Effects of pre-treatments on bulk stable isotope ratios in fish samples: A cautionary note for studies comparisons

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ABSTRACT

Rationale: Stable isotope analysis (SIA) has revolutionized ecological studies over the past thirty years. One of the major fields where SIA is applied in the marine environment, is related to the definition of ecosystem structure and function. With marine top predators such as sharks, SIA is a method of choice because tissue samples can be collected without the sacrifice of the animal. In elasmobranch research, the influence of molecules such as urea, trimethylamine oxide and lipids must be considered when using stable isotopes as ecological markers. Currently, a range of pre-treatments are used to chemically remove these molecules prior to SIA.

Methods This study investigated the impact of 11 commonly used pre-treatments on carbon and nitrogen contents and C:N atomic ratio, as well as carbon and nitrogen SI ratios in elasmobranch tissues and its prey, measured by isotope ratio mass spectrometry. Three tissues were tested: blood and muscle of the ragged-tooth shark *Carcharias taurus*, and muscle of one teleost species, the Cape knifejaw *Oplegnathus conwayi*.

Results: Compared with untreated samples, no trend or generalization could be highlighted with the influence of pre-treatments being species-, tissues- and chemical element-dependent. For the $\delta^{13}C$ and $\delta^{15}N$ values, differences among pre-treatments were as high as 3 ‰, therefore potentially leading to erroneous ecological interpretation.

Conclusion: The chemical properties of molecules (e.g. urea, lipids) combined with the polarity of solutions (e.g. water, solvents) explained a large part these observations. This study highlights that pre-treatments need to be considered especially when comparing carbon and nitrogen stable isotope ratios between studies. The results of this study provide a call to all stable isotope researchers to make a concerted effort to standardize pre-treatment methods. This is crucial as global reviews are becoming increasingly more informative.

Key words: Elasmobranch, urea, lipids, *Carcharias taurus*, *Oplegnathus conwayi*

INTRODUCTION

The use of biomarkers to study the trophic ecology of predators has enhanced tremendously the understanding of such interactions in the last thirty years. A favoured method is the measurement of naturally occurring stable isotopes in an animal's tissues. In the marine environment, measurements of carbon ($^{13}C/^{12}C$; $\delta^{13}C$ values) and nitrogen ($^{15}N/^{14}N$; $\delta^{15}N$ values) stable isotope ratios are the most common indicators of carbon flow pathways and consumer's trophic position, respectively.[1] The stable isotope ratios of these elements in a consumer's tissues result from an equilibrium between diet and physiology.[2]

Similarly to other fields, fish scientists are increasingly using stable isotopes as spatial and trophic indicators and studies have increased tremendously over the last 10 years, bringing new insights into fish trophic ecology (e.g.[3]), migration patterns (e.g.[4]), and physiology (e.g.[5]). Studies of the foraging ecology of elasmobranchs are no exception with a recent increase in published papers using the stable isotope approach (e.g.[6-9]). Due to the life history of elsmobranchs, their wide ranging migration patterns and concern about their population decline[10], the stable isotope approach for their study is particularly useful, as samples can be collected non-lethally (e.g. biopsies), and by sampling various tissues of different metabolic rates from the same individual, trophic information can be obtained at various time scales (e.g. muscle vs blood[11,12]). However, the elevated urea ($(NH_2)_2CO$) and trimethylamine N-oxide (C_3H_9NO; TMAO) concentrations in elasmobranch tissues used to combat osmotic stress[13-15] complicate the interpretation of stable isotope ratios when used as ecological indicators. Typically, urea is an end product of nitrogen metabolism which is enriched in ^{14}N.[16] Tissues with high urea content will thus exhibit lower $\delta^{15}N$ values and C:N ratios than those for the same tissue types with low urea contents. This factor is particularly important for lipid rich tissues because lipids are known to artificially increase C:N ratios and lower $\delta^{13}C$ values.[17-20] Tissues rich in both lipids and urea may therefore exhibit completely acceptable C:N ratios when in fact their $\delta^{15}N$ and $\delta^{13}C$ values are affected by urea and lipid contents, respectively.[21]

Numerous different protocols are currently being used to chemically remove urea and lipids from elasmobranch tissues. To date, little is known about how these various protocols compare in their efficiency of lipid and urea removal. This is likely to affect carbon and nitrogen contents and $\delta^{13}C$ and $\delta^{15}N$ values, as well as C:N ratios. Critically important information for comparisons between studies (e.g.[22]) is thus missing. To investigate this, we tested the influence of 11 pre-treatments commonly used in published studies on carbon and nitrogen percentages and C:N atomic ratios, as well as carbon and nitrogen stable isotope

ratios, from muscle tissue for two fish species: one elasmobranch species, the ragged-tooth shark (*Carcharias taurus* Rafininesque 1810), and one teleost species, the Cape knifejaw (*Oplegnathus conwayi* Richardson 1840). In addition, we tested whether the influence of pre-treatments was tissue-dependent by comparing treated -muscle and -blood samples from individual ragged-tooth sharks. Due to their different biochemical compositions, we hypothesised that the influence of pre-treatments on stable isotope ratios and C:N ratios would be species- and tissue- specific.

MATERIAL AND METHODS

SAMPLE COLLECTION

Muscle samples of Cape knifejaw (n = 5), and whole blood and muscle samples of ragged-tooth sharks (n = 8) were collected between March 2012 and October 2014 along the south-east coast of South Africa. Cape knifejaw samples were collected from dead fish (stranded individuals killed by an anoxic phytoplankton bloom), as part of a larger study looking at the ecological role of sharks in the area. Ragged-tooth shark samples were mostly collected from living animals that were captured in an ultrasonic tagging study to investigate their movement pattern. When sharks were in tonic immobility, muscle tissue was collected from with a corer to remove a tissue sample ventral to the dorsal fin, and blood was collected from the caudal vein using a disposable syringe and needle. Sampled ragged-tooth shark were released within 10 min of capture once all samples had been collected. Two of the ragged-tooth sharks were sampled freshly dead (one stranded and one caught as bycatch by local commercial fisheries). Samples were stored on ice in the field and then frozen to -20 °C on return to the laboratory. They were later oven dried at 50 °C for 48 h. Samples were then finely ground prior to further analysis.

EXPERIMENTAL

The homogenised samples were sub-divided into seven sub-samples before being subjected to a range of pre-treatment methods to remove urea and lipids from the various tissue types (Figure 1).

Set 1 of the samples was left untreated and are referred to as *Bulk* samples. The other six sample sets were treated using a range of lipid and urea extraction methods commonly used in fish and elasmobranch studies (e.g.[23-25]).

