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# Effect of sample preparation techniques for carbon and nitrogen stable isotope analysis of hydroxyapatite structures in the form of elasmobranch vertebral centra

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**RATIONALE:** Bulk stable isotope analysis (SIA) provides an important tool for the study of animal ecology. Elasmobranch vertebral centra can be serially sampled to obtain an isotopic history of an individual over ontogeny. The measured total  $\delta^{13}$ C value, however, may be misinterpreted due to the inclusion of the <sup>13</sup>C-rich inorganic portion. Hydrochloric acid (HCl) is commonly used to remove the inorganic portion of hydroxyapatite structures before undertaking SIA, but more recently ethylenediaminetetraacetic acid (EDTA) has been recommended for elasmobranch vertebrae. These acid treatments may introduce uncertainty on measured  $\delta^{13}$ C and  $\delta^{15}$ N values above instrument precision and the effect of small sample size remains untested for elasmobranch vertebrae.

**METHODS:** Using a non-dilution program on an isotope ratio mass spectrometer the minimum sample weight of vertebrae required to obtain accurate isotopic values was determined for three shark species: white (*Carcharodon carcharias*), tiger (*Galeocerdo cuvier*), and sand tiger (*Carcharias taurus*). To examine if acid treatment completely removes the inorganic component of the vertebrae or whether the technique introduces its own uncertainty on measured  $\delta^{13}$ C and  $\delta^{15}$ N values, vertebrae samples were analyzed untreated and following EDTA treatment.

**RESULTS:** The minimum sample weight required for accurate stable isotope values and the percentage sample yield following EDTA treatment varied within and among species. After EDTA treatment, white shark vertebrae were all enriched in <sup>13</sup>C and depleted in <sup>15</sup>N, tiger shark vertebrae showed both enrichment and depletion of <sup>13</sup>C and <sup>15</sup>N, and sand tiger shark vertebrae were all depleted in <sup>13</sup>C and <sup>15</sup>N.

**CONCLUSIONS:** EDTA treatment of elasmobranch vertebrae produces unpredictable effects (i.e. non-linear and noncorrectable) among species in both the percentage sample yield and the measured  $\delta^{13}$ C and  $\delta^{15}$ N values. Prior to initiating a large-scale study, we strongly recommend investigating (i) the minimum weight of vertebral material required to obtain consistent isotopic values and (ii) the effects of EDTA treatment, specific to the study species and the isotope ratio mass spectrometer employed. Copyright © 2014 John Wiley & Sons, Ltd.

Bulk stable isotope ratios of carbon ( $\delta^{13}$ C values) and nitrogen ( $\delta^{15}$ N values) in animal tissues are commonly used to study the movement, foraging, and trophic ecology of species, including elasmobranchs (sharks, skates, and rays).<sup>[11]</sup> For most organisms, the  $\delta^{13}$ C value of a tissue has been shown to reflect diet and primary productivity (i.e. indicative of foraging base)<sup>[2,3]</sup> and is often used as a chemical tracer of species movement.<sup>[4,5]</sup> The  $\delta^{15}$ N values exhibit a more marked increase between predator and prey, such that the relative trophic position and feeding behavior of the individual can be inferred.<sup>[3,6,7]</sup> Combined quantitative analysis of  $\delta^{13}$ C and  $\delta^{15}$ N data for single or multiple species

has led to novel insights into seasonal migrations,<sup>[4]</sup> dietary specialization within a population,<sup>[8,9]</sup> and community-wide trophic structure.<sup>[10]</sup>

Standardized sample storage and preparation methods prior to stable isotope analysis are important to ensure consistent results among studies and populations. For elasmobranch muscle tissue the effects of ethanol preservation and lipid extraction have been found to alter  $\delta^{13}$ C and  $\delta^{15}$ N values, respectively.<sup>[1,11]</sup> Inconsistent effects on stable isotope values following storage and treatment methods indicate that these factors need to be addressed on an individual tissue and species basis. Tissues such as blood and muscle can be obtained non-lethally, providing valuable data for an individual, but typically represent a single snapshot value and, because of relatively fast tissue turnover rates, indicate only recent feeding behavior.<sup>[12,13]</sup> Biomineralized structures in vertebrate species (e.g., whale baleen, turtle scutes, and elasmobranch vertebrae) are metabolically inert<sup>[4,9,14]</sup> and



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have the potential to provide important and novel ecological information, but have received less attention.<sup>[4,8,9]</sup> Elasmobranch vertebrae are biomineralized structures, which form annual growth rings in many species,<sup>[15]</sup> that can be individually sampled to provide an isotopic history throughout ontogeny (including pre-natal information formed during development).

