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Urea and lipid extraction treatment effects on $\delta^{15}N$ and $\delta^{13}C$ values in pelagic sharks

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RATIONALE: Stable isotope analysis (SIA) provides a powerful tool to investigate diverse ecological questions for marine species, but standardized values are required for comparative assessments. For elasmobranchs, their unique osmoregulatory strategy involves retention of ^{15}N-depleted urea in body tissues and this may bias $\delta^{15}N$ values. This may be a particular problem for large predatory species, where $\delta^{15}N$ discrimination between predator and consumed prey can be small.
METHODS: We evaluated three treatments (deionized water rinsing [DW], chloroform/methanol [LE] and combined chloroform/methanol and deionized water rinsing [LE+DW]) applied to white muscle tissue of 125 individuals from seven pelagic shark species to (i) assess urea and lipid effects on stable isotope values determined by IRMS and (ii) investigate mathematical normalization of these values.
RESULTS: For all species examined, the $\delta^{15}N$ values and C:N ratios increased significantly following all three treatments, identifying that urea removal is required prior to SIA of pelagic sharks. The more marked change in $\delta^{15}N$ values following DW (1.3 ± 0.4‰) and LE+DW (1.2 ± 0.6‰) than following LE alone (0.7 ± 0.4‰) indicated that water rinsing was more effective at removing urea. The DW and LE+DW treatments lowered the %N values, resulting in an increase in C:N ratios from the unexpected low values of <2.6 in bulk samples to ~3.1 ± 0.1, the expected value of protein. The $\delta^{13}C$ values of all species also increased significantly following LE and LE+DW treatments.
CONCLUSIONS: Given the mean change in $\delta^{15}N$ (1.2 ± 0.6‰) and $\delta^{13}C$ values (0.7 ± 0.4‰) across pelagic shark species, it is recommended that muscle tissue samples be treated with LE+DW to efficiently extract both urea and lipids to standardize isotopic values. Mathematical normalization of urea and lipid-extracted $\delta^{15}N_{LE+DW}$ and $\delta^{13}C_{LE+DW}$ values using the lipid-extracted $\delta^{15}N_{LE}$ and $\delta^{13}C_{LE}$ data were established for all pelagic shark species. Copyright © 2015 John Wiley & Sons, Ltd.

The stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in shark tissues provide a powerful tool to investigate important ecological questions regarding movement,[1,2] foraging strategies,[3,4] trophic position,[5,6] reproduction[7] and multi-species interactions.[8,9] Their application is based on the premise that, as predators consume prey, the carbon and nitrogen stable isotope ratio values of those predators fractionate systematically throughout the food web. Specifically, the change in $\delta^{13}C$ values of the predator are conservative (0–1‰), relative to the prey eaten, allowing identification of basal productivity or foraging locations;[10] while the $\delta^{15}N$ values show a more prominent increase per trophic step (2–5‰), providing a method to quantify the trophic position (TP) of predators and to estimate food chain length.[11–13]

When examining carbon stable isotope ratios, lipids in animals' tissues are a source of measurement uncertainty.[14] Lipids are depleted in ^{13}C relative to protein and carbohydrates;[15] consequently, the higher the tissue lipid content, the more negative the $\delta^{13}C$ value of the organism irrespective of diet or foraging location. Lipid removal or correction is therefore recommended to standardize data among species within a food web.[16] This procedure is widely adopted across a range of aquatic animal groups including teleost fish,[17,18] cephalopods,[19] crustaceans,[20] marine mammals[21] and elasmobranchs.[1,22,23]

Compared with most aquatic species, elasmobranchs adopt a unique osmoregulation mechanism.[24] Elasmobranchs maintain urea ($CO(NH_2)_2$) and trimethylamine oxide (TMAO; C_3H_9NO) in their tissues for osmotic balance. These soluble nitrogenous compounds may artificially lower $\delta^{15}N$ values in shark tissues confounding data interpretation as they are considered to be ^{15}N-depleted.[3,25,26] Furthermore, inter/intra-specific variations in the concentrations of urea and TMAO in body tissues of different species/life-stages that fluctuate depending on ambient salinity can bias comparisons among species.[27–29] This may be a particular problem for large predatory species, where $\delta^{15}N$ discrimination between predator and consumed prey can be small.[30] Similar to lipid extraction, the removal of urea and TMAO from shark muscle is therefore recommended prior to SIA.[25]