Set 2 was treated using repeated washes with distilled water (referred to as H_2O) to remove urea from the various tissues, based on the methodology used by Li et al.[25] and Kim and

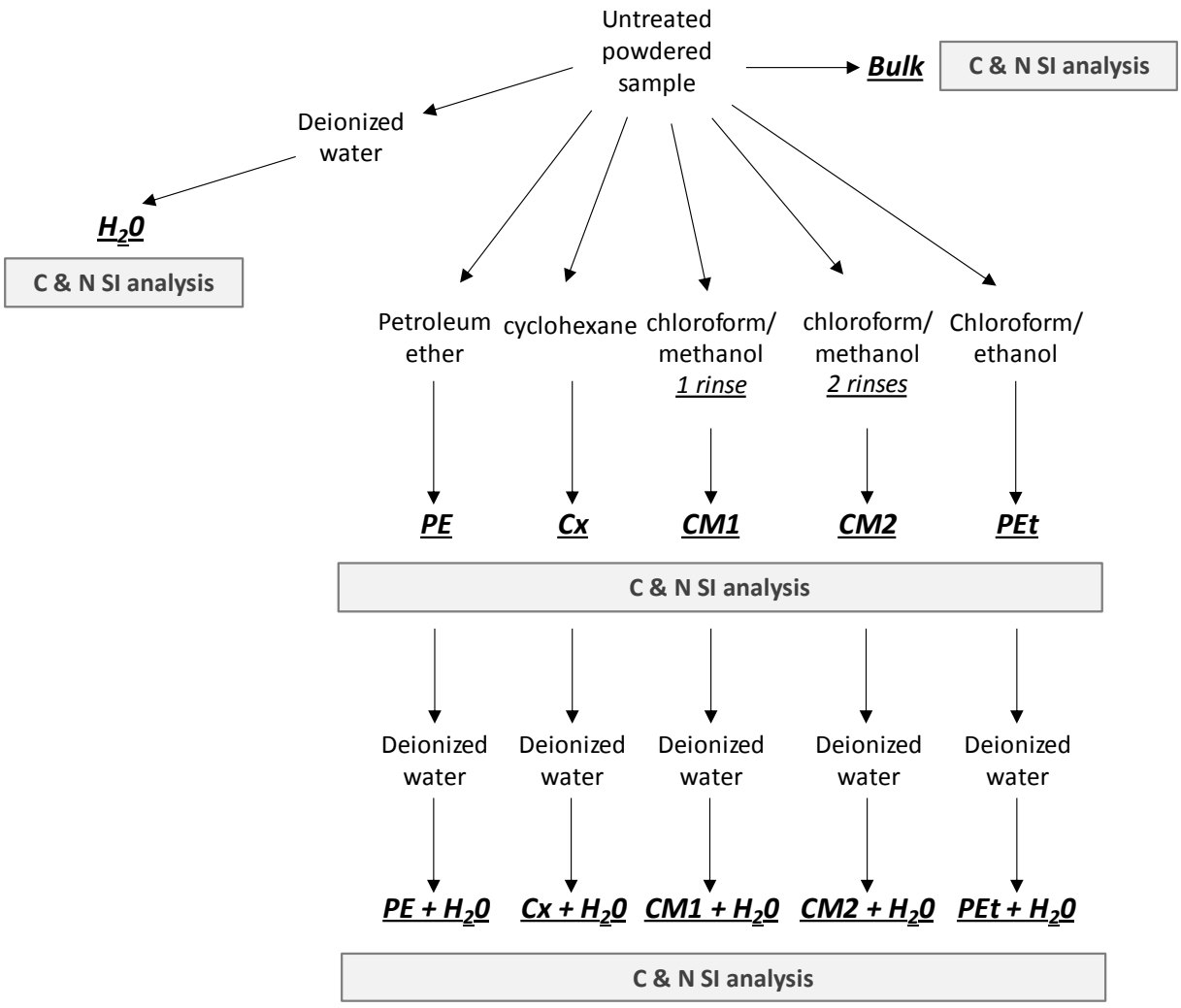

FIGURE 1

Figure 1. Schematic representation of the various pre-treatments tested. SI: stable isotope.

Koch[23]. Aliquots of tissue were place in 5-mL graduated centrifuge tubes and 4 mL of distilled water added and vortexed (IKA MS 3 basic; IKA®-Werke, Staufen, Germany) for 1 min. They were placed on a Coulter Mixer (Coulter Electronics Ltd, Harpenden, UK) and rotated for 24 h, centrifuged at 5000 rpm for 5 min and the water removed with a syringe. This was repeated a further two times and the samples were then dried at 50°C.

Set 3 was treated with petroleum ether, the solvent used by Kim and Koch[23]. Aliquots of the samples were placed in 1.5-mL micro-centrifuge tubes with 1.2 mL of petroleum ether (referred to as *PE*; Minema P1630, Spellbound Laboratory Solutions, Port Elizabeth, South Africa) and vortexed for 1 min, placed on a Coulter Mixer for 1 h, centrifuged at 5000 rpm for 5 min and the petroleum ether decanted. This was repeated a second time and the samples were dried at 50 °C.

Set 4 was treated with cyclohexane, the solvent used by Kiszka et al[24]. Aliquots of the samples were placed in 1.5-mL micro-centrifuge tubes with 1.2 mL of cyclohexane (referred to as *Cx*; SAAR1763000LC, UniLab, Merck (Pty) Ltd, Modderfontein, South Africa) and vortexed for 1 min, placed on a Coulter Mixer for 1 h, centrifuged at 5000 rpm for 5 min and the cyclohexane decanted. This was repeated a second time and the samples were then dried at 50 °C.

Sets 5 and 6 were both treated a 2:1 chloroform/methanol mixture adapted from Hussey et al[26-28]. Lipid extraction on sample set five was performed using a single rinse of the 2:1 chloroform/methanol solution (referred to as *CM1*; chloroform: SAAR1595040LC uniVar; methanol: SAAR4146080LC uniVar; Merck (Pty) Ltd, Modderfontein, South Africa), while set six received two rinses (referred to as *CM2*). Aliquots of sample were placed in 1.5-mL micro-centrifuge tubes with 1.2 mL of the 2:1 chloroform/methanol solution and vortexed for 1 min, placed on a Coulter Mixer for 1 h, centrifuged at 5000 rpm for 5 min and the chloroform/methanol decanted. This was repeated a second time for sample set six and the samples were then dried at 50 °C.

Set 7 was pre-treated in a similar manner to *CM1*, but using a 2:1 chloroform/ethanol solution (referred to as *CEth*; ethanol rectified 96% SAAR2233510LP uniVar, Merck (Pty) Ltd) with the samples receiving a single rinse. This is currently the lipid extraction method used by the Mammal Research Institute Stable Isotope Laboratory at the University of Pretoria (South Africa).[29]

Five additional pre-treatments were then prepared to test the effect of additional water rinses on the solvent washed tissues as in Li et al[25]. Solvent washed samples *PE*, *Cx*, *CM1*, *CM2*

and *CEth* were subjected to a series of distilled water washes over a period of three days as per the method used for the *H_2O* samples. These water-treated samples are referred to as *PE H_2O, Cx H_2O, CM1 H_2O, CM2 H_2O* and *CEth H_2O,* respectively (Figure 1).

STABLE ISOTOPE ANALYSIS

All sub-samples of ragged-tooth shark whole blood and muscle and Cape knifejaw muscle (*Bulk*, *H_2O*, *PE*, *Cx*, *CM1*, *CM2*, *CEth*, *PE H_2O*, *Cx H_2O*, *CM1 H_2O*, *CM2 H_2O*, *CEth H_2O*) were then analysed for carbon and nitrogen stable isotopic composition. Aliquots of 0.55 to 0.60 mg of each sub-sample were weighed into tin capsules which had been pre-cleaned in toluene (SAAR6081040LC uniVar, Merck (Pty) Ltd).

