tissue discrimination factor of 2.3 ± 0.2‰ for muscle tissue of large sharks (Hussey et al., 2010a,b), a mean $\delta^{15}$N

Author's personal copy



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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{15}N$ 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 $\delta^{13}C$ 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 $\delta^{15}N$ values increased following lipid extraction, total percent nitrogen decreased and C:N ratios rebalanced to those expected for pure protein, the data strongly suggests that soluble urea in elasmobranch tissue can significantly alter $\delta^{15}N$ values. Similar to $\delta^{13}C$, the $\delta^{15}N$ 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 $\delta^{15}N$ 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.

## References[RH]


Arrington, D.A., Winemiller, K.O., 2002. Preservation effects on stable isotope analysis of fish muscle T. Am. Fish. Soc. 131, 337–342.

Ballantyne, J.S., 1997. Jaws: the inside story. The metabolism of elasmobranch fishes. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 118, 703–742.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Physiol. Pharmacol. 37, 911–917.

Bone, Q., Roberts, B.L., 1969. The density of elasmobranchs. J. Mar. Biol. Assoc. UK 49, 913–937.

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

Christie, W.W., 1993. Preparation of lipid extracts from tissues. In: Christie, W.W. (Ed.), Advances in Lipid Methodology — Two. Oily Press, Dundee, pp. 195–213.

Costanzo, J.P., Lee, R.E., 2008. Urea loading enhances freezing survival and postfreeze recovery in a terrestrially hibernating frog. J. Exp. Biol. 211, 2969–2975.

Dale, J.J., Wallsgrove, N., Popp, B.N., Holland, K.N., 2011. Nursery habitat and foraging ecology of the brown stingray *Dasyatis lata* determined from stomach contents, bulk and amino acid stable isotopes. Mar. Ecol. Prog. Ser. 433, 221–236.

Davidson, B., Zayed, M., Zayed, S., Cliff, G., 2011. Liver lipids of Indian and Atlantic Ocean spinner *Carcharhinus brevipinna* and blacktip *Carcharhinus limbatus* sharks. Afr. J. Mar. Sci. 33, 115–118.

DeNiro, M.J., 1987. Stable isotopy and archaeology. Am. Sci. 75, 182–191.

DeNiro, M.J., Epstein, S., 1977. A mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197, 261–263.

DeNiro, M.J., Epstein, S., 1981. Influence of diet on the distribution of nitrogen isotopes in animals. Geochim. Cosmochim. Acta 45, 341–351.

Dennis, C.A., MacNeil, M.A., Rosati, J.Y., Pitcher, T.E., Fisk, A.T., 2010. Diet-tissue discrimination factors are inversely related to $\delta^{15}N$ and $\delta^{13}C$ values of food for fish under controlled conditions. Rapid Commun. Mass Spectrom. 24, 3515–3520.

Devadoss, P., 1984. Nutritive values of sharks, skates and rays from Portonovo coast. Indian J. Fish. 31, 156–161.

Dudley, S.F.J., Cliff, G., Zungu, M.P., Smale, M.J., 2005. Sharks caught in protective gill nets off KwaZulu-Natal, South Africa. 10. The dusky shark, *Carcharhinus obscurus*, (Lesueur 1818). Afr. J. Mar. Sci. 27, 107–127.

Eastman, J.T., DeVries, A.L., 1982. Buoyancy studies of notothenioid fishes in McMurdo Sound, Antarctica. Copeia 1982, 385–393.

Fagan, K.A., Koops, M.A., Arts, M.T., Power, M., 2011. Assessing the utility of C:N ratios for predicting lipid content in fishes. Can. J. Fish. Aquat. Sci. 68, 374–385.

Fisk, A.T., Tittlemier, S.A., Pranschke, J.L., Norstrom, R.J., 2002. Using anthropogenic contaminants and stable isotopes to assess the feeding ecology of Greenland sharks. Ecology 83, 2162–2172.

Freidrich, C., Hagen, W., 1994. Lipid contents of five species of notothenioid fish from high-Antarctic waters and ecological implications. Polar Biol. 14, 359–369.

Goldstein, L., Walter Oppelt, W., Maren, T.H., 1968. Osmotic regulation and urea metabolism in the lemon shark, *Negaprion brevirostris*. Am. J. Physiol. 215, 1493–1497.

Gordievskaya, V.S., 1973. Shark flesh in the fish industry. Israel Program for Scientific Transl. IPST Cat. No. 60080 2.

Heupel, M.R., Yeiser, B.G., Collins, A.B., Ortega, L., Simpfendorfer, C.A., 2010. Long term presence and movement patterns of juvenile bull sharks, *Carcharhinus leucas*, in an estuarine river system. Mar. Freshwater Res. 61, 1–10.