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Solvents such as chloroform/methanol are widely used to treat tissue samples prior to SIA to remove lipids.[31] For elasmobranchs, species-specific effects of lipid removal on carbon isotope values have been reported, indicating a need to determine if lipid extraction is required on a species by species basis.[1] These species-specific effects probably relate to the typically low lipid content of muscle tissue across elasmobranch species.[32] For urea, isotopic experimental work to date on elasmobranchs has revealed conflicting results. Using water washing to remove urea from muscle tissue and blood of spiny dogfish (*Squalus acanthias*), Logan and Lutcavage[33] observed no treatment effect on δ^{15}N values. In contrast, Kim and Koch[25] observed significant shifts in δ^{15}N values in leopard shark (*Triakis semifasciata*) muscle tissue following lipid extraction and water rinsing, and these authors concluded that deionized water rinsing was the most effective treatment to remove urea. Standard chloroform/methanol treatment for lipid extraction, however, has also been found to reduce %N, and increase both C:N ratios and δ^{15}N values in elasmobranch muscle tissue, suggesting removal of urea.[1,26]

More than 40% of the global shark fin trade is composed of pelagic species, raising concern over their future conservation status.[34,35] Their ecological role as apex predators in oceanic ecosystems is recognized,[36,37] but limited data is available on their trophic dynamics and to understand the effects of their removal.[8] Given the potential and increasing use of SIA to examine the trophic dynamics of pelagic sharks in open ocean ecosystems,[38,39] improved preparation methods prior to SIA are necessary to standardize data for comparative analyses.

In this study, three treatments were performed on muscle tissue of seven pelagic shark species prior to SIA to: (1) assess the necessity of urea removal using deionized water rinsing, (2) evaluate the effects of lipid extraction (chloroform/methanol) and urea removal (water rinsing) on δ^{13}C, δ^{15}N, and C:N values, and (3) investigate the potential for all species and species-specific mathematical normalization for urea effects using lipid-extracted isotope data.

EXPERIMENTAL

Sampling methods

Lipid extraction and urea removal treatments were undertaken on a total of 125 individuals from seven pelagic shark species: the silky (*Carcharhinus falciformis*), blue (*Prionace glauca*), smooth hammerhead (*Sphyrna zygaena*), scalloped hammerhead (*Sphyrna lewini*), oceanic whitetip (*Carcharhinus longimanus*), shortfin mako (*Isurus oxyrinchus*), and pelagic thresher shark (*Alopias pelagicus*). Shark samples were obtained from the bycatch of the Chinese tuna longline fleets operating in the Northeast Central Pacific (8°–10°N, 115°–125°W) between June and November 2014. For each individual, the precaudal length (PCL) was measured to the nearest cm and a white muscle tissue sample was excised from the muscle block anterior to the anal fin and adjacent to the vertebral column, and immediately frozen (Table 1).

Sample preparation

All samples were freeze-dried at –55°C for 48 h using a Christ Alpha 1-4 LD plus freeze dryer (Martin Christ, Osterode am Harz, Germany) and homogenized using a Mixer Mill MM 400 (Retsch, Haan, Germany). Each powdered muscle tissue sample was then equally divided into four subsamples and a treatment assigned to each prior to SIA: (i) no treatment (referred to as 'Control'); (ii) urea removal (referred to as treatment 'DW') where the subsample was vortexed in a 5 mL Effendorf tube with 4.0 mL deionized water for 1 min, and soaked for 24 h at room temperature. The sample was then centrifuged for 5 min and the water removed using a medical needle. The above process was repeated three times, the sample freeze-dried and then analyzed; (iii) lipid extraction (referred to as treatment 'LE') where the subsample was immersed in a 2:1 mixture of chloroform and methanol. The mixture was vortexed for 1 min and left undisturbed overnight at room temperature, centrifuged for 10 min and decanted. This process was repeated three times and the samples re-dried overnight to eliminate excess solvent;[31] and (iv) combined lipid extraction and urea removal (referred to as treatment 'LE+DW') following the protocols detailed in (iii) and then (ii).

Lipid quantification

To quantify tissue lipid content, 0.2 ± 0.0001 g of each untreated muscle tissue sample was loaded into a 5 mL Effendorf tube, to which 3.5 mL of 2:1 chloroform/methanol was added. The mixture was vortexed for 1 min, heated in a water bath at 60°C for 10 min, and then left undisturbed overnight at room temperature. Following centrifuging at 5000 *g* for 10 min, the supernatant was separated and