Several samples were run in duplicate to ensure reproducibility of the results. Isotopic analysis was carried out by continuous-flow isotope ratio mass spectrometry via an elemental analyzer (Flash EA 1112 Series) coupled to a Delta V Plus stable light isotope mass spectrometer via a ConFlo IV system (all equipment supplied by Thermo Fisher, Bremen, Germany), housed at the Stable Isotope Laboratory, Mammal Research Institute, University of Pretoria, Pretoria, South Africa.

Two in-house laboratory running standards, Merck Gel ($\delta^{13}C$ = -20.57 ‰, %C = 43.83, $\delta^{15}N$ = 6.80 ‰, %N = 14.64) and DL-valine ($\delta^{13}C$ = -10.57 ‰, %C = 41.28, $\delta^{15}N$ = -6.15 ‰, %N = 15.29), as well as a blank sample were run after every 11 unknown samples. The analytical accuracy of these laboratory running standards was validated against international standards (National Institute of Standards & Technology; NIST, Gaithersburg, MD, USA): NIST 1557b (bovine liver), NIST 2976 (mussel tissue) and NIST 1547 (peach leaves). All samples were measured and calibrated using the above mentioned laboratory running standards. The percentages carbon and nitrogen and atomic C:N ratios for each sample were calculated using a mass balance equation utilizing the sample and Merck Gel weights, and the known C (41.28%) and N (15.29%) percentages for the Merck Gel. Each run comprised 96 unknown samples, 20 laboratory standards and 10 blanks. All results are referenced to the internationally used standards of V-PDB (Vienna Pee-dee Belemnite) for carbon isotope values, and to atmospheric air for nitrogen isotope values. Results are expressed in delta (δ) notation using the standard equation:

$$\delta X\ (‰) = (R_{sample} / R_{standard}) - 1$$

where X = ^{13}C or ^{15}N and R represents $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

The analytical precision, based on the results for the DL-valine standard, across multiple runs was < 0.1 ‰ for both $\delta^{13}C$ and $\delta^{15}N$ values.

STATISTICAL ANALYSIS

The effect of pre-treatments on carbon and nitrogen percentages, C:N atomic ratios and carbon and nitrogen stable isotope ratios was approached by calculating the difference between treated tissues and *Bulk* for all three sample types. Either paired Student t-test or Wilcoxon signed rank tests were then used to assess whether the differences observed were significant. Paired Student t-tests were used when normality (Shapiro test) and homoscedasticity (Levene's test) assumptions were verified. Differences among pre-treatments were then tested separately on each tissue using analyses of variance (ANOVA) for repeated sampling when residuals followed a normal distribution. When this assumption was not verified, data were rank transformed and repeated ANOVAs were then conducted. Both were followed by Tukey post-hoc tests using a Bonferroni correction factor.
The level of significance was set at 0.05 or less in the case of Bonferroni correction for multiple comparisons. All statistical analyses were conducted using PAST v3.0[30] and R[31] software.

RESULTS

ELEMENTAL COMPOSITION

The water rinsing of *Bulk* tissues had mixed effects on carbon and nitrogen percentages as well as C:N ratios depending on tissue and species (Figure 2).

Rinsing of Cape knifejaw muscle did not change the carbon (48.3 ± 3.5 % vs 48.3 ± 0.4 %) or nitrogen (13.5 ± 0.1 % vs 13.2 ± 0.8 %) percentages but significantly increased the C:N ratios (4.2 ± 0.1 vs 4.3 ± 0.1; Table 1a). For ragged-tooth shark muscle, water rinsing significantly lowered nitrogen percentage (14.9 ± 0.9 % vs 13.7 ± 1.2 %), and increased C:N ratios (2.9 ± 0.1 vs 4.1 ± 0.1) (Table 1b). The water treatment on untreated samples (*Bulk*) significantly affected the three parameters for ragged-tooth shark blood (increased % C: 36.9 ± 2.8 % vs 50.6 ± 0.6 % and C:N atom: 2.5 ± 0.3 vs 4.3 ± 0.1, or decreased % N: 17.1 ± 0.7 % vs 13.7 ± 0.8 %) (Table 1c).

Compared with *Bulk*, no trend or generalization could be highlighted as the influence of pre-treatments was species-, tissues- and chemical element-dependent (Table 2). The tissue most affected by pre-treatments was ragged-tooth shark blood with 26 out of 30 outcomes of comparisons being significant (Table 2c).

Most subsequent water rinses of tissues pre-treated with solvents did not affect the carbon and nitrogen percentages in Cape knifejaw muscle samples except for the *CM1*, *CM2* and

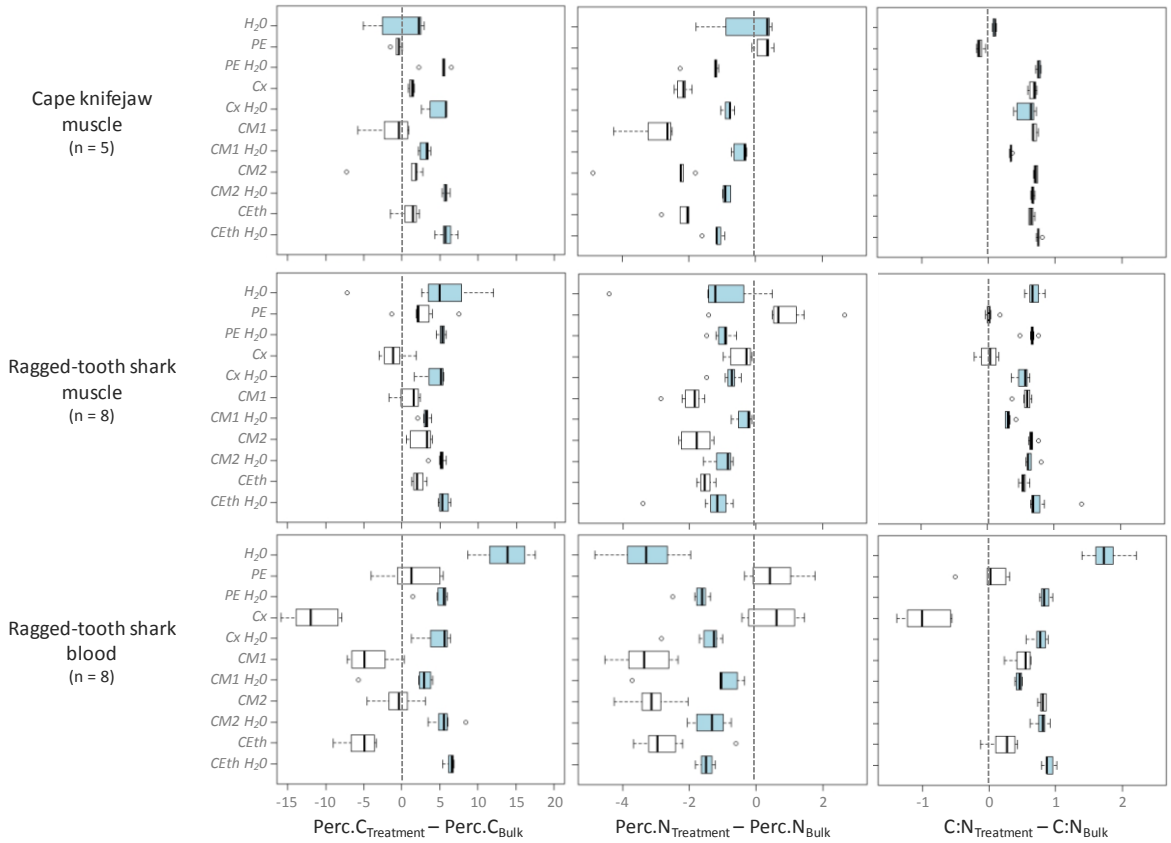

FIGURE 2

Figure 2. Changes in carbon and nitrogen percentages (Perc. C and Perc. N, respectively) and C:N ratios after the 11 pre-treatments relative to the untreated *Bulk* samples (Cape knifejaw muscle, ragged-tooth shark muscle and blood). The data for water rinsed tissues are in blue. (Refer to Figure 1 for abbreviations).