Elasmobranch vertebral centra consist of an inorganic (mineralized) portion in the form of areolar calcification,<sup>[16]</sup> and an organic portion (collagen), but also include proteoglycans and water.<sup>[17]</sup> The inorganic portion may have a different  $\delta^{13}$ C value from the organic portion of interest, which can result in a mixed measured  $\delta^{13}$ C value,<sup>[11,18]</sup> an effect previously reported in terrestrial and marine mammal bones.<sup>[19]</sup> A common technique to address this issue is to decalcify the structure using hydrochloric acid (HCl), thus removing the inorganic portion, prior to bulk stable isotope analysis.<sup>[20-22]</sup> In the first isotopic studies on elasmobranch vertebrae multiple preparation methods were used prior to analysis including drying samples<sup>[23]</sup> and decalcifying by HCl with lipid extraction.<sup>[24]</sup> In an attempt to standardize these pre-treatment methods, Kim and Koch<sup>[11]</sup> recommended isolating the collagen elasmobranch vertebrae through treatment in with ethylenediaminetetraacetic acid (EDTA), a method that has successfully been used to isolate collagen in human bone<sup>[25]</sup> and ostrich eggshells.<sup>[26]</sup> Kim and Koch<sup>[11]</sup> suggested that EDTA was a more suitable method for removing the inorganic component of elasmobranch vertebrae rather than HCl due to rapid decalcification when using the latter. Consequently, a higher percentage yield of collagen is retained in samples treated with EDTA than in those treated with HCl, which is beneficial when only small amounts of material are available for analysis.<sup>[11,25]</sup>

A key advantage of using elasmobranch vertebrae for stable isotope analysis is the ability to examine retrospective ontogenetic profiles. Sample size (i.e. weight of individual sample), however, may be limited due to the size of the vertebrae (related to the size of the species) and therefore the width of the growth rings that can be sampled. The extent to which elasmobranch vertebrae are mineralized, which is known to vary among species,<sup>[17]</sup> will also affect the amount of untreated sample that is available for analysis, as the inorganic component contains less carbon per unit volume than the organic portion.<sup>[17,19,27]</sup> Therefore, samples with more mineralization will require a higher weight of untreated sample to be analyzed. This, combined with the amount of sample available from each growth ring, may become a limiting factor for serial stable isotope analysis of vertebrae to provide reliable  $\delta^{13}$ C and  $\delta^{15}$ N values. Determining the appropriate sample mass required for viable isotopic analysis of elasmobranch vertebrae is therefore warranted.

Typically, samples for  $\delta^{15}$ N analysis are untreated based on the assumption that inorganic N does not contribute to the total sample N.<sup>[28]</sup> However, with the increased use of dualmode stable isotope analysis, which requires the input of only one sample to determine both  $\delta^{13}$ C and  $\delta^{15}$ N values, samples for both C and N analysis are commonly acid treated. It is therefore necessary to establish if the  $\delta^{15}$ N value of a sample is affected by EDTA treatment, given that several studies have reported effects of HCl treatment on measured  $\delta^{15}$ N values.<sup>[20,28,29]</sup>

Considering the global archived collections of elasmobranch vertebrae for age and growth work, there is vast potential to undertake stable isotope analysis on these samples to reconstruct migration patterns and to examine foraging behavior of sharks over ontogeny. This would allow analysis of current movement and feeding behaviors of species plus allow retrospective analysis of historical samples to examine human and/or potential climate mediated effects. Prior to their use, an understanding of how acid treatment affects measured  $\delta^{13}\!\dot{C}$  and  $\delta^{15}\!N$  values of elasmobranch vertebral material is required. In this study, the effect of EDTA treatment on stable isotope values in elasmobranch vertebrae was investigated in three shark species: white (Carcharodon carcharias), tiger (Galeocerdo cuvier), and sand tiger (Carcharias taurus). Specifically, we examined (i) the minimum starting sample weight required for viable isotope data (EDTA-treated and untreated), (ii) the percentage sample yield following EDTA extraction, (iii) the  $\delta^{13}$ C values in the inorganic and organic portions of the vertebrae, and (iv) the effect of EDTA treatment on  $\delta^{13}$ C and  $\delta^{15}$ N values.