Table 1. Species, sample sizes and length of pelagic shark included in the study

Species	Common name	Code	*n*	PCL (cm) Mean	PCL (cm) Range
Carcharhinus falciformis	Silky shark	FAL	46	106	57–167
Prionace glauca	Blue shark	BSH	28	153	130–205
Sphyrna zygaena	Smooth hammerhead shark	SPZ	20	152	103–218
Sphyrna lewini	Scalloped hammerhead shark	SPL	6	161	106–131
Carcharhinus longimanus	Oceanic whitetip shark	OCS	7	110	97–122
Isurus oxyrinchus	Shortfin mako shark	SMA	5	148	109–195
Alopias pelagicus	Pelagic thresher shark	PTH	13	151	95–184

n represents the sample size, PCL is the precaudal length

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approximately 1.5 mL of 0.9% saline solution was added. The mixture was then shaken vigorously, and allowed to separate. The bottom layer was drained into a pre-weighed aluminum dish. The contents were evaporated at 70°C overnight using a vacuum drying oven, cooled to room temperature, and weighed to the nearest 0.0001 g. The mass of lipid per 0.2 g of dry muscle tissue was calculated by subtracting the weight of each aluminum dish.

Urea concentration determination

For each pelagic shark species, 0.06 ± 0.02 g of untreated and lipid-extracted muscle tissue powder from two to six individuals was randomly selected for urea concentration determination (UCD). The powdered samples were water rinsed as detailed above and then filtered using a Whatman No.1 (9 cm) filter paper and diluted ×50. Urea concentrations were determined using a Urea/Ammonia kit (Megazyme, Bray, Ireland) with the spectrophotometer set at 340 nm.

Stable isotope analysis

Approximately 1.0–1.5 mg of prepared sample were weighed into 0.3 mg tin capsules and analyzed using a model 100 isotope ratio mass spectrometer (IsoPrime Corporation, Cheadle, UK) and a vario ISOTOPE cube elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany) at Shanghai Ocean University Stable Isotope Laboratory. The standard reference materials for C and N were Pee Dee Belemnite carbonate and atmospheric N_2, respectively. Reference standards USGS 24 [Graphite, –16.05 ± 0.04‰ vPDB (Mean ± SD)] and USGS 26 [Ammonium Sulfate, 53.7 ± 0.4‰ vN_2 (Mean ± SD)] obtained from the International Atomic Energy Agency (IAEA, Vienna, Austria) were analyzed in triplicate for every new reference gas. Every tenth sample was also run in triplicate with a laboratory reference standard, i.e. Protein (–26.98‰ vPDB and 5.96‰ vN_2), to assess the within-run precision, and a blank sample run every ten samples to clear off residual gases. The analytical error of the δ^{13}C and δ^{15}N values was approximately 0.1‰. All C:N ratios were calculated based on atomic mass.

Statistical analysis

Paired Student's t-tests or Wilcoxon signed rank tests were used to examine whether the stable isotope values and C:N ratios were significantly different or not among treatments. Student's t-tests were used when the data were of equal variance and normally distributed;[40] Wilcoxon signed rank tests were used when the sample size was <10. Mathematical normalization of urea- and lipid-extracted $\delta^{15}N_{LE+DW}$ and $\delta^{13}C_{LE+DW}$ values using the lipid-extracted stable isotopic values, i.e. the $\delta^{15}N_{LE}$ and $\delta^{13}C_{LE}$ values, was established using linear models for the three shark species with sample sizes larger than 20 and all sharks combined:

$$\delta^{15}N_{LE+DW} = a_1 \times \delta^{15}N_{LE} + b_1 \quad (1)$$

$$\delta^{15}C_{LE+DW} = a_2 \times \delta^{15}C_{LE} + b_2 \quad (2)$$

where a_1, b_1, a_2, and b_2 are the slope and intercept parameters in the linear models. All statistical analyses were conducted using R version 3.1.2.[41]

RESULTS

The mean and standard deviation of the δ^{15}N and δ^{13}C values and the C:N ratio for each of the four treatments, Control, DW, LE and LE+DW, are shown in Table 2. Treatment DW resulted in an increase in the mean δ^{15}N and δ^{13}C values and C:N ratios of all sharks by 1.3 ± 0.4‰ (mean ± SD), 0.3 ± 0.4‰ and 0.7 ± 0.2, respectively. At the species level, the largest increase in δ^{15}N values was found in the blue shark (1.4 ± 0.4‰), while the shortfin mako shark showed the smallest change of 0.9 ± 0.3‰. Following treatment LE, the mean δ^{15}N and δ^{13}C values and C:N ratios across all shark species increased by 0.7 ± 0.4‰, 0.5 ± 0.4‰ and 0.2 ± 0.1, respectively. Consistent with the DW treatment results, with the LE treatment the blue shark yielded the largest shift in δ^{15}N values (0.9 ± 0.3‰) and the shortfin mako had the smallest increase (0.4 ± 0.4‰), but the shifts were smaller in magnitude than with the DW treatment. For the combined LE+DW treatment, an average shift of 1.2 ± 0.7‰ for δ^{15}N values, 0.7 ± 0.4‰ for δ^{13}C values and 0.5 ± 0.1 for the C:N ratios was found across all species. The maximum and minimum increases in δ^{15}N values following the LE+DW treatment were for the blue shark (1.6 ± 0.5‰) and the pelagic thresher shark (1.0± 0.5‰), respectively.