Table 1. Effect of the water rinsing on untreated samples (*Bulk*) or subsequent to solvent washing on carbon and nitrogen percentages, C:N ratios, and $\delta^{13}C$ and $\delta^{15}N$ values in the three fish tissues. (Refer to Figure 1 for abbreviations). Significant results are in bold, W values are in italics.

	H_2O		*PE*		*Cx*		*CM1*		*CM2*		*CEth*	
	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p
a) Cape knifejaw muscle (n = 5)												
Percent C	-0.01	0.995	-1.80	0.146	*15*	0.063	-3.98	**0.016**	*15*	0.063	-18.28	**<0.001**
Percent N	0.64	0.556	-0.99	0.379	*15*	0.063	-8.97	**<0.001**	*15*	0.063	-17.21	**<0.001**
C:N atomic	-6.78	**0.002**	-5.24	**0.006**	*12*	0.313	27.5	**<0.001**	4.15	**0.014**	-12.51	**<0.001**
$\delta^{13}C$	-1.93	0.126	6.93	**0.002**	3.33	**0.029**	-6.19	**0.003**	-6.08	**0.004**	12.25	**<0.001**
$\delta^{15}N$	-18.6	**<0.001**	-18.21	**<0.001**	-3.97	**0.017**	-29.07	**<0.001**	-5.89	**0.004**	-7.22	**0.002**
b) Ragged-tooth shark muscle (n = 8)												
Percent C	-2.33	0.052	-6.49	**<0.001**	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	-8.85	**<0.001**
Percent N	*33*	**0.036**	5.12	**0.002**	4.7	**0.002**	*36*	**0.012**	-5.05	**0.002**	*28*	0.161
C:N atomic	*36*	**0.012**	*28*	**0.018**	-8.11	**<0.001**	*36*	**0.012**	27.5	0.184	*36*	**0.012**
$\delta^{13}C$	-0.85	0.421	4.09	**0.006**	2.36	0.051	-0.89	0.403	-4.85	**0.002**	*35*	**0.017**
$\delta^{15}N$	-6.35	**<0.001**	-6.02	**<0.001**	-4.68	**0.002**	-17.22	**<0.001**	-8.58	**<0.001**	-2.80	**0.026**
c) Ragged-tooth shark blood (n = 8)												
Percent C	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	*35*	**0.017**	-6.17	**<0.001**	*36*	**0.012**
Percent N	*36*	**0.012**	*36*	**0.012**	5.96	**<0.001**	-3.99	**0.005**	-6.66	**<0.001**	-3.17	**0.016**
C:N atomic	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	0.96	0.366	0.42	0.685	-8.81	**<0.001**
$\delta^{13}C$	16.4	**<0.001**	14.73	**<0.001**	9.85	**<0.001**	-6.99	**<0.001**	-11.7	**<0.001**	3.08	**0.018**
$\delta^{15}N$	7.1	**<0.001**	8.17	**<0.001**	8.07	**<0.001**	-12.52	**<0.001**	-3.08	**0.018**	3.25	**0.014**

Table 2. Changes in carbon and nitrogen percentages, C:N ratios, and $\delta^{13}C$ and $\delta^{15}N$ values from the 10 pre-treatments relative to untreated (*Bulk*) muscle (Cape knifejaw, ragged-tooth shark) and blood (ragged-tooth shark) samples. (Refer to Figure 1 for abbreviations). Significant results are in bold, W values are in italics.

	PE		*PE H 20*		*Cx*		*Cx H 20*		*CM1*		*CM1 H 20*		*CM2*		*CM2 H 20*		*CEth*		*CEth H 20*	
	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p	t or *W*	p
a) Cape knifejaw muscle (n = 5)																				
Percent C	2.23	0.089	-1.31	0.260	*15*	**0.043**	-1.02	0.364	4.24	**0.013**	3.44	**0.026**	*15*	**0.043**	-8.87	**0.001**	4.29	**0.013**	-3.45	**0.026**
Percent N	-2.08	0.106	-1.64	0.176	3.26	**0.031**	-13.00	**<0.001**	2.77	0.051	*15*	**0.043**	*14*	0.080	-14.1	**<0.001**	1.43	0.227	-4.00	**0.016**
C:N atomic	4.81	**0.009**	2.04	0.111	6.84	**0.002**	3.96	**0.017**	4.68	**0.009**	*15*	**0.043**	3.74	**0.020**	4.49	**0.011**	7.43	**0.002**	2.61	0.059
$\delta^{13}C$	-1.66	0.172	7.61	**0.002**	-3.96	**0.017**	2.03	0.112	1.94	0.125	-2.76	0.051	4.15	**0.014**	-0.99	0.380	-5.7	**0.005**	0.29	0.789
$\delta^{15}N$	*11*	0.345	-24.4	**<0.001**	-4.34	**0.012**	-4.61	**0.010**	-6.79	**0.002**	-49.1	**<0.001**	-11.2	**<0.001**	-23.2	**<0.001**	-0.26	0.806	-19.8	**<0.001**
b) Ragged-tooth shark muscle (n = 8)																				
Percent C	-3.06	**0.018**	*28*	**0.018**	0.11	0.914	-5.64	**0.001**	*30*	0.093	*36*	**0.012**	-5.33	**0.001**	*36*	**0.012**	36	**0.012**	-6.31	**<0.001**
Percent N	-1.87	0.104	1.95	0.099	*14*	1.000	1.38	0.210	4.64	**0.002**	*20*	0.779	4.14	**0.004**	1.65	0.143	33	**0.036**	2.08	0.076
C:N atomic	-4.96	**0.002**	*28*	**0.018**	0.94	0.380	-7.79	**<0.001**	-21.1	**<0.001**	-6.17	**0.001**	*36*	**0.012**	-12.1	**<0.001**	36	**0.012**	-8.95	**<0.001**
$\delta^{13}C$	0.78	0.460	3.53	**0.012**	1.07	0.321	2.41	**0.047**	1.73	0.128	*19*	0.889	2.27	0.058	*30*	0.093	0.03	0.978	1.84	0.108
$\delta^{15}N$	-1.37	0.212	-6.5	**0.001**	-7.53	**<0.001**	-5.92	**0.001**	-5.21	**0.001**	-14.5	**<0.001**	-3.43	**0.011**	-6.01	**0.001**	-1.54	0.167	-5.77	**0.001**
c) Ragged-tooth shark blood (n = 8)																				
Percent C	-1.31	0.232	*36*	**0.012**	6.31	**<0.001**	*36*	**0.012**	5.89	**0.001**	*36*	**0.012**	-13.6	**<0.001**	*36*	**0.012**	-4.26	**0.004**	*36*	**0.012**
Percent N	-1.98	0.089	*36*	**0.012**	3.49	**0.010**	8.31	**<0.001**	*36*	**0.012**	6.97	**<0.001**	*36*	**0.012**	10.52	**<0.001**	*36*	**0.012**	*36*	**0.012**
C:N atomic	-0.36	0.727	*36*	**0.012**	1.21	0.266	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	*36*	**0.012**	7.13	**<0.001**	*36*	**0.012**
$\delta^{13}C$	0.93	0.383	23.69	**<0.001**	0.34	0.741	16.92	**<0.001**	14.27	**<0.001**	12.71	**<0.001**	22.64	**<0.001**	13.46	**<0.001**	6.43	**<0.001**	18.72	**<0.001**
$\delta^{15}N$	-0.76	0.471	8.93	**<0.001**	-2.24	0.060	10.16	**<0.001**	9.41	**<0.001**	1.65	0.144	10.26	**<0.001**	7.75	**<0.001**	-9.83	**<0.001**	8.89	**<0.001**