# **EXPERIMENTAL**

All samples were obtained from individuals that had been incidentally caught in beach protection nets off KwaZulu-Natal, South Africa (for further details, see Cliff and Dudley<sup>[30]</sup>). Vertebrae were excised from five individuals of each species (white, tiger, and sand tiger sharks) anterior to the first dorsal fin during routine dissections by KwaZulu-Natal Sharks Board staff and stored frozen. Prior to analysis, the samples were defrosted, cleaned of excess tissue and dried for 48 h at 40 °C. Bow-tie sections were cut from each vertebral centrum using an IsoMet® low-speed diamond saw (Beuhler Canada, Whitby, ON, Canada). The entire corpus calcareum was separated from the intermediala for each individual and ground into a fine powder. To create a homogenized mixture and remove any potential ontogenetic effects on measured stable isotope values the resulting powder was placed on an orbital shaker (VWR OS-500, VWR International, Mississauga, ON, Canada) for 8 h. The powder for each individual corpus calcareum was then split between two treatments: no treatment and EDTA-treated.

## **EDTA treatment**

To compare EDTA-treated samples with untreated samples, three replicate samples per individual (five individuals per species) weighing 3 mg each were taken from the homogeneous mixture and treated following the procedure outlined in Kim and Koch.<sup>[11]</sup> Briefly, sample powder was placed in a 2 mL cryovial and 1.5 mL of 0.5 M EDTA (pH 8.0, Fisher Scientific, Waltham, MA, USA) was added. The samples were then vortexed for 1 min. After allowing the reaction to proceed for 1 week at room temperature, the samples were centrifuged for 15 min and the EDTA was removed, replaced, and the process was repeated. The samples were washed with Milli-Q water (Milli-Q RG, EMD Millipore Corporation, Billerica, MA, USA) 10 times and sonicated for 10 min after the 1<sup>st</sup>, 5<sup>th</sup>, and 10<sup>th</sup> washes. The samples were soaked overnight after the 5<sup>th</sup> and 10<sup>th</sup> wash, then freeze-dried for 48 h, and the remaining sample weighed into tin capsules.



# Minimum sample weight for isotopic analysis of elasmobranch vertebrae

Untreated samples were weighed in tin capsules in triplicate for each individual shark in 200  $\mu$ g increments from 200  $\mu$ g to 1600  $\mu$ g and analyzed for  $\delta^{13}$ C and  $\delta^{15}$ N.

The use of multiple consecutive weights of the homogenized untreated samples enabled an investigation of (i) the effect of sample weight on isotope values, and (ii) an appropriate minimum sample weight for viable isotope data, and also enabled (iii) a comparison of stable isotope data of untreated samples with those treated with EDTA.

To investigate the minimum sample weight required for EDTA-treated vertebrae, 20 mg of the homogenized mixture from one individual per species was treated with EDTA. After EDTA treatment multiple consecutive weight samples increasing in mass from 200 µg to 1200 µg were weighed as described above. The minimum sample weight to obtain viable isotopic values for both EDTA-treated and untreated vertebrae was determined as the point at which the  $\delta^{13}C$  and  $\delta^{15}N$  values reached an asymptote.

#### Stable isotope analysis

All samples were analyzed with an elemental analyzer (Costech Instruments, Valencia, CA, USA) interfaced to a Thermo Finnigan Delta<sup>Plus</sup> mass spectrometer (Thermo Finnigan, San Jose, CA, USA) at the Great Lakes Institute for Environmental Research, University of Windsor (Windsor, ON, Canada). Stable isotope ratios are expressed in delta ( $\delta$ ) values as the ratio of an unknown sample to a recognized standard and are expressed in parts per thousand (or per mil, ‰) using the following equation:

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

where X is the element, b is the mass of the heavy isotope (less abundant) and  $R_{sample}$  and  $R_{standard}$  are the heavy to light isotope ratios (e.g.; carbon:  ${}^{13}C/{}^{12}C$ , nitrogen:  ${}^{15}N/{}^{14}N$ ) of the sample and standard, respectively.<sup>[3]</sup> To measure analytical precision the standard deviation of a National Institute of Standards and Technology (NIST) (Gaithersburg, MD, USA) standard (NIST standard 8414-bovine liver) and an internal laboratory standard (fish muscle, tilapia (*Oreochromis niloticus*)) were used. The standard deviation was 0.2 ‰ for  $\delta^{13}C$  values (n=80) and 0.1 ‰ for  $\delta^{15}N$  values (n=80) for both standards. The accuracy of the NIST standards for  $\delta^{13}C$  (NIST 8542 and NIST 8573) was within 0.17 ‰ and 0.09 ‰ of the certified values, respectively, and for  $\delta^{15}N$  (NIST 8547 and NIST 8549) was within 0.12 ‰ and 0.10 ‰, respectively.