Hobson, K.A., 1999. Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia 120, 314–326.

Hussey, N.E., Brush, J., McCarthy, I.D., Fisk, A.T., 2010a. $\delta^{15}N$ and $\delta^{13}C$ diet discrimination factors for large sharks under semi-controlled conditions. Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 155, 445–453.

Hussey, N.E., MacNeil, M.A., Fisk, A.T., 2010b. The requirement for accurate diet-tissue discrimination factors for interpreting stable isotopes in sharks. Hydrobiologia 654, 1–5.

Hussey, N.E., MacNeil, M.A., Olin, J.A., McMeans, B.C., Kinney, M., Chapman, D.D., Fisk, A.T., 2012. Stable isotopes and elasmobranchs: tissue types, methods, applications and assumptions. J. Fish Biol. 80, 1449–1484.

Ingram, T., Mathews, B., Harrod, C., Stephens, T., Grey, J., Markel, R., 2007. Lipid extraction has little effect on the $\delta^{15}N$ of aquatic consumers. Limnol. Oceanogr.-Meth. 5, 338–343.

Jackson, A.L., Inger, R., Parnell, A.C., Bearhop, S., 2011. Comparing isotopic niche widths among and within communities: SIBER — stable isotope Bayesian ellipses in R. J. Anim. Ecol. 80, 595–602.

Kilijunen, M., Grey, J., Sinisalo, T., Harrod, C., Immonen, H., Jones, R.I., 2006. A revised model for lipid normalising $\delta^{13}C$ values from aquatic organisms, with implications for isotope mixing models. J. Appl. Ecol. 43, 1213–1222.

Kim, S.L., Koch, P.L., 2011. Methods to collect, preserve, and prepare elasmobranch tissues for stable isotope analysis. Environ. Biol. Fish. http://dx.doi.org/10.1007/s10641-011-9860-9.

Knip, D.M., Heupel, M.R., Simpfendorfer, C.A., Tobin, A.J., Moloney, J., 2011. Ontogenetic shifts in movement and habitat use of juvenile pigeye sharks *Carcharhinus amboinensis* in a tropical nearshore region. Mar. Ecol. Prog. Ser. 425, 233–246.

Layman, C.A., Quattrochi, J.P., Peyer, C.M., Allgeier, J.E., 2007a. Niche width collapse in a resilient top predator following ecosystem fragmentation. Ecol. Lett. 10, 937–944.

Layman, C.A., Arrington, D.A., Montana, C.G., Post, D.M., 2007b. Can stable isotope ratios provide for community-wide measures of trophic structure? Ecology 88, 42–48.

Lesage, V., Morin, Y., Rioux, E., Pomerleau, C., Ferguson, S.H., Pelletier, E., 2010. Stable isotopes and trace metals as indicators of diet and habitat use in cetaceans: predicting errors related to preservation, storage and normalisation. Mar. Ecol. Prog. Ser. 419, 249–265.

Logan, J.M., Lutcavage, M.E., 2008. A comparison of carbon and nitrogen stable isotope ratios of fish tissues following lipid extraction with non-polar and traditional chloroform/methanol solvent systems. Rapid Commun. Mass Spectrom. 22, 1081–1086.

Logan, J.M., Lutcavage, M.E., 2010. Stable isotope dynamics in elasmobranch fishes. Hydrobiologia 644, 231–244.

Logan, J.M., Jardine, T.D., Miller, T.J., Bunn, S.E., Cunjak, R.A., Lutcavage, M.E., 2008. Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modeling methods. J. Anim. Ecol. 77, 838–846.

MacNeil, M.A., McMeans, B.C., Hussey, N.E., Vecsei, P., Svavarsson, J., Kovacs, K., Lydersen, C., Treble, M., Skomal, G.B., Ramsay, M., Fisk, A.T., 2012. Biology of the Greenland shark *Somniosus microcephalus* Bloch & Schneider 1801. J. Fish Biol. 80, 991–1018.

Mathew, S., Shamasundar, B.A., 2002. Effects of ice storage on the functional proteins from shark (*Scoliodon laticaudus*) meat. Nahrung Food 46, 220–226.

Mathew, S., Karthikeyan, M., Shamasundar, B.A., 2002. Effects of water washing of shark (*Scoliodon laticaudus*) meat on the properties of proteins with special reference to gelation. Nahrung Food 46, 78–82.

Author's personal copy




Matich, P., Heithaus, M.R., Layman, C.A., 2010. Contrasting patterns of individual specialization and trophic coupling in two marine apex predators. J. Anim. Ecol. 80, 294–305.