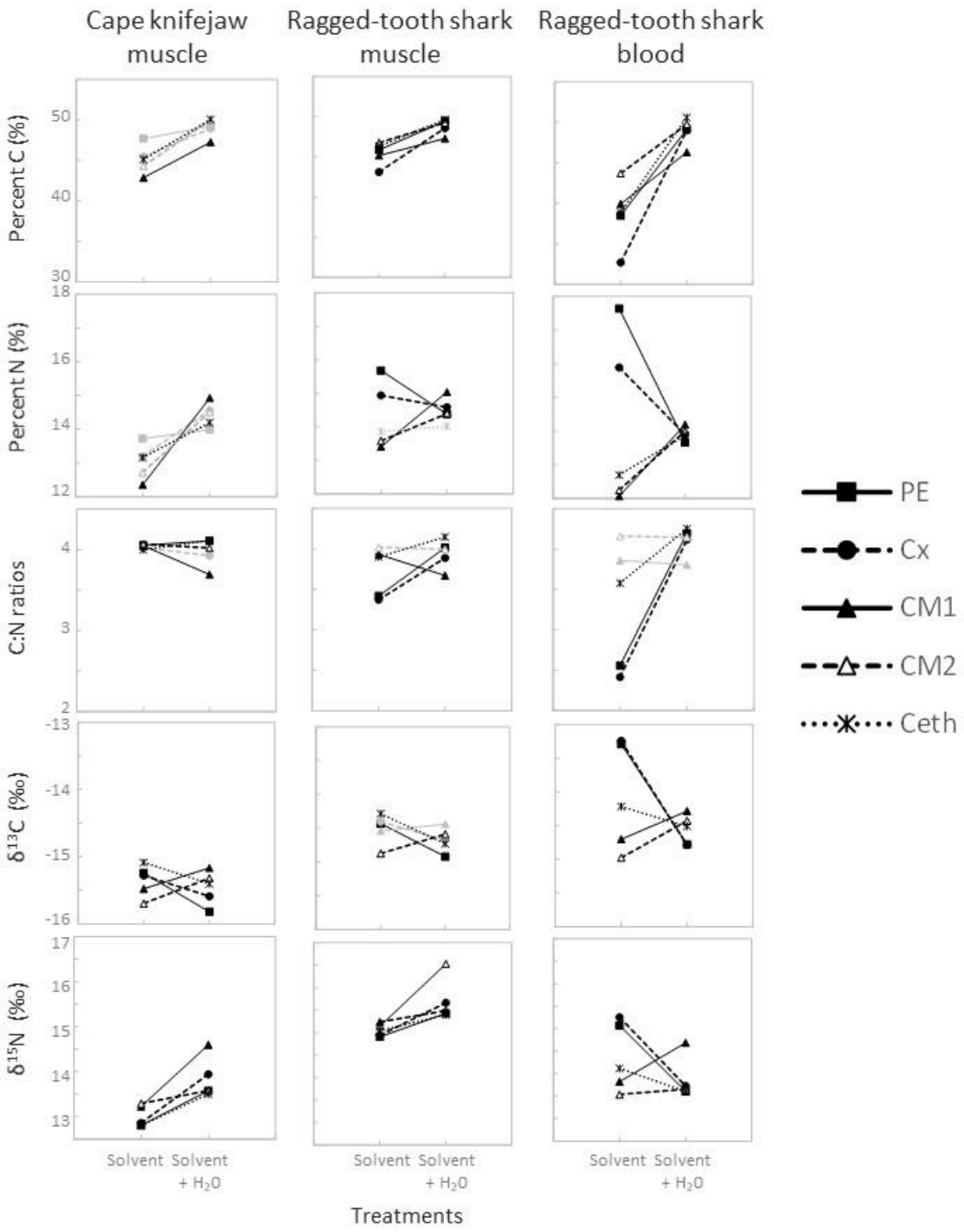

FIGURE 3

Figure 3. Effect of subsequent water wash after initial solvent wash on carbon percentage, nitrogen percentage, C:N atomic ratios, and $\delta^{13}C$ and $\delta^{15}N$ values. Significant changes are in black. (Refer to Figure 1 for abbreviations).

Table 3. Comparisons across pre-treatments for the five parameters in three tissues. Significant results are in bold.

	Cape knifejaw muscle (n = 5)		Ragged-tooth shark muscle (n = 8)		Ragged-tooth shark blood (n = 8)	
	F	p	F	p	F	p
Percent C	11.47	**< 0.001**	18.28	**< 0.001**	55.48	**< 0.001**
Percent N	24.35	**< 0.001**	27.82	**< 0.001**	25.15	**< 0.001**
C:N atomic	27.77	**< 0.001**	44.97	**< 0.001**	65.50	**< 0.001**
$\delta^{13}C$	21.5	**< 0.001**	5.56	**< 0.001**	64.44	**< 0.001**
$\delta^{15}N$	38.82	**< 0.001**	27.68	**< 0.001**	44.01	**< 0.001**

CEth pre-treatments (6 out of 10 outcomes of the comparisons were significant; Table 2a, Figure 3). In contrast, water rinses on solvent pre-treated samples significantly affected most carbon and nitrogen contents in both ragged-tooth shark tissues except for muscle nitrogen content with the *CEth* pre-treatment (Table 2b, 2c, Figure 3). The C:N ratios were diversely significantly affected depending on pre-treatments and tissues (Table 2, Figure 3).

The final values of carbon and nitrogen percentages as well as C:N ratios were significantly affected by pre-treatments (all three ANOVAs on ranks $p < 0.001$; Table 3). Most outcomes of the pair-wise comparisons were significant for the three tissues (Tables S1 - S3, supporting information).