#### Statistical analysis

The percentage sample yield, a proxy for the amount of collagen in the vertebrae, was calculated as the amount of vertebral material present after EDTA treatment compared with the amount of vertebral material before EDTA treatment, following Kim and Koch.<sup>[11]</sup> To determine the percent C (%C) in the inorganic and organic portions of the vertebrae and the  $\delta^{13}$ C value of the inorganic C, a mass balance approach was adopted:

$$W^*c_u = C_t \tag{2}$$

where W is the bulk sample weight,  $c_u$  is the total %C in the untreated sample (organic and inorganic) and  $C_t$  is the total amount of C. The amount of organic C in the sample was calculated as:

$$Y^*W^*c_e = C_o \tag{3}$$

where Y is equal to the percentage sample yield,  $c_e$  is the %C in the EDTA-treated sample (organic) and  $C_o$  is the amount of organic C. To determine the amount of inorganic C we subtracted the organic C from the total C and converted these values into percentages. The  $\delta^{13}$ C value of the inorganic portion of the vertebrae was calculated as:

$$z = \frac{T - (x * p)}{q} \tag{4}$$

where z is the  $\delta^{13}$ C value for inorganic carbon, T is the  $\delta^{13}$ C value of the untreated sample, x is the  $\delta^{13}$ C value of the treated sample, p is the %C in the organic portion, and q is the %C in the inorganic portion.

Due to replicates and unequal sample sizes between untreated and EDTA-treated vertebral samples, the effects of EDTA treatment on measured  $\delta^{13}C$  and  $\delta^{15}N$  values was examined using linear mixed effects models (LMEs). Three models were constructed and contrasted and the model with the lowest Akaike information criterion (AIC) was selected. The first model measured the variance in  $\delta^{13}$ C and  $\delta^{15}$ N values between individuals. The second model included EDTA treatment as a fixed effect to test between treatments among individuals, with all individuals assumed to be equally affected by EDTA treatment. The third model assumed random EDTA treatment effects (i.e., individuals respond differently to EDTA treatment). For all species and for both  $\delta^{13}$ C and  $\delta^{15}$ N values, the third model had the lowest AIC score in all cases and the results for this are presented. For the LMEs, no evidence of an effect of EDTA treatment on measured  $\delta^{13}$ C and  $\delta^{15}$ N values in the samples was indicated by confidence intervals overlapping zero while evidence of an effect was indicated by confidence intervals that did not overlap zero. The strength of the effect is shown by the distance from zero: with a larger value from zero indicating strong evidence for an effect and a value near zero indicating modest evidence of a small effect. The global statistic accounts for the unobserved portion of the population. The difference in  $\delta^{13}$ C and  $\delta^{15}$ N values (± standard error (SE)) between the EDTA-treated and untreated vertebral samples for each species was calculated both for individual sharks and for the species overall. It was expected that EDTA treatment would decrease  $\delta^{13}$ C values, a result of removing the inorganic mineralized component, but that  $\delta^{15}N$  values would remain consistent given limited nitrogen in inorganic material. All statistical analyses were conducted using R version 2.15.1.<sup>[31]</sup>

# **RESULTS AND DISCUSSION**

#### Minimum sample weight

Untreated samples had higher measured  $\delta^{13}$ C values with increasing carbon amplitude until reaching an asymptote at an amplitude of ~1000 mV (Fig. 1(A)). This value is in agreement





**Figure 1.** (A)  $\delta^{13}$ C values (±SE) of untreated and EDTA-treated vertebral samples versus isotope ratio mass spectrometer carbon amplitude from three species of sharks. Samples from individual sharks are represented by a different color. Black squares represent the EDTA-treated sample. Red vertical line indicates where values reach an asymptote. (B) Isotope ratio mass spectrometer carbon amplitude versus sample weight analyzed for untreated and EDTA-treated vertebral samples from three species of sharks. Samples from individual sharks are represented by a different color. Black squares represented by a different color. Black squares from three species of sharks. Samples from individual sharks are represented by a different color. Black squares indicate EDTA-treated sample. Red dashed lines indicate minimal weight required to obtain a carbon amplitude of 1000 mV.

with the recommendation of the manufacturer of the isotope ratio mass spectrometer for obtaining consistent and reproducible results. The minimum untreated sample weight required to obtain a C amplitude of 1000 mV varied by species: ~800  $\mu$ g for white shark, ~800  $\mu$ g for tiger shark and ~1000  $\mu$ g for sand tiger shark vertebrae (Fig. 1(B)). The minimum sample weight to obtain an amplitude of 1000 mV for EDTA-treated samples was lower for all species, reflecting the removal of the mineralized component:  $\sim$ 300 µg –white,  $\sim$ 300 µg –tiger,  $\sim$ 350 µg – sand tiger (Fig. 1(B)).