Paired t-tests and Wilcoxon signed rank tests revealed significant shifts in the δ^{15}N values between Control and both treatment DW and treatment LE for all shark species (Table 3), indicating urea removal by either water rinsing or lipid extraction. The actual urea concentrations measured in water rinses of the bulk and LE tissues were 5.4 ± 1.2% and 1.9 ± 0.6% of dry weight, respectively (Table 4), in agreement with the above observations. Significant differences in δ^{15}N values between the DW and LE treatments, however, indicated that deionized water rinsing was more effective at removing urea than chloroform/methanol (Table 3 and Fig. 1). No observed difference in δ^{15}N values between the DW and LE +DW treatments for all shark species, but a significant difference between treatments LE and LE+DW (Table 3), further supports the proposal that water rinsing is more effective at urea removal (Table 3).

The δ^{13}C values for all shark species increased between Control and treatment LE and between treatments DW and LE+DW (Table 3). In both comparisons, the shortfin mako shark yielded the largest shift in δ^{13}C values (1.1 ± 0.7‰ and 1.2 ± 0.5‰) and the silky shark showed the smallest change (0.4 ± 0.2‰ and 0.3 ± 0.2‰). More variable changes in δ^{13}C values were seen between Control and treatment DW, between treatments LE and LE+DW, and between treatments DW and LE (Table 3). The lipid content of pelagic shark muscle tissue (dry mass) ranged from 2.4% to 15.0% with a mean of 5.9 ± 2.7% (n = 125) (Table 2). There was no significant relationship between the difference in δ^{13}C values following treatment LE vs lipid content (p = 0.20) or treatment LE+DW vs lipid content (p = 0.65). These data indicate that lipid removal occurred but, given the low content, the effect of treatment on the δ^{13}C values was variable among species (Tables 2 and 3).

Significant relationships were found for the δ^{15}N and δ^{13}C values between the LE and LE+DW treatment tissues for all three shark species with sample sizes >20 and all three species combined. Species-specific mathematical normalizations are

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Table 2. Comparison of the four different treatments, Control – Bulk, DW – Deionized water rinsing , LE – Lipid extraction and LE+DW – Lipid extraction and deionized water rinsing, on the isotope values of muscle tissue of pelagic sharks sampled from the northeast Central Pacific. The mean and (standard deviation) are presented

Code	n	Lipids (%)	Parameter	Treatments (‰)			
				Control	DW	LE	LE+DW
FAL	46	7.0 (2.8)	$\delta^{15}N$	14.7 (0.8)	16.0 (0.8)	15.4 (0.8)	15.9 (0.8)
			$\delta^{13}C$	–16.9 (0.3)	–16.7 (0.3)	–16.5 (0.3)	–16.4 (0.3)
			C:N	2.5 (0.1)	3.2 (0.1)	2.8 (0.1)	3.1 (0.1)
BSH	28	5.8 (2.7)	$\delta^{15}N$	14.7 (1.2)	16.1 (1.3)	15.6 (1.1)	16.2 (1.3)
			$\delta^{13}C$	–18.6 (0.6)	–18.1 (0.9)	–18.0 (0.7)	–17.8 (0.7)
			C:N	2.6 (0.1)	3.3 (0.1)	2.8 (0.1)	3.2 (0.03)
SPZ	20	4.1 (1.4)	$\delta^{15}N$	14.8 (1.3)	16.1 (1.3)	15.2 (1.3)	15.9 (1.3)
			$\delta^{13}C$	–16.8 (0.2)	–16.4 (0.5)	–16.3 (0.3)	–15.9 (0.2)
			C:N	2.7 (0.1)	3.3 (0.04)	2.9 (0.1)	3.2 (0.04)
SPL	6	5.1 (1.1)	$\delta^{15}N$	16.0 (0.4)	17.3 (0.6)	16.5 (0.4)	17.3 (0.5)
			$\delta^{13}C$	–17.1 (0.2)	–16.9 (0.5)	–16.6 (0.1)	–16.3 (0.3)
			C:N	2.77 (0.04)	3.3 (0.03)	3.0 (0.1)	3.2 (0.03)
OCS	7	5.6 (2.0)	$\delta^{15}N$	15.2 (0.5)	16.4 (0.5)	15.7 (0.6)	16.8 (0.9)
			$\delta^{13}C$	–16.9 (0.3)	–16.6 (0.1)	–16.4 (0.4)	–16.2 (0.1)
			C:N	2.6 (0.1)	3.3 (0.2)	2.9 (0.1)	3.1 (0.02)
SMA	5	8.6 (4.4)	$\delta^{15}N$	14.6 (1.3)	15.5 (1.0)	15.0 (1.2)	15.7 (1.0)
			$\delta^{13}C$	–16.7 (0.4)	–16.7 (0.3)	–15.6 (0.6)	–15.5 (0.5)
			C:N	2.6 (0.2)	3.5 (0.2)	2.9 (0.1)	3.1 (0.04)
PTH	13	4.9 (2.3)	$\delta^{15}N$	15.2 (0.9)	16.3 (0.8)	15.8 (0.9)	16.2 (0.9)
			$\delta^{13}C$	–17.4 (0.3)	–17.3 (0.5)	–16.7 (0.4)	–16.6 (0.3)
			C:N	2.6 (0.1)	3.3 (0.2)	2.9 (0.1)	3.1 (0.02)