STABLE ISOTOPIC COMPOSITION

The water rinse of *Bulk* tissues did not significantly affect the $\delta^{13}C$ values for muscle tissue of either Cape knifejaw (-15.4 ± 0.2 ‰ vs -15.2 ± 0.3 ‰) or ragged-tooth shark (-14.3 ± 0.9 ‰ vs -14.2 ± 0.7 ‰), as opposed to the $\delta^{15}N$ values which significantly increased in muscle tissue samples of both Cape knifejaw (12.8 ± 0.4 ‰ vs 13.7 ± 0.3 ‰) and ragged-tooth shark (14.8 ± 0.7 ‰ vs 15.5 ± 0.7 ‰; Table 1a and 1b, Figure 4). Both $\delta^{13}C$ and $\delta^{15}N$ values decreased significantly in ragged-tooth shark blood after water rinsing compared with untreated *Bulk* samples ($\delta^{13}C$: -13.2 ± 0.7 ‰ vs -14.1 ± 0.6 ‰; $\delta^{15}N$: 15.0 ± 0.7 ‰ vs 13.7 ± 0.8 ‰; Table 1c).

The water rinsing after solvent wash significantly affected $\delta^{13}C$ and $\delta^{15}N$ values in Cape knifejaw muscle and ragged-tooth shark blood (except for the *PE* pre-treatment of Cape knifejaw muscle; Table 2). The direction of the effect (increased or decreased) was, however, species-, tissue- and chemical element-dependent (Figure 3). For ragged-tooth shark muscle, the water rinsing also significantly increased $\delta^{15}N$ values compared with tissues pre-treated with solvents, but $\delta^{13}C$ values were only affected by water rinsing after the pre-treatments *PE, CM2* and *CEth* (Table 2, Figures 3 and 4).

Pre-treatments had significant effects on the final $\delta^{13}C$ and $\delta^{15}N$ values in all 3 tissues (Table 3, Figure 4). For both species' muscle samples, the difference in $\delta^{13}C$ values among all pre-treatments added up to 0.7 ‰, while the differences in $\delta^{15}N$ values added up to 1.6 ‰ and 1.8 ‰ for ragged-tooth shark and Cape knifejaw, respectively (Figure 4). For ragged-tooth shark blood, the $\delta^{13}C$ and $\delta^{15}N$ ranges were 1.8 ‰ and 1.7 ‰, respectively (Figure 4).

Difference among pre-treatments varied with tissues with few pairwise comparisons being significant for $\delta^{13}C$ values of ragged-tooth shark muscle, in contrast to the other tissues and $\delta^{15}N$ values where most pairwise comparisons between pre-treatments were found to be

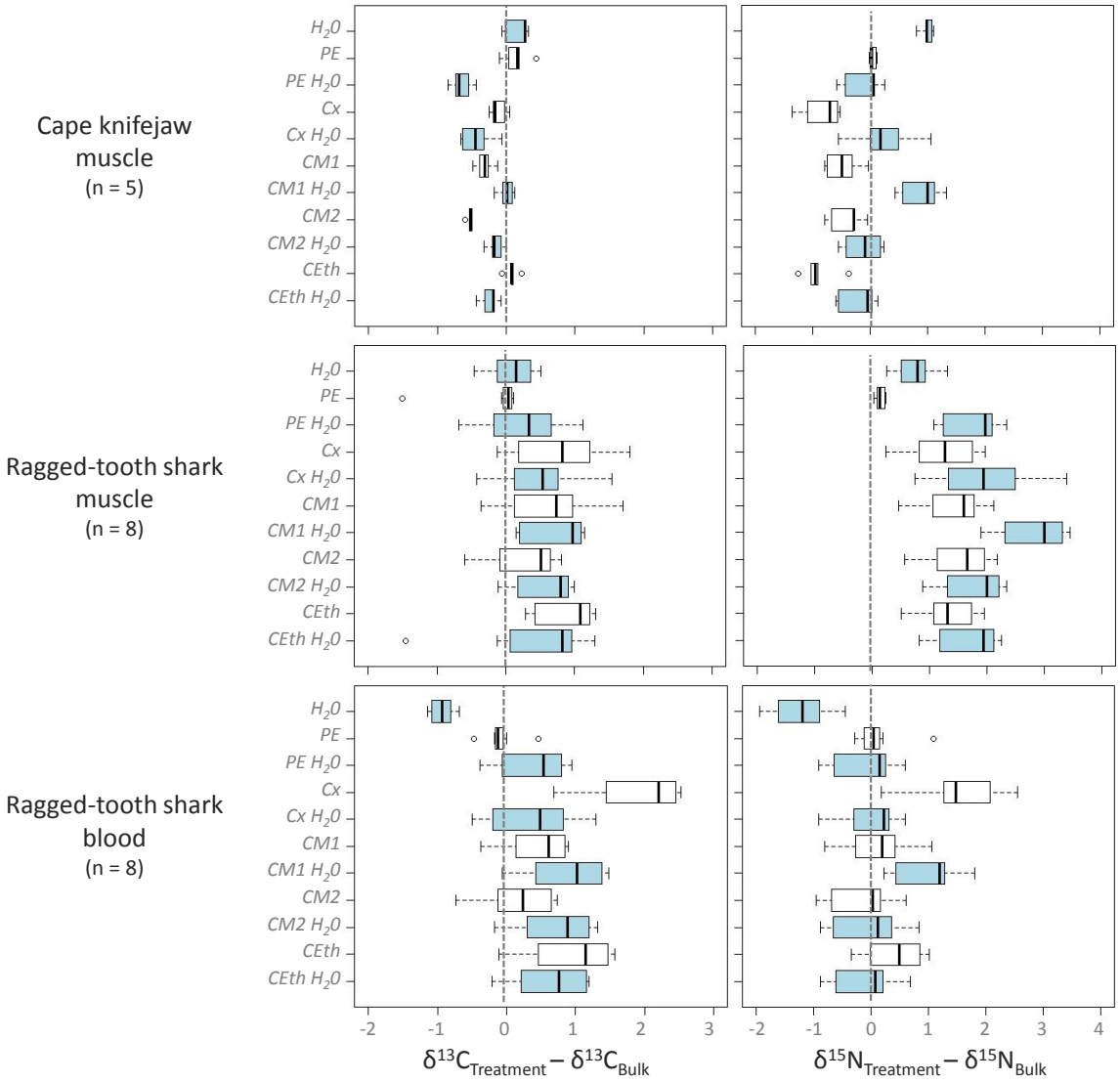

FIGURE 4

Figure 4. Changes in $\delta^{13}C$ and $\delta^{15}N$ values after the 11 pre-treatments relative to the untreated bulk samples of the 3 tissues (Cape knifejaw muscle, ragged-tooth shark muscle and blood. The data for water rinsed tissues are in blue. (Refer to Figure 1 for abbreviations).