In contrast to the  $\delta^{13}$ C values, the untreated samples had lower measured  $\delta^{15}$ N values with increasing N amplitude, reaching an asymptote at an amplitude of ~1500 mV (Fig. 2(A)), a value above the manufacturer's recommendation of 1000 mV. This



**Figure 2.** (A)  $\delta^{15}$ N values (±SE) of untreated and EDTA-treated vertebral samples versus isotope ratio mass spectrometer nitrogen amplitude from three species of sharks. Samples from individual sharks are represented by a different color. Black squares represent the EDTA-treated sample. Red vertical line indicates where values reach an asymptote. (B) Isotope ratio mass spectrometer nitrogen amplitude versus sample weight analyzed for untreated and EDTA-treated vertebral samples from three species of sharks. Samples from individual sharks are represented by a different color. Black squares represented vertebral samples from three species of sharks. Samples Red dashed lines indicate minimal weight required to obtain a nitrogen amplitude of 1500 mV.

trend was observed in all individuals and for all three species (Fig. 2(A)). To obtain an N amplitude of 1500 mV the minimum untreated sample weight was ~700  $\mu$ g for white sharks and ~800  $\mu$ g for tiger and sand tiger sharks (Fig. 2(B)). For EDTA-treated samples, ~250  $\mu$ g of sample material was required for all three species (Fig. 2(B)).

These results indicate that measured elasmobranch vertebrae isotopic values may be artificially lower for  $\delta^{13}C$ by ~0.6 % ( $\delta^{13}$ C values; range 0.3–1.3) or higher for  $\delta^{15}$ N by ~0.9 ‰ ( $\delta^{15}$ N values; range 0.1–1.3) when samples are below the minimum required weight. This is in agreement with previous work that reported unpredictable higher and lower delta values for low weight samples.<sup>[22]</sup> These observed trends in measured stable isotope values associated with sample weight are a result of the total carbon and nitrogen levels in the sample material being below that of the instrument baseline, specific to the isotope ratio mass spectrometer used. Given that the erroneous isotope values occurred within the manufacturer's recommended levels (i.e. 1000 mV for  $\delta^{15}$ N values), it is necessary to report initial test runs of sample weight versus vertebrae isotope data, and to report accurate final sample weights analyzed.

## Percentage sample yield

The percentage of vertebral material (i.e. collagen) present after EDTA treatment varied within and among species (Table 1). The percentage collagen yields following EDTA extraction were slightly below previously reported values,<sup>[11]</sup> but in agreement with quantitative collagen measurements.<sup>[17]</sup> Following Kim and Koch,<sup>[11]</sup> this demonstrates that EDTA is effective at removing the mineralized component within elasmobranch vertebrae. Due to the inter- and intra-species variability in percentage sample yield following EDTA treatment, a starting sample weight of 3 mg of untreated material is recommended for EDTA extraction of all three species studied to obtain sufficient collagen weight for viable isotopic analysis.

**Table 1.** Mean (± standard error (SE)) percentage sample yield after EDTA treatment of vertebrae in three species of sharks

Species	Individual	n	Mean percentage sample yield (±SE)	Species mean sample percentage yield (±SE)
white	1	3	$16.9 \pm 1.36$	$16.1 \pm 1.24$
	2	3	$21.0 \pm 2.43$	
	3	3	$17.1 \pm 0.57$	
	4	3	$17.8 \pm 0.84$	
	5	3	$7.0 \pm 0.99$	
tiger	1	3	$12.4 \pm 1.32$	$15.5 \pm 0.78$
Ŭ	2	3	$14.2 \pm 2.07$	
	3	3	$14.3 \pm 1.64$	
	4	3	$16.2 \pm 1.54$	
	5	3	$18.4 \pm 0.96$	
sand tiger	1	3	$10.8 \pm 2.42$	$11.4 \pm 1.15$
Ŭ	2	3	$15.6 \pm 0.42$	
	3	3	$10.8 \pm 2.42$	
	4	3	$14.3 \pm 2.90$	
	5	3	$7.2 \pm 1.92$	