Table 3. Paired Student's t-tests (n >10) and Wilcoxon signed rank tests (n <10) comparing stable isotope ratio values ($\delta^{15}N$ and $\delta^{13}C$) of muscle tissue among different treatments

CODE	n	Parameter	Paired Student's t-tests (n>10) and Wilcoxon signed rank tests (n<10)					
			Control vs DW	Control vs LE	LE vs LE+DW	DW vs LE	DW vs LE+DW	Control vs LE+DW
FAL	46	$\delta^{15}N$	*	*	*	*	p=0.21	*
		$\delta^{13}C$	*	*	*	*	*	*
		C:N	*	*	*	*	*	*
BSH	28	$\delta^{15}N$	*	*	*	*	p=0.25	*
		$\delta^{13}C$	*	*	*	p=0.13	*	*
		C:N	*	*	*	*	*	*
SPZ	20	$\delta^{15}N$	*	*	p=0.12	*	p=0.12	*
		$\delta^{13}C$	*	*	*	p=0.09	*	*
		C:N	*	*	*	*	*	*
SPL	6	$\delta^{15}N$	*	*	*	*	p=0.69	*
		$\delta^{13}C$	p=0.44	*	p=0.22	p=0.16	p=0.06	*
		C:N	*	*	*	*	*	*
OCS	7	$\delta^{15}N$	*	*	*	*	p=0.16	*
		$\delta^{13}C$	*	*	p=0.08	p=0.22	*	*
		C:N	*	*	*	*	*	*
SMA	5	$\delta^{15}N$	p=0.06	p=0.06	p=0.06	p=0.06	p=0.06	p=0.06
		$\delta^{13}C$	p=0.95	p=0.06	p=0.63	p=0.06	p=0.06	p=0.06
		C:N	p=0.06	p=0.06	p=0.06	p=0.06	p=0.06	p=0.06
PTH	13	$\delta^{15}N$	*	*	*	*	p=0.38	*
		$\delta^{13}C$	p=0.21	*	p=0.18	*	*	*
		C:N	*	*	*	*	*	*

*represent statistical significance (p <0.05)

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Table 4. Urea concentrations (% of dry weight) measured in the rinses of bulk (Control) and lipid-extracted (LE) samples

		Urea in bulk sample rinses (%)		Urea in lipid-extracted sample rinses (%)	
Code	*n*	Mean	SD	mean	SD
FAL	2	5.3, 4.1	-	0.5	-
BSH	3	6.8	0.7	2.8	0.6
SPZ	2	4.9, 4.5	-	3.4	-
SPL	3	4.3	2.3	1.1	0.2
OCS	3	5.5	0.1	-	-
SMA	3	5.6	0.5	3.3	0.3
PTH	6	5.8	1.0	1.2	0.1

provided in Table 5 and Fig. 2. The mathematical normalization for all three pelagic shark species combined is also presented. Both nitrogen and carbon isotopic values derived from mathematical normalizations were not statistically different from the original $\delta^{15}N_{LE+DW}$ and $\delta^{13}C_{LE+DW}$ values for the three shark species. However, the slopes and intercepts of the species-specific normalization linear regression models varied.