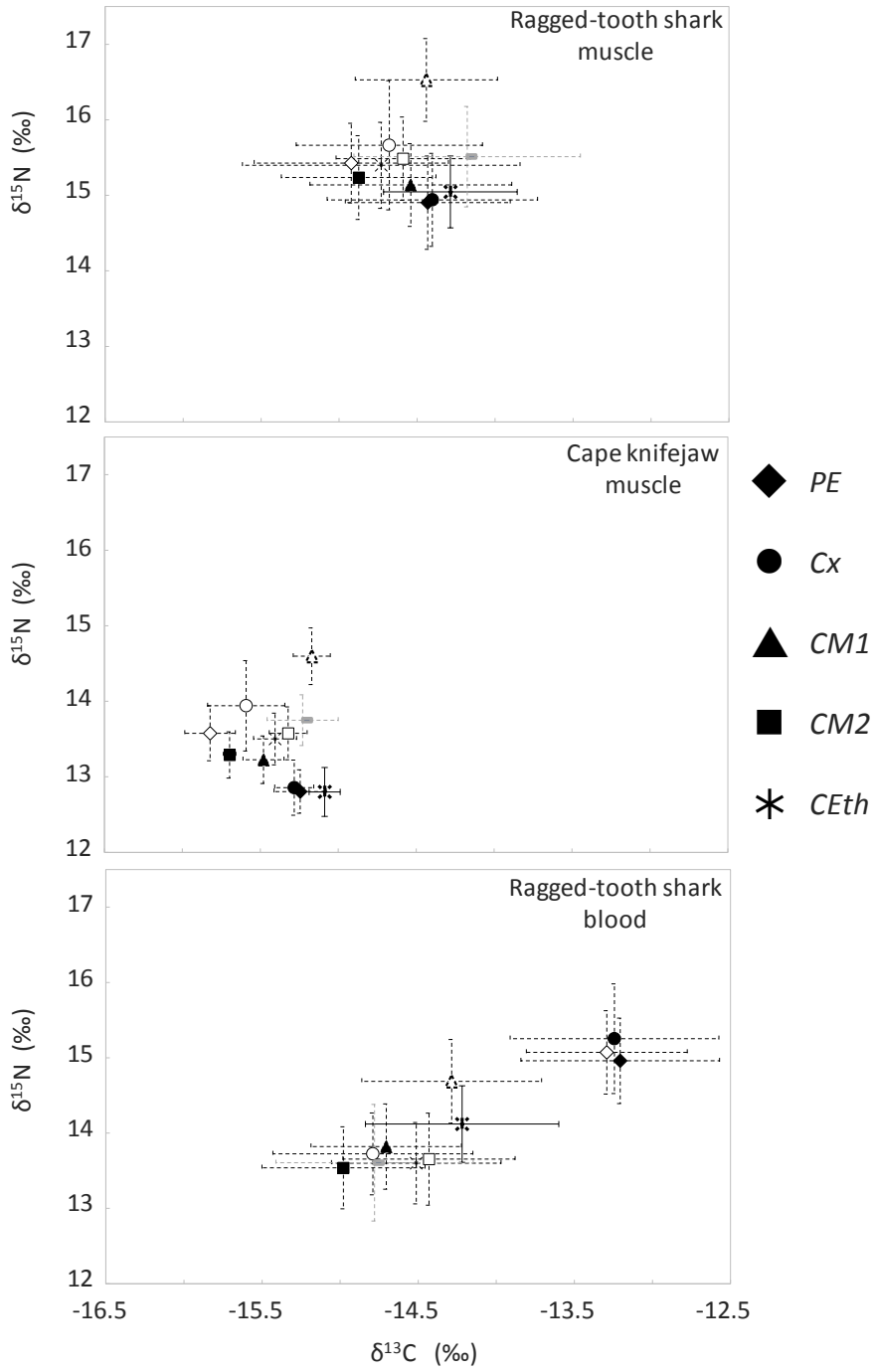

FIGURE 5

Figure 5. Carbon and nitrogen stable isotope ratios (mean ± SD) of ragged-tooth shark muscle and blood, and Cape knifejaw after water treatment (grey), solvent treatment (black), solvent and water treatments (dotted line). (Refer to Figure 1 for abbreviations).

significant (Table 4, and Tables S1 – S3, supporting information). Overall, *H_2O* washed muscle samples in both species had significantly higher $\delta^{15}N$ values than their counterpart solvent washed samples (Figure 5). The pre-treatment *CM1 H_2O* exhibited the most different $\delta^{15}N$ values in the muscle samples of both species compared with the other 10 pre-treatments. In ragged-tooth shark blood, the $\delta^{13}C$ and $\delta^{15}N$ values were most affected by three pre-treatments (i.e. *PE, PE H_2O, Cx*) which particularly exhibited higher $\delta^{13}C$ values than the other eight pre-treatments (Figure 5).

DISCUSSION

A review of the literature using SIA to determine the trophic ecology of elasmobranchs highlighted that, despite several studies dealing with pre-treatment issues of lipid and/or urea rich tissues (e.g.[23,25,28]), a consensus has yet to be reached on the preferred pre-treatment. A number of recently published studies used either no pre-treatment (e.g.[8,32-34]), a distilled water rinse followed by a chloroform:methanol 2:1 rinse (e.g.[35]), or cyclohexane rinses (e.g.[36]). The use of different pre-treatments can be problematic with the emergence of studies investigating the trophic ecology of shark species worldwide.[22] Indeed, these pre-treatments may not be equivalent in removing biological molecules such as lipids and urea, and thus they may influence the carbon and nitrogen contents and the C:N ratios, as well as the $\delta^{13}C$ and $\delta^{15}N$ values. While acknowledging the limited sample size, the 11 pre-treatments tested in this study significantly influenced the five measured parameters (carbon and nitrogen percentages, C:N ratios, and $\delta^{13}C$ and $\delta^{15}N$ values; Table 3). The effects were tissue, species and pre-treatment-dependent which thus precluded any simple mathematical normalization.

The differences between pre-treatments are likely to result from a combination of the chemical properties of the rinsing solution and the biological molecules removed (e.g. lipids, urea) that dictate their solubility. The solutions tested can be placed on a polarity scale with water being the most polar solution followed by chloroform:methanol, chloroform:ethanol, cyclohexane, and petroleum ether in decreasing polarity. Similarly, the polarity of biological molecules depends on their chemical composition and conformation. Urea and TMAO are particularly soluble in polar solutions such as water and less so in solvents such as cyclohexane or petroleum ether.[37,38] On the contrary, lipids in general are insoluble in water but highly soluble in organic solvents.[39] The solubility of lipids in solvents also depends on lipid classes, with neutral lipids such as triacylglycerols being particularly soluble in less

polar solvents (e.g. cyclohexane) and phospholipids in more polar solvents (e.g. chloroform).[39]

This suggests that the pre-treatment *H_2O* removed all water-soluble molecules including urea and TMAO. The removal of urea, depleted in ^{15}N[40] and probably enriched in ^{13}C[41], would artificially increase $\delta^{15}N$ values and potentially lower $\delta^{13}C$ values in urea-rich tissues such as in elasmobranchs.[23] The stronger effect of *H_2O* rinse was observed in ragged-tooth shark blood for C and N percentages as well as C:N ratios than in the two muscle samples (Figure 2). However, an increase of $\delta^{15}N$ values was observed in muscle but not in blood of ragged-tooth sharks where the contrary was observed (Figure 4). Measured urea contents are relatively homogenous within elasmobranch individuals with very little variation among tissues[14,42]; one would thus expect the effect of urea removal to be similar in blood and muscle. Our results suggest that other water-soluble molecules were removed from the blood concurrently with the urea/TMAO. Elasmobranch blood is characterised by high free amino acid contents compared with muscle.[42] Most of these are soluble in water[37] and would therefore be leached with the *H_2O* rinse. Their disappearance would then influence any following chemical analyses. Amino acid contents and compound-specific SIAs would identify whether this amino acid leaching is the reason for differences between blood and muscle in ragged-tooth tissues.