On average the %C in the EDTA-treated samples increased, due to the removal of inorganic carbon, relative to the untreated samples for each species: white shark %C increased from  $16.6\% \pm 0.1$  to  $41.8\% \pm 0.1$ , tiger shark %C increased from  $15.2\% \pm 0.05$  to  $42.0\% \pm 0.1$ , and sand tiger shark %C increased from  $12.5\% \pm 0.1$  to  $41.4\% \pm 0.1$ . The level of mineralization in shark vertebrae is known to vary among species,<sup>[17]</sup> such that shortfin mako (Isurus oxyrinchus) vertebrae have 39.2% dry mass mineralization, whereas gulper shark (Centrophorus granulosus) vertebrae have 55.1% dry mass mineralization.<sup>[17]</sup> These differences in mineralization among species account for different material properties in the vertebrae required to accommodate different life strategies. As expected, the variability observed in the percentage sample yield data suggests good correspondence with the level of mineralization in the vertebrae, accepting that these structures also include proteoglycans and water. Following removal of the mineralized component by EDTA treatment, the %C in all three species was more similar and less variable, as expected. Sand tiger sharks had the lowest percentage sample yield of collagen indicating they had the highest level of mineralization and therefore a larger untreated sample weight was required for both untreated and EDTA-extracted samples to obtain accurate isotope data (as evidenced above). Knowledge of the extent to which a species' vertebrae are mineralized will help predict the minimum untreated sample amount required.

# Effects of EDTA treatment on $\delta^{13}C$ and $\delta^{15}N$ values

EDTA treatment had variable effects on measured  $\delta^{13}$ C values in the vertebrae of the three shark species examined relative to untreated samples (Table 2). White shark vertebrae treated with EDTA were enriched in <sup>13</sup>C relative to untreated samples (Fig. 3(A)). The LME found that EDTA treatment had an overall effect (Global statistic) on  $\delta^{13}$ C values; however, at the individual level there was no treatment effect in one individual and the effect was minimal for two individuals. Tiger shark EDTA-treated vertebral samples were both enriched and depleted in <sup>13</sup>C compared with untreated samples, indicating that within-species variation was occurring (Fig. 3(A)). The variation was within machine precision and therefore cannot be attributed to machine- or EDTA-induced error. For tiger sharks, the LME found no overall effect of EDTA treatment on the  $\delta^{13}$ C values but there was a minor effect of treatment at the individual level (Table 2). Alternatively, sand tiger shark EDTA-treated vertebral samples were all depleted in <sup>13</sup>C compared with the untreated samples (Fig. 3(A)) and the LME detected an effect of the EDTA treatment (both overall and at the individual level; Table 2). The enrichment of <sup>13</sup>C in EDTA-treated vertebrae of white shark and some tiger shark samples compared with untreated samples was contradictory. It would be expected that vertebrae would be depleted in <sup>13</sup>C due to the removal of the <sup>13</sup>C-rich inorganic portion,<sup>[27]</sup> potentially suggesting a bias caused by the EDTA treatment for these two species and/or the effect being less than machine precision.

The effects of EDTA as a pre-treatment agent for inorganic C removal prior to stable isotope measurements in elasmobranch vertebrae have not been investigated previously. However, significant effects on stable isotopic values have been documented for blood and muscle samples from quail (*Coturnix coturnix japonica*),<sup>[32]</sup> blood from sheep



**Table 2.** Results of linear mixed effects model for  $\delta^{13}C$  and  $\delta^{15}N$  values between EDTA-treated and untreated vertebral samples from three species of sharks

Sample	Estimate	95% Confidence Interval	Sample	Estimate	95% Confidence Interval		
white shark $\delta^{13}$ C			white shark $\delta^{15}$ N				
Global	0.16	0.06, 0.27	Global	-0.39	-0.69, -0.09		
1	0.28	0.18, 0.36	1	-0.91	-1.02, -0.80		
2	0.14	0.05, 0.23	2	-0.22	-0.33, -0.11		
3	0.10	0.01, 0.19	3	-0.33	-0.43, -0.22		
4*	0.05	-0.04, 0.14	4* -0.04		-0.15, 0.07		
5	0.25	0.16, 0.34	5	-0.45	-0.56, -0.34		
tiger shark $\delta^{13}$ C			tiger shark $\delta^{15}$ N				
Global*	-0.27	-1.01, 0.46	Global*	-0.27	-0.58, 0.05		
1	-0.14	-0.27, -0.01	1	-0.70	-0.83, -0.57		
2	0.30	0.18, 0.42	2	-0.27	-0.39, -0.14		
3	-1.69	-1.82, -1.57	3	-0.34	-0.46, -0.21		
4*	-0.08	-0.21, 0.04	4	-0.28	-0.41, -0.16		
5	0.26	0.13, 0.38	5	0.26	0.13, 0.38		
sand tiger shark $\delta^{13}$ C			sand tiger shark $\delta^{15}N$				
Global	-0.54	-0.66, -0.43	Global	-0.44	-0.62, -0.27		
1	-0.63	-0.69, -0.56	1	-0.38	-0.47, -0.28		
2	-0.57	-0.64, -0.51	2	-0.27	-0.37, -0.17		
3	-0.49	-0.55, -0.42	3	-0.74	-0.83, -0.64		
4	-0.50	-0.56, -0.43	4	-0.47	-0.57, -0.38		
5	-0.53	-0.59, -0.46	5	-0.36	-0.45, -0.26		
*indicates the	ere is no evidenc	e of an effect of the EDTA treatm	ent on the samp	ole			