McConnaughey, T., McRoy, C., 1979. Food-web structure and the fractionation of carbon isotopes in the Bering Sea. Mar. Biol. 53, 257–262.

Minagawa, M., Wada, E., 1984. Stepwise enrichment of 15-N along food chains: further evidence and the relation between 15-N and animal age. Geochim. Cosmochim. Acta 48, 1135–1140.

Mintenbeck, K., Brey, T., Jacob, U., Knust, R., Struck, U., 2008. How to account for the lipid effect on carbon stable-isotope ratio ($\delta^{13}C$): sample treatment effects and model bias. J. Fish Biol. 72, 815–830.

Murry, B.A., Farrell, J.M., Teece, M.A., Smyntek, P.M., 2006. Effect of lipid extraction on the interpretation of fish community trophic relations determined by stable carbon and nitrogen isotopes. Can. J. Fish. Aquat. Sci. 63, 2167–2172.

Newsome, S.D., Tinker, M.T., Monson, D.H., Oftedal, O., Ralls, K., Fogel, M.L., Estes, J.A., 2009a. Using stable isotopes to investigate individual diet specialization in California sea otters (*Enhydralutris nereis*). Ecology 90, 961–974.

Newsome, S.D., Etnier, M.A., Monson, D.H., Fogel, M.L., 2009b. Retrospective characterization of ontogenetic shifts in killer whale diets via $\delta^{13}C$ and $\delta^{15}N$ analysis of teeth. Mar. Ecol. Prog. Ser. 374, 229–242.

Olson, K.R., 1999. Rectal gland and volume homeostasis. In: Hamlett, W.C. (Ed.), Sharks, Skates, and Rays: The Biology of Elasmobranch Fishes. The John Hopkins University Press, Baltimore, Maryland, USA, pp. 329–352.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18, 293–320.

Pillans, R.D., Good, J.P., Anderson, W.G., Hazon, N., Franklin, C.E., 2005. Freshwater to seawater acclimation of juvenile bull sharks (*Carcharhinus leucas*): plasma osmolytes and $Na^{+}/K^{+}$-ATPase activity in gill, rectal gland, kidney and intestine. J. Comp. Physiol. 175, 37–44.

Pinnegar, J.K., Polunin, N.V.C., 1999. Differential fractionation of $\delta^{13}C$ and $\delta^{15}N$ among fish tissues: implications for the study of trophic interactions. Funct. Ecol. 13, 225–231.

Post, D.M., Layman, C.A. Albrey, Arrington, D., Takimoto, G., Quattrochi, J., Montana, C.G., 2007. Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152, 179–189.

Reum, J.C.P., 2011. Lipid correction model of carbon stable isotopes for a cosmopolitan predator, spiny dogfish *Squalus acanthias*. J. Fish Biol. 79, 2060–2066.

Sidell, B.D., Crockett, E.L., Driedzic, W.R., 2005. Antarctic fish tissues preferentially catabolise monoenoic fatty acids. J. Exp. Zool. 271, 73–81.

Simpfendorfer, C.A., Freitas, C.G., Wiley, T.R., Heupel, M.R., 2005. Distribution and habitat partitioning of immature bull sharks (*Carcharhinus leucas*) in a southwest Florida estuary. Estuaries 28, 78–85.

Sotiropoulos, M.A., Tonn, W.M., Wassenaar, L.I., 2004. Effects of lipid extraction on stable carbon and nitrogen isotope analyses of fish tissues: potential consequences for food web studies. Ecol. Freshwater Fish 13, 155–160.

Sweeting, C.J., Polunin, N.V.C., Jennings, S., 2006. Effects of chemical lipid extraction and arithmetic lipid correction on stable isotope ratios of fish tissues. Rapid Commun. Mass Spectrom. 20, 595–601.

Vander Zanden, H.B., Bjorndal, K.A., Reich, K.J., Bolten, A.B., 2010. Individual specialists in a generalist population: results from a long-term stable isotope series. Biol. Lett. http://dx.doi.org/10.1098/rsbl.2010.0124.

Vaudo, J.J., Matich, P., Heithaus, M.R., 2010. Mother–offspring isotope fractionation in two species of placentatrophic sharks. J. Fish Biol. 77, 1724–1727.

Withers, P.C., Morrison, G., Hefter, G.T., Pang, T.S., 1994. Role of urea and methylamines in buoyancy of elasmobranchs. J. Exp. Biol. 188, 175–189.

Wolf, N., Carleton, S.A., Martinez del Rio, C., 2009. Ten years of experimental isotopic ecology. Funct. Ecol. 23, 17–26.

Wood, C.M., 1993. The Physiology of Fishes. CRC Press, Boca Raton, FL, pp. 379–425.