DISCUSSION

Understanding the effects of urea and lipid extraction on the stable isotope values of pelagic shark muscle tissue is important for accurately interpreting data in food-web studies. In the present study, deionized water rinsing was undertaken to remove urea from the muscle tissue of seven pelagic shark species, following the standard method of treating shark flesh for human consumption by removing non-protein nitrogen.[42] Because urea and potentially TMAO are depleted in ^{15}N,[3,25] the observed increase in δ^{15}N values for all pelagic shark species following treatment DW was expected. Similar shifts in δ^{15}N values also occurred for all species after treatment LE, although the sample sizes for some species limited the strength of the statistical comparisons. This is also due to urea extraction (Table 5);[1,24] however, the potential for chloroform and methanol to remove extra lipophilic amino acids and polar lipids that are bound to membrane proteins cannot be ruled out.[31,43] The urea concentration determined in water rinses from treatments LE+DW (Table 4), and an increase in δ^{15}N values of 0.7 ± 0.3‰, between treatments DW and LE, identify that treatment LE+DW is the most effective for urea removal, in agreement with Kim and Koch.[25] This, combined with notable shifts in δ^{13}C values following LE, identifies that LE+DW is the required treatment for pelagic shark muscle tissue to address the isotopic bias of urea and lipids.

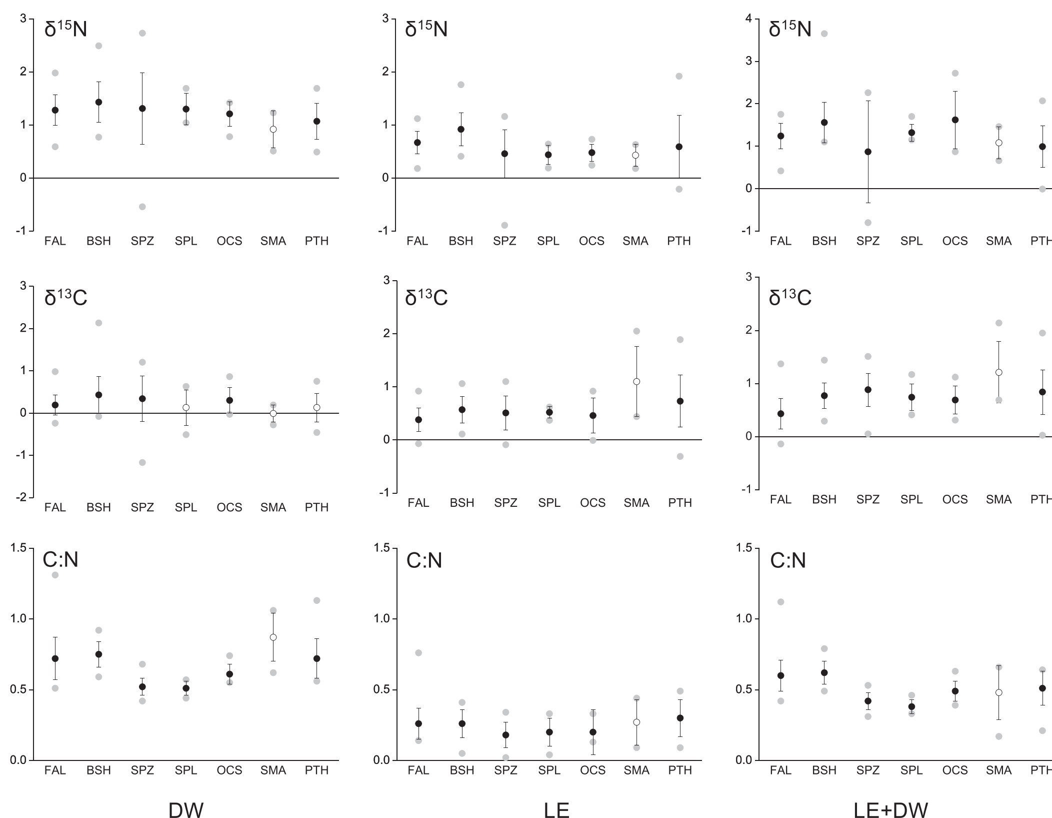

Figure 1. Calculated differences in δ^{15}N and δ^{13}C values and C:N ratios among treatments (deionized water (DW)), lipid extraction (LE), lipid extraction combined with deionized water rinsing (LE+DW) and untreated (Control) shark muscle tissue for each species. Solid grey circles are minimum and maximum values for each species. Solid black circles and open black circles are mean values (±SD) with significant and non-significant paired Student's t-tests or Wilcoxon signed rank tests, respectively (Table 3). For species codes and sample sizes, see Table 1.