When lipid removal is necessary before SIA, not all lipid extraction protocols are equivalent in terms of quantity and composition of lipids removed (e.g.[43]). Coarsely, lipids can be separated into neutral lipids such as storage lipids (glycerides, wax esters), squalene, sterols, free fatty acids, and structural polar lipids (glycol-, phospho-, sphingo-lipids) which are linked to proteins.[39] Due to the apolarity (or very low polarity) of neutral lipids, they are better extracted with solvents of low polarity (e.g. petroleum ether, cyclohexane), while structural polar lipids may be extracted with more polar solvents such as chloroform. When removing lipids prior to SIA in lipid-rich tissues, it is preferable to target storage lipids which are highly variable within and among individuals[44], while preserving the integrity of cell membranes to avoid the co-extraction of associated amino acids. The five solvent mixes tested in this study had varying effects on the five measured parameters (carbon and nitrogen percentages, C:N ratios, and $\delta^{13}C$ and $\delta^{15}N$ values). Overall, the least polar solvent (*PE*) had the least influence on the five parameters measured in the three tissue types (Figures 2 and 4). A *PE* rinse alone did not change either the $\delta^{13}C$ or the $\delta^{15}N$ values in any of the three

analysed tissues. Interestingly, the other non-polar solvent, *Cx*, had the most significant effect on the C percentage and C:N ratios, as well as $\delta^{13}C$ and $\delta^{15}N$ values in ragged-tooth shark blood. A direct influence of *Cx* carbons into the results can be ruled out as this pattern is not observed in muscle tissues. These results suggest that there may be a carbon-rich molecule/component present in blood but not in muscle, and insoluble in *PE*, that could affect the five measured parameters following a *Cx* extraction. The identity of this molecule is currently unknown and would require further research.

Instead of using chemicals, mathematical normalizations are sometimes used after a *H2O* rinse to remove the impact of lipids on $\delta^{13}C$ values.[45] Several mathematical models exist (e.g. [19,46,47]) and they are not all equivalent.[48] When testing two widely used models developed using multispecies data[19,46] with our results, the models consistently predicted higher $\delta^{13}C$ values (up to 2 ‰) for all three tissues and solvent tested except for whole blood rinsed with *PE* and *Cx* where the $\delta^{13}C$ values were lower than expected with both models (-0.6 ‰). As pointed previously (e.g. [46,48,49]), this stresses the need for species- and tissue-specific equations developed on a subset of samples. Importantly, our work also shows that these equations will also depend on the chemicals used to remove the lipids as not all pre-treatments are equivalent (see above).

The comparison of pre-treatments consisting of the solvent rinse followed by the water rinse showed that this appraoch reduced the difference among treatments, particularly with regard to the carbon and nitrogen percentages in blood. The comparison of $\delta^{13}C$ and $\delta^{15}N$ values among pre-treatments showed that the measured differences were higher than any instrument errors for all three tissues (Figures 4 and 5). The differences in $\delta^{13}C$ values between the various pre-treatments may be as high as 3 ‰, but most often are about 1 ‰ (Figure 4). In both ragged-tooth shark tissues, the corrected $\delta^{13}C$ values were ~1 ‰ higher than those in bulk tissues, while the contrary was true for Cape knifejaw muscle. $\delta^{13}C$ values provide insight into the source production at the base of the food chain[50], and modelling studies using isoscapes to retrospectively locate predators are multiplying (e.g.[51]). An artificial variation of $\delta^{13}C$ value due to laboratory protocols may therefore lead to erroneous ecological interpretation. The variability among pre-treatments was higher for $\delta^{15}N$ values. The most notable impact was observed in $\delta^{15}N$ values of ragged-tooth shark muscle with differences of up to 3 ‰ (i.e. a trophic level[52]) between *PE* and *CM1* treatments. With regard to the results from the other two tissues, the effects of the pre-treatments resulted in significant differences

of about 2 ‰. The range of these differences is likely to influence ecological interpretations in global studies, particularly when $\delta^{15}N$ values rather than trophic levels are compared.

In addition to the influence of pre-treatments on the five parameters measured, the variability among samples within the three sample types was also noticeable (Figures 2 and 4). Patterns of enrichment were particularly variable for $\delta^{13}C$ and $\delta^{15}N$ values (Figure 4). This was probably linked to variable urea, TMAO and lipid contents in the individual fish analysed. For example, as all the sharks were captured over a period of 18 months, they were variable in length (188 to 287 cm total length; Smale unpubl.), and sex (2 males, 4 females; Smale unpublished), and these factors may have contributed to differing quantities of urea, TMAO and lipid being present in the tissues. This would have influenced how much of these molecules was removed by the pre-treatments and thus introduced variability in the impact of these pre-treatments on the corrected values.

Finally, the high C:N ratios observed in Cape knifejaw muscle (C:N ≥ 4.1) indicated that lipid extraction had to be undertaken to remove the lowering effect of lipids on $\delta^{13}C$ value.[19,20] Initial low C:N ratios observed in ragged-tooth shark tissues (C:N < 3.5) suggested that a lipid extraction was not necessary. However, after removing water soluble molecules, the C:N ratios increased to above 4 in both shark tissues, suggesting a potential confounding effect of lipids on the $\delta^{13}C$ values. Such results indicate that care must be taken to ensure that the most appropriate pre-treatment is used prior to SIA. The lipid content of muscle tissue from ragged-tooth sharks is particularly high in comparison with that of other shark species found in South African waters.[53] Our study confirms the inadequacy of using raw C:N ratios as an indication of lipid contents in elasmobranchs.[21,25]

CONCLUDING REMARKS

Although lipid and urea removals are used in most food web studies including elasmobranchs, the various pre-treatments yield statistically significant different results.

- Caution is therefore needed when using SI values from the literature because the pre-treatments vary widely between studies; the compared SI data thus reflect the intrinsic differences in the tissue composition combined with the influence of the pre-treatments which may confound ecological interpretation;

- Our study, conducted on a limited number of samples, shows that the difference among pre-treatments extends beyond the elasmobranch species and should therefore be confirmed with tests on additional species;
- The pre-treatment comparative approach that we followed for ragged-tooth sharks and Cape knifejaws could be adapted for other species prior to large scale studies to provide conversion factors;
- The next step would entail the evaluation of the pre-treatments in their abilities in removing solely the biological molecules of interest (urea, TMAO, lipids). This would allow us to choose the best pre-treatment which interferes least with the amino acid composition;
- We reiterate the call for a standardised approach to pre-treatments which would greatly benefit larger scale comparisons of SI studies, particularly when global comparisons of wide ranging species are starting to emerge (e.g.[22]).

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Ethics

This work was undertaken under an annual research permit issued by the Department of Agriculture, Forestry and Fisheries and Department of Environmental Affairs: Oceans and Coasts (DEA: O&C). Animal handling was according to the Standard Operating Procedure: Shark Tagging, accepted by Bayworld and DEA: O&C Ethics Committees.

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