(*Ovis aries*),<sup>[32]</sup> and epidermis from turtles,<sup>[33]</sup> that were preserved in dimethyl sulfoxide (DMSO) containing EDTA. Previously, acid treatment using HCl has resulted in variable effects by species such that a significant lowering of  $\delta^{13}$ C values in invertebrates<sup>[27]</sup> and algae and cyanobacteria<sup>[34]</sup> was reported, while no significant differences were found in molluscs,<sup>[34]</sup> winter flounder (*Pleuronectes americanus*) or mud shrimp (*Crangon septemspinosa*).<sup>[35]</sup> The variable effects of EDTA treatment on measured stable isotope values in this study mirror those reported for HCl treatment. The imprecision associated with the treatment method is typically not reported; however, if it is higher than the instrument imprecision (which is normally reported), there could be implications for interpreting stable isotope values for ecological studies.

The percentage of inorganic and organic C in vertebrae from all three species was similar (Table 3), indicating that, while sand tiger sharks had the highest amount of mineralization, the amount of inorganic C contained within the mineralized portion was similar to that of white and tiger sharks. The  $\delta^{13}$ C values for the inorganic portion in white and tiger sharks were similar to those of the organic portion (Table 3), consistent with the small differences observed in  $\delta^{13}$ C values between EDTA-treated and untreated samples (~0.2 ‰ (Fig. 4)). This value would not be considered ecologically significant for most applications, questioning the need for EDTA treatment in these two species. The sand tiger sharks, however, had a larger difference between the  $\delta^{13}$ C values for organic and inorganic portions (Table 3) than the white and tiger sharks, which may be caused by differences in feeding strategies among species. The organic C in collagen is derived from the proteins of the individual's diet, while the inorganic C in the mineralized portion is derived from the carbohydrates and lipids of an individual's diet.<sup>[36]</sup> Therefore, the observed differences in  $\delta^{13}$ C values between the organic and inorganic portions of the vertebrae may be driven by the individuals' metabolic pathways and/ or growth requirements. There is potential for future research examining the differences observed between  $\delta^{13}$ C values in the inorganic and organic portions of shark vertebrae to investigate trophic level effects on the growth and energy pathways of a species.

When considering  $\delta^{15}$ N values, the white shark EDTAtreated vertebral samples were depleted in <sup>15</sup>N relative to untreated samples (Fig. 3(B)). The LME found an effect of EDTA treatment both overall and at the individual level in white shark vertebrae (Table 2). The tiger shark EDTA-treated vertebral samples were both enriched and depleted in <sup>15</sup>N relative to untreated samples (Fig. 3(B)), but there was no overall effect of EDTA treatment on the  $\delta^{15}\!N$  values. The treated sand tiger shark samples were depleted in <sup>15</sup>N relative to untreated samples (Fig. 3(B)) and the LME found that there was both an overall and an individual effect of EDTA treatment on  $\delta^{15}$ N values (Table 2). The  $\delta^{15}$ N values should not be affected by the removal of the inorganic component, as it is assumed that inorganic N does not contribute to the total N.<sup>[19,28]</sup> Brodie et al.,<sup>[22,28]</sup> however, compared the effects of different acidification methods on  $\delta^{15}$ N values in terrestrial and aquatic organisms and concluded that variability within and among methods/tissue analyzed complicates comparisons between species and studies. In addition, depletion and enrichment in <sup>15</sup>N following acidification of soil samples<sup>[28]</sup> and seagrass,<sup>[29]</sup> and shrimp,<sup>[29]</sup> respectively, have been observed.



**Figure 3.** Stable isotope values (±SE) of EDTA-treated versus untreated vertebral samples for white, tiger and sand tiger sharks: (A)  $\delta^{13}$ C values and (B)  $\delta^{15}$ N values. Black solid line is linear regression of isotope values between treatments. Black dashed line indicates the one to one relationship.