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Table 5. Results of mathematical normalization of urea- and lipid-extracted $\delta^{15}N$ and $\delta^{13}C$ values using the lipid-extracted isotope values

Code	Parameter	*n*	Equation	R^2	*p* value
FAL	$\delta^{15}N$	46	$\delta^{15}N_{LE+DW}=0.955\times\delta^{15}N_{LE}+1.268$	0.92	<0.001
	$\delta^{13}C$	46	$\delta^{13}C_{LE+DW}=0.885\times\delta^{13}C_{LE}-1.835$	0.66	<0.001
BSH	$\delta^{15}N$	28	$\delta^{15}N_{LE+DW}=1.032\times\delta^{15}N_{LE}+0.136$	0.80	<0.001
	$\delta^{13}C$	28	$\delta^{13}C_{LE+DW}=1.011\times\delta^{13}C_{LE}+0.396$	0.88	<0.001
SPZ	$\delta^{15}N$	20	$\delta^{15}N_{LE+DW}=0.984\times\delta^{15}N_{LE}+2.063$	0.89	<0.001
	$\delta^{13}C$	20	$\delta^{13}C_{LE+DW}=0.581\times\delta^{13}C_{LE}-6.435$	0.57	<0.001
ALL	$\delta^{15}N$	125	$\delta^{15}N_{LE+DW}=0.856\times\delta^{15}N_{LE}+2.813$	0.65	<0.001
	$\delta^{13}C$	125	$\delta^{13}C_{LE+DW}=0.954\times\delta^{13}C_{LE}-0.615$	0.91	<0.001

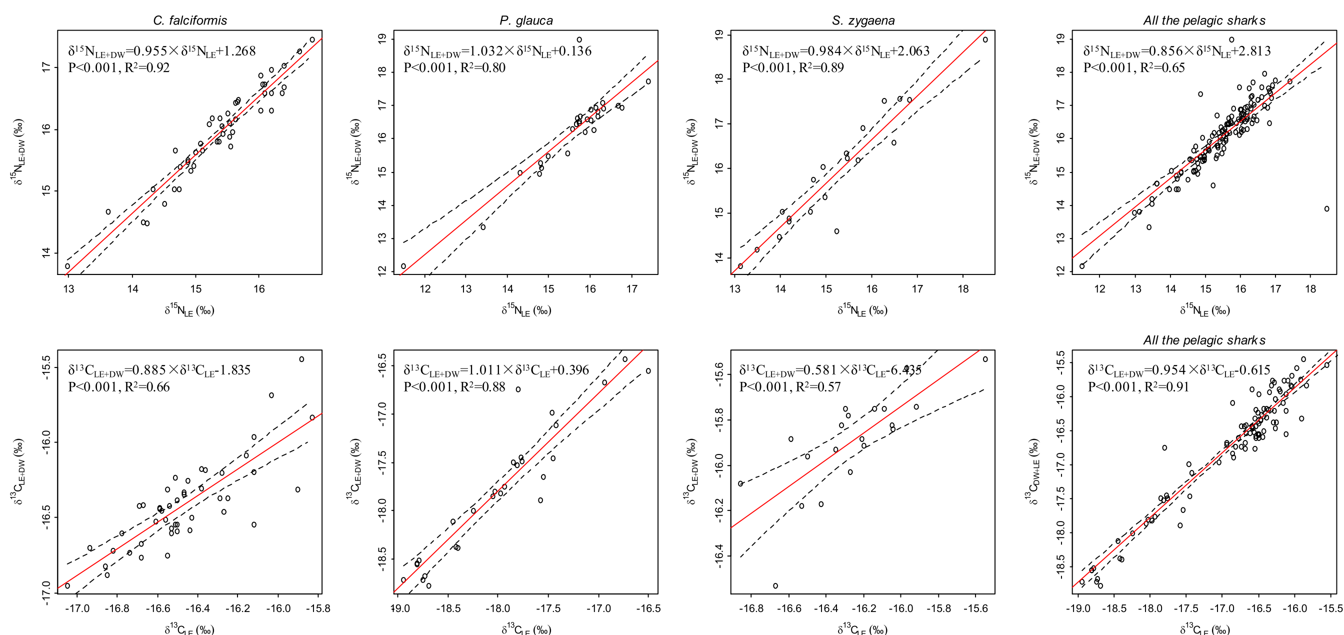

Figure 2. Relationship between urea- and lipid-extracted (LE+DW) and lipid-extracted (LE) $\delta^{15}N$ values (upper panels) and urea- and lipid-extracted (LE+DW) and lipid-extracted (LE) $\delta^{13}C$ values (lower panels) of muscle tissue sampled from *C. falciformis*, *P. glauca*, *S. zygaena* and all three species combined. Dotted lines indicate 95% confidence intervals for linear regressions.

The mean change in $\delta^{15}N$ values across all seven shark species in this study of 1.3 ± 0.4‰ (n = 125) after treatment DW showed that urea/TMAO can have a marked effect on $\delta^{15}N$ values when interpreting the ecological and trophic role of sharks. Assuming a diet-tissue discrimination factor of 2.3 ± 0.2 for large sharks,[44] this difference in $\delta^{15}N$ values would adjust the estimates of trophic position by ~0.5 of a trophic level. Moreover, Hussey *et al.*[30] reported decreasing isotopic discrimination at higher trophic levels when animals consume prey with higher $\delta^{15}N$ values. Narrowing discrimination with increasing trophic position would consequently result in even larger shifts in the estimated trophic position (TP) of pelagic sharks following urea removal. Underestimation of TP values of top predators may affect methods using TP to monitor temporal trends in global fisheries,[45] to measure levels of omnivory, and to identify critical trophic interaction strengths.[6,28,46] Alternatively, not accounting for inter- and intra-specific variation in muscle urea concentration may also confound TP comparisons among species and individuals of different sizes, i.e. juveniles vs adults.[30]

Chloroform/methanol (LE) treatment significantly increased $\delta^{13}C$ values in the muscle tissue of all pelagic shark species, indicating that although the lipid content is low (Table 2),[1] ^{13}C-depleted lipids were removed. In contrast to Post *et al.*,[16] there was no significant relationship observed between the difference in $\delta^{13}C$ values after treatments LE and LE+DW and Control, and the lipid content. A possible explanation is that the simultaneous removal of nitrogenous compounds, i.e. urea and TMAO, masked the overall relationship because their concentrations may be highly variable, related to species life histories and ambient environmental salinity.[28,29] In this study, the increase in the C:N ratio following DW, LE+DW, and LE indicated that all treatments remove urea and TMAO. If only lipids were removed, the C:N ratio would be expected to decrease, similar to teleost muscle tissue,[43] but the opposite effect for pelagic shark species was observed. This was a result of removing a higher proportion of nitrogenous waste from the sample relative to the low lipid content.[1,44]

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The mean shift in $\delta^{13}C$ values of 0.7 ± 0.4‰ in the muscle tissue of pelagic sharks after treatment LE+DW was similar to results reported by Hussey *et al*.[1] for small and large shark species commonly encountered on the continental shelf (0.6 ± 1.2‰). One possible reason for these moderate shifts could be the relatively low lipid content (5.9 ± 2.7% of dry mass) in pelagic shark muscle tissue compared with in other shark species.[47] Hussey *et al*.[1] reported marked increases in $\delta^{13}C$ values in Greenland shark (+5.0‰) and whale shark (+3.0‰) muscle tissue following lipid extraction, attributed to the high lipid content of these species. An alternative explanation could relate to the ^{13}C-enriched values of urea and TMAO, which might balance out the lower $\delta^{13}C$ values of lipid. By pooling five water rinse residues of a silky shark, the $\delta^{13}C$ value of the mixture of nitrogenous compounds and lipid removed by water was found to be –17.6‰. As the $\delta^{13}C$ values of lipid are considered to be 6–8‰ less than those of pure protein,[14] it is possible that the urea and/or TMAO are ^{13}C-enriched relative to lipid. Nevertheless, significant differences in $\delta^{13}C$ values between LE+DW and Control for some species indicate that it is sensible to undertake lipid extraction to standardize carbon stable isotopic values for inter- and intra-specific comparisons of species within food webs.

The differences in isotopic data between the LE and LE+DW treatments of pelagic shark muscle tissue, although relatively small, were significant. Species-specific mathematical corrections showed strong positive correlations between the LE and LE+DW treatments, identifying their potential applicability. However, these mathematical corrections should be used with caution. Only when sufficient sample numbers are available and the study question is focused on understanding broad inter-species relationships at the ecosystem level, should mathematical correction be used to save cost and time in sample preparation. However, for investigating the spatial and temporal variation in isotope values of individuals within each shark species, normalization is not recommended.

CONCLUSIONS

The $\delta^{15}N$ and $\delta^{13}C$ values of pelagic shark muscle tissue displayed a marked shift following treatment LE+DW compared with treatment LE, indicating the suitability and efficiency of this dual treatment for urea and lipid removal. Although LE sample preparation for correcting lipid bias on $\delta^{13}C$ values can remove urea, the change in $\delta^{15}N$ values between LE and LE+DW treatments, as well as the measured urea concentrations in the LE+DW rinses, highlights the importance of supplementary urea removal. Treatment LE+DW is therefore recommended as the most suitable sample preparation method to obtain accurate shark $\delta^{15}N$ and $\delta^{13}C$ values. Future work should focus on urea and lipid separation in the rinses from treatments DW and DW+LE, and further investigate the $\delta^{15}N$ and $\delta^{13}C$ values of different nitrogenous compounds. The fact that treatments DW and LE may have incidentally removed some low molecular protein cannot be ruled out; consequently, future work is required to examine amino acid compositions following the different treatments.

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