**Table 3.** Measured  $\delta^{13}$ C values (± SE) for the total and organic portions and calculated  $\delta^{13}$ C values (± SE) for the inorganic portion in vertebrae from three species of sharks. The calculated percentage (± SE) organic and inorganic C is also presented

Species	Untreated $\delta^{13}C$ (‰)	Organic $\delta^{13}C$ (‰)	Inorganic $\delta^{13}C(\%)$	Difference between organic and inorganic $\delta^{13}C(\%)$	Organic C (%)	Inorganic C (%)
white	$-11.9 \pm 0.16$	$-11.7 \pm 0.14$	$-12.0 \pm 0.19$	0.3	$40.6 \pm 6.2$	$59.4 \pm 6.2$
tiger	$-12.0 \pm 0.33$	$-12.2 \pm 0.26$	$-11.9 \pm 0.39$	-0.3	$38.6 \pm 1.6$	$61.4 \pm 1.6$
sand tiger	$-11.0 \pm 0.17$	$-11.6 \pm 0.15$	$-10.7 \pm 0.15$	-0.9	$40.3 \pm 4.3$	$59.7 \pm 4.3$

In this study, EDTA treatment resulted in both the depletion (white, tiger, sand tiger sharks) and the enrichment (tiger sharks) of <sup>15</sup>N in shark vertebrae. Overall, the mean difference in  $\delta^{15}$ N values between EDTA-treated and untreated samples was less than 0.5 ‰ for all species (Fig. 4), but the effect of EDTA treatment was variable among species

and larger than the effect of EDTA on measured  $\delta^{13}$ C values for the white and tiger shark (Fig. 4). This would suggest that EDTA extraction is not required for these species and might result in measured  $\delta^{15}$ N values with larger uncertainties. Moreover, following EDTA extraction the  $\delta^{15}$ N values did not show a systematic increase or decrease, indicating that



Figure 4. Mean difference (±SE) in measured stable isotope values between EDTA-treated and untreated vertebral samples for white, tiger and sand tiger sharks. Gray circles indicate difference for  $\delta^{13}$ C values and black circles indicate difference for  $\delta^{15}$ N values.

the use of a correction factor is not suitable (i.e. the effect is non-linear, sensu Brodie et al.<sup>[28]</sup>). For the sand tiger shark, it would appear EDTA extraction is required to correct  $\delta^{13}C$ values, but the effect on  $\delta^{15}$ N values must be acknowledged. Specifically, for higher trophic level species an increase in the  $\delta^{15}$ N values of 1.2–1.8 ‰ between predator and prey has been described,<sup>[37]</sup> indicating that the unpredictable enrichments and depletions in <sup>15</sup>N caused by EDTA treatment may cause the incorrect trophic level to be assigned to a species. In addition, several studies have used stable isotope analysis of multiple tissues (e.g. vibrissae, turtle scute, muscle, and blood)<sup>[8,9,13]</sup> to investigate the degree of dietary specialization within a population. If this method were applied to elasmobranch vertebrae treated with EDTA the degree of specialization might be misinterpreted. Samples in this study were run using dual-mode analysis but to correct for the effect of EDTA treatment on the  $\delta^{15}N$  values of sand tiger vertebrae would require the analysis of paired samples per individual: one acid-treated sample for C analysis and one untreated sample for N analysis, again assuming that inorganic N does not contribute to total N. This approach might be restricted by the sample weight available (such as serially sampled elasmobranch vertebrae), as a larger sample will be required. Consequently, examining fine-scale ontogenetic changes in isotope values of growth bands may be limited for certain species, but coarse-level profiles would be possible.

## CONCLUSIONS

The unpredictable enrichment and depletion of <sup>13</sup>C and significant effect on  $\delta^{15}$ N values in white, tiger, and sand tiger shark vertebrae following EDTA treatment raises questions over the suitability of this approach to remove inorganic C material from samples prior to bulk stable isotope analysis. Importantly, to obtain robust stable isotopic data from untreated and EDTA-treated vertebral material required the analysis of species-specific weights of starting sample material. For the white and tiger shark, EDTA treatment resulted in minimal effects on  $\delta^{13}$ C values, but had a more marked change on  $\delta^{15}$ N values and is therefore not recommended. A correction factor is not suitable as the effect of EDTA treatment on measured  $\delta^{13}$ C and  $\delta^{15}$ N values is

non-linear and varies both within and among species. For sand tiger sharks, there was a consistent effect of EDTA treatment on  $\delta^{13}$ C values suggesting that treatment is required, but its effects on  $\delta^{15}$ N values must be accounted for. The differences in  $\delta^{13}$ C values between the inorganic and organic portions of the vertebrae observed among species indicate that there is potential to use these profiles to investigate metabolic pathways among species of different trophic levels and phylogeny.

When examining stable isotopes in elasmobranch vertebrae we recommend preliminary analysis of EDTA-treated and untreated samples for the study species across different sample weights to identify (i) if EDTA treatment is required to obtain viable  $\delta^{13}C$  data and (ii) to determine the sample weight required (EDTA-treated or untreated) for analytical precision. For studies where EDTA treatment is required for C analysis, untreated samples should be analyzed for N